NEURAL CONTROL OF THE CARDIAC RESPONSE

OF THE PEKIN DUCK

(ANAS PLATYRHYNCHOS)

TO FORCED SUBMERSION

by

GEOFFREY ROY JULIAN GABBOTT
B.Sc.(Hons) University of East Anglia, Norwich, 1976

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Department of ___ZOOLOGY_____

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

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ABSTRACT

Cardiovascular responses evoked during forced submersion enable the Pekin duck (<u>Anas</u> <u>platyrhynchos</u>) to survive protracted periods of asphyxia. The responses include an extraordinary bradycardia and intense peripheral vasoconstriction with the result that blood flow is favoured to those organs most susceptible to lack of oxygen. These adjustments appear to be mediated via the caudal brainstem following stimulation of peripheral and central arterial chemoreceptors.

The minor role that baroreceptors play in the generation of these responses was demonstrated by the persistance of the cardiovascular changes following peripheral arterial baroreceptor denervation. Isolation of the cephalic circulation from the systemic circulation enabled a series of experiments to assess the relative contributions from peripheral chemoreceptors, located in the carotid bodies, and from unidentified central chemoreceptors within the cranial circulation. A declining arterial PO_2 in the systemic circulation appeared especially potent in evoking bradycardia during submersion. Increased arterial PCO_2, likewise, resulted in a reduced heart rate. Similar changes in the blood gas levels of the cephalic circulation did not elicit significant bradycardia. However, both receptor groups responded to arterial hypoxic hypercapnia by activating substantial reduction in peripheral blood flow, as reflected by the rise in hind limb vascular resistance. Although baroreceptors may continue to mitigate changes in arterial blood pressure and cause some

change in heart rate and vascular resistance, chemoreceptors appear to be predominantly responsible for the changes during submersion.

The cardiac response to chemoreceptor stimulation during submersion was discovered to habituate following repetitive diving. Habituation was so pronounced in some ducks that after several training sessions the bradycardia during 40-second forced dives was abolished. Habituation of the cardiac response appeared dependent on the intensity of chemoreceptor stimulation. With severe arterial hypoxia, produced by either prolonging dive times or by reducing the pre-dive inspired oxygen content, little or no cardiac habituation was observed.

Tests were conducted to demonstrate efficacy of the cardioinhibitory efferent discharge. Maintained sensitivity of chemoreceptors was suggested by the lack of change in oxygen breathing tests before and after training. Furthermore, the persistence of stimulus intensity was established and these observations led to the suggestion that the locus of habituation is within the CNS.

The demonstration that the level of bradycardia was dependent on arterial PO_2 in both naive and habituated animals argues against the contention that the diving response is a fear response. Further evidence agaist this view was provided by the demonstration that the diving response remains essentially intact following transection in the rostral mesencephalon below the level of the hypothalamus.

It is concluded that chemoreceptor-driven cardiovascular

changes evoked as part of the diving response are mediated by regions of the CNS below the rostral brainstem. Modification of these responses can be produced in the intact animal by simple forms of learning. However, it remains uncertain at what level this influence arises.

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ACKNOWLEDGEMENTS

The experiments in Section 1. were performed in collaboration with D.R.Jones and W.K.Milsom although neither necessarily agrees with my interpretation of the results of these experiments.

GENERAL INTRODUCTION

It has long been known that diving animals can remain submerged for an inordinate period of time. Paul Bert pioneered research in this field in 1870 when he demonstrated that a duck of equivalent size and weight to a chicken will survive as much as seven times longer than the chicken when both are held under water. A few years later Charles Richet (1894) established that water played a key role in triggering extraordinary defensive mechanisms against asphyxia. He discovered that ducks would succumb far sooner if they were asphyxiated by tracheal occlusion than by forced head immersion. However, Laurence Irving, in collaboration with Per Scholander, was the first to report studies that began to unravel this problem. Scholander, who was concerned with special properties in the blood of diving seals, discovered that lactate concentration in the blood showed a remarkable increase following a dive (Scholander 1940). This increase was particularly unusual because throughout the dive lactate concentration hardly increased at all. Irving, on the other hand, was concerned with the strategy of the cardiovascular system to cope with imminent asphyxia. It was known that the central nervous system (CNS) and the myocardium are most susceptible to oxygen deprivation; thus Irving postulated a differential distribution of blood flow during hypoxia so that the brain and heart were favoured with a steady ration of blood. Other tissues received either a considerably reduced flow or none at all (Irving 1937,1938). The post-dive appearance of high lactate could thus be explained by the fact

that during a dive, lactate produced by the muscles remained sequestered as blood flow was stagnant, and only upon surfacing when normal blood flow was able to flush the muscles did the lactate appear (Irving et al. 1942; Scholander et al. 1942). The comprehensive and ground-breaking studies of Irving and Scholander remain as the greatest contributions to diving physiology and subsequent work has continued to characterise the details of the cardiovascular changes that are elicited by immersion in water.

The Responses

In addition to apnoea, the most easily observed feature during submergence is the marked fall in heart rate. In most species the rate falls to as little as 10 to 20% of pre-submergence levels. This fall reflects a similar change in cardiac output because, in most animals studied, stroke volume remains unchanged (Franklin et al. 1962; Johansen and Aakhus 1963; Elsner et al. 1964; Shelton and Jones 1965; Jones and Holeton 1972). In conjunction with the bradycardia is an increase in the resistance to flow in many systemic vascular beds (Andersen 1959; Butler and Jones 1971; Daly 1972; Jones et al. 1979; Zapol et al. 1979; McKean 1982). The intensity of this reduction in flow is manifest in the cessation of kidney function during forced submersion (Murdaugh et al. 1961; Sykes 1966). Cerebral blood flow, by contrast, remains constant (Kerem and Elsner 1973; Zapol et al. 1979) or may even increase

during the dive (Irving 1938; Dormer et al. 1977; Jones et al. 1979). Some uncertainty surrounds data of blood flow to the brain because most measurements have been taken indirectly and consequently are not amenable to rigorous analysis. Information on flow resistance in the pulmonary circulation is sparse, but it appears to increase though considerably less than the systemic circulation (Jones and Holeton 1972; Sinnet et al. 1978; Jones 1981). Coronary flow during forced submersion remains unchanged in the duck (Jones et al. 1979) but in the seal it appears to decrease in proportion to the reduction in cardiac output (Blix et al. 1976; Zapol et al. 1979). As with CNS tissue, cardiac muscle is unable to sustain itself by anaerobiosis and consequently cardiac effort remains subject to oxygen availability (Olsson 1981). In addition to the brain and the heart, the adrenal glands may be the only regions with vascular beds remaining patent in a dive (Elsner et al. 1978; Jones et al. 1979; Zapol et al. 1979; McKean 1982). The predominant result of the vascular changes is a selective restriction in tissue perfusion which acts to conserve available oxygen almost exclusively for the CNS and the heart. The fall in cardiac output and the rise in systemic vascular resistance are in sufficient harmony that mean arterial blood pressure is usually unaltered (Irving et al. 1942; Hollenberg and Uvnas 1963; Johansen and Aakhus 1963; Butler and Jones 1968, 1971; Jones and Johansen 1972; Angell-James et al. 1978). (FIGURE 1)

In comparison to their more strictly terrestrial homologues, diving animals possess enhanced oxygen stores in the

Figure 1.   Cardiovascular responses to forced submersion of a
     Pekin duck (<u>Anas</u> <u>platyrhynchos</u>).   'Peripheral
     resistance' refers to the resistance to blood flow in
     one leg.   Blood oxygen tension was measured in the
     brachiocephalic artery.   (from Butler and Jones 1982)

air flow
(L/min)

+3
-3

heart rate
(beats/min)

400

200

0

peripheral
resistance
(pru)

20

0

arterial blood
pressure
(mm Hg)

230

115

0

blood oxygen
tension
(mm Hg)

150

75

0

dive   surface   30sec
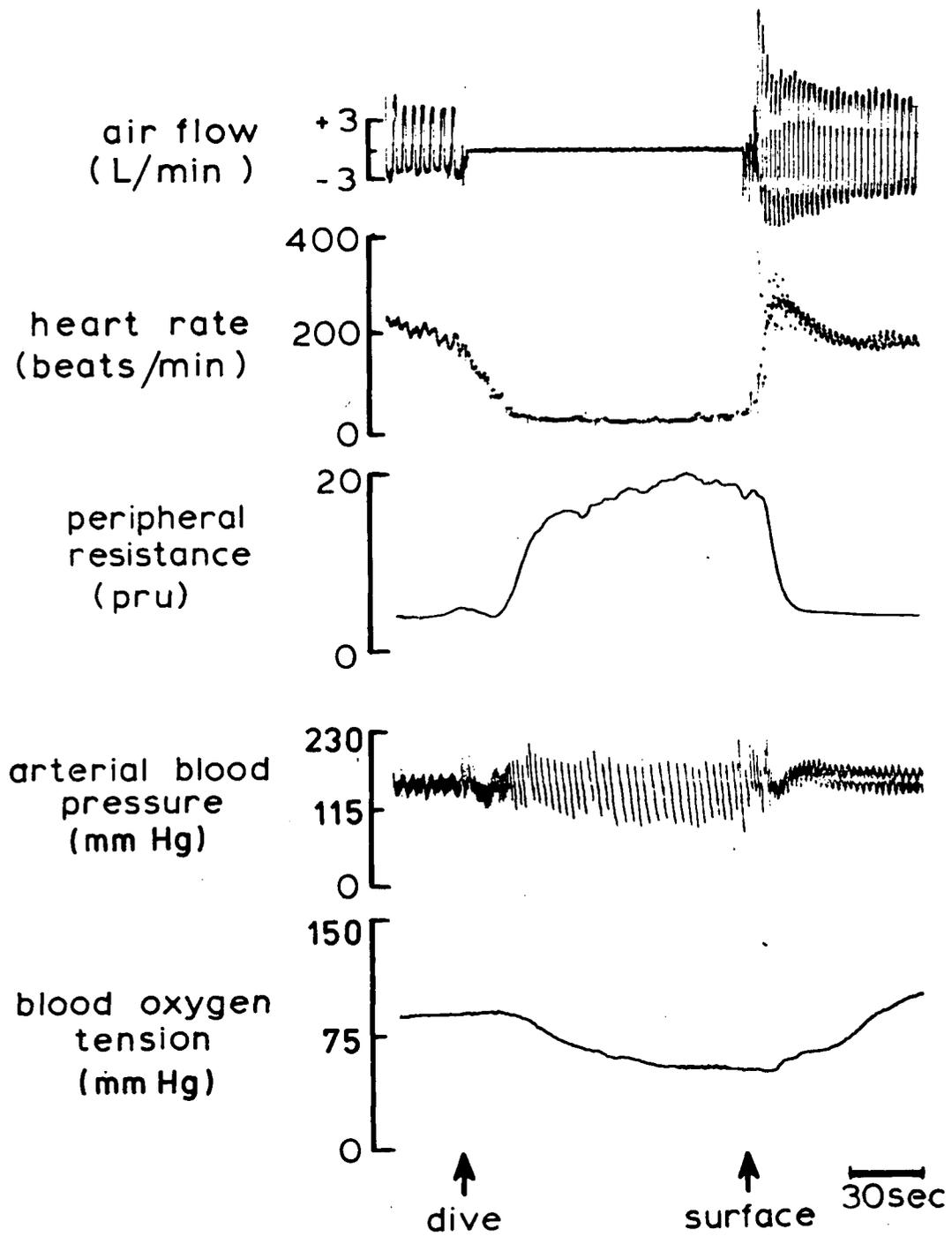
form of large blood volumes and high haemoglobin concentrations
(Irving 1939; Milsom et al. 1973; Lenfant et al. 1969). Birds
also possess large stores of oxygen in their air sacs.
Although, for diving birds, this represents a potentially
important source of oxygen, its usefulness during diving is
uncertain. Gas exchange does not occur across the air-sac
membranes; therefore, to be available to the animal, oxygen in
the respiratory system must be passed through the lungs.
However, it is not known if "stirring" of gases between air sacs
occurs in the actively swimming bird. Furthermore, like many
diving mammals, diving birds may expire before submergence. As
buoyancy is a primary concern, many diving birds possess reduced
air sac volumes compared to their non-diving relatives (Jones
and Furilla: in preparation). But by far and away the most
important strategy against asphyxia is the change that occurs in
the circulation. This is best demonstrated by work in which
this change is blocked by pharmacological substances.

Under normal circumstances, the domestic duck will easily
withstand forced submersion for 8 to 10 minutes without any
indication of damage to its CNS and after sufficient recovery
will seem alert, responsive and well prepared for another
submersion. Indeed as Richet noted, on average his animals
would finally succumb after protracted dives of 23 minutes. Yet
if the vascular moiety of the diving response is prevented by
inhibition of the sympathetic system with a-receptor blockade,
ducks will barely survive 1 minute under water (Kobinger and Oda
1969; Andersen and Blix 1974). Such severe effect on diving

performance also occurs if the vagally mediated bradycardia is abolished by atropine (Richet 1894; Butler and Jones 1968, 1971). Bryan and Jones (1980) demonstrated a greater decrease in surface oxygen tension of the cerebral hemispheres and a significant increase in cerebral NADH during submersion in ducks treated with atropine compared to controls.

Response Initiation

Considerable difficulty has been encountered in efforts to define the precise mechanisms whereby submersion is able to set in train these cardiovascular defences against asphyxia. This is largely because at the moment of submersion there are so many diverse sensory inputs impinging upon the animal, all of which may play a significant role in the initiation of the responses. The effect of water itself as a stimulus to facial or narial receptors has been strongly suspected (Koppanyi and Dooley 1928; Andersen 1963a,b,c; Harrison and Kooyman 1968; Bamford and Jones 1974; Blix et al. 1975) particularly because of their sensitivity to physical contact and because of the apnoea and cardiovascular reflexes so often provoked by stimulation of these regions (Lombroso 1913; Tchobroutsky et al. 1969; White and McRitchie 1973). Postural changes, such as those executed by voluntarily diving animals, may also contribute to the development of diving responses and this has long been investigated in the laboratory by mimicking the changes in head and body position that occur with submersion (Huxley 1913; Paton

1913; Koppanyi and Kleitman 1927). The cessation of central respiratory activity and pulmonary feedback, both of which are a consequence of the apnoea, may serve to depress cardiovascular activity (Irving et al. 1941; Lin et al. 1972; Butler and Taylor 1973; Bamford and Jones 1976). Analysis of the importance of any one of these sensory inputs is further confounded by the possibility that interaction may occur between them (Butler and Jones 1982). It is also apparent that considerable interspecies variation may exist in the preferred afferent pathway, and this is best demonstrated by the variation in response patterns elicited by submersion of different animals.

Some of the earliest studies showed that marine mammals responded to face immersion with almost immediate bradycardia and this strongly suggested reflex activity via possible facial or narial receptors (Angell-James and Daly 1969; Folkow et al. 1971; Dykes 1974; Drummond and Jones 1979). Similar studies have shown that certain diving birds also exhibit rapid onset of bradycardia indicative of the reflex action of immersion per se on the heart rate response (Catlett and Johnston 1974; Butler and Woakes 1978; Mangalam and Jones 1984). The profile of diving bradycardia in the Mallard or domestic duck, however, shows a gradually falling heart rate and this argues against a neural link between the water stimulus and the cardiac response. An alternative hypothesis was put forward that bradycardia was due to progressive hypoxia and hypercapnia that develops as a consequence of the apnoea which is elicited by submergence

(Feigl and Folkow 1963; Andersen 1963). This was supported by Jones and Purves (1970) who discovered that denervation of the carotid body chemoreceptors almost totally disrupted the heart rate response to forced diving. Butler and Woakes (1982) have more recently shown that even with free-swimming diving ducks, such as tufted ducks, the cardiac response in the later stages of voluntary dives is attenuated following carotid body denervation. It was also established that the chemoreceptive nature of the cardiac response was by no means exclusive to the domestic duck. Studies showed that though marine mammals responded with immediate bradycardia upon immersion, perpetuation of the lowered heart rate was dependent upon carotid body stimulation (Huang and Peng 1976; Daly et al. 1977, 1979).

The activation of vascular responses during diving has been less well studied than activation of cardiac change. Murdaugh and co-workers (1968) conducted experiments that indicated systemic vasoconstriction in seals can occur in the absence of bradycardia. In ducks the independence of vasoconstriction was also implicated by the substantial rise in blood pressure during forced submersion, in spite of the abolition of any change in heart rate (Butler and Jones 1971). Moreover, it has been shown in dogs that chemoreceptor stimulation by hypoxic and hypercapnic blood will cause an increase in total peripheral vascular resistance when pulmonary ventilation is held constant (Daly and Scott 1963; Daly and Ungar 1966).

On the strength of the observations that vasoconstriction

might be the direct consequence of chemoreceptor activation, the causal relation between these receptors and the cardiac chronotropic response was also called into question (Andersen and Blix 1974; Blix et al. 1974, 1975). It was proposed that cardiomotor and vasomotor responses are sequentially activated so that diving bradycardia is merely the expression of a barostatic reflex which responds to an incipient rise in blood pressure caused by a chemoreceptor-driven vasoconstriction. Pharmacological experiments supporting this contention demonstrated the abolition of the bradycardiac response when vasoconstriction was prevented by α-adrenergic blockade (Blix et al. 1974). These results, however, are at odds with the earlier demonstration that surgical denervation of systemic arterial baroreceptors leaves the bradycardia virtually intact in chronically denervated ducks (Jones 1973).

Whether or not the cardiomotor response and the vasomotor response are elicited independently or whether there is a causal relation between them is further confounded by the contribution of two other factors to vasoconstriction. First, evidence has demonstrated the possibility of vasomotor activity derived from chemoreceptor activation of the defence reaction (Bizzi et al. 1961; Hilton and Joels 1964; Marshall 1981; Hilton and Marshall 1982). In the decerebrate cat with an intact hypothalamus, chemoreceptor stimulation will evoke cholinergic vasodilatation in the hind limb along with vasoconstriction in the mesentery and kidney. It could, therefore, be reasoned that during submersion, chemoreceptor- driven vasoconstriction may be

attributed to activation of the defense reaction (Marshall
1981). Second, certain evidence suggests the existence of an
unidentified receptor group which stimulates vasoconstriction
upon activation. If both peripheral chemoreceptor and
baroreceptor groups are acutely denervated in the duck, a
significant rise in mean arterial blood pressure persists when
the animal is submerged (Jones and West 1978; Lillo and Jones
1982).

Higher Centre Influence

Reflexogenic effects notwithstanding, it is well documented
how pervasive is the extent of "higher CNS" control. In fact,
in any experiment conducted to discern the contributions of
receptor groups toward the diving bradycardia, due attention
should be paid to the "psychological condition" of the animal
both before and during diving.

Once again, Irving and Scholander were first to note that
the disposition and nervous state of seals was more important
for full development of forced diving responses than physical
factors (Irving et al. 1941). Folkow and co-workers (1967)
have also concluded that "calm" ducks show more intense diving
responses than "alarmed" animals. Moreover, in man, mental
stress or activity, in the form of mental arithmetic, prevents
most, if not all, of the cardioinhibitory effects of face
immersion (Paulev 1969; Wolf et al. 1975; Wolf 1978; Ross and
Steptoe 1980).

In this regard, the advances in radiotelemetric technology have had considerable impact. It is now possible to obtain physiological data from unrestrained animals behaving more "naturally" during voluntary submergence. When recording from tufted ducks, the striking observation during voluntary diving, was that the heart never displayed the extraordinary reductions in rate so characteristic of the forcibly submerged animals. Instead, the heart rate rose briefly just before diving and then, in the dive, fell back to levels similar to that when the animals were swimming actively on the surface (Butler and Woakes 1979; Butler 1980). Similar recordings showing a lack of intense bradycardia were taken from freely diving cormorants and Canada geese (Kanwisher et al. 1981). The bradycardia exhibited by forcibly submerged Papua penguins was reported to be slightly more intense than when these animals were diving voluntarily; however, the fall in mean femoral blood flow was more rapid in voluntary dives compared with forced dives (MIllard et al. 1973).

Telemetrically recorded data from free-diving mammals has proven to be equally as conflicting as that from the birds. Reports indicate that in the case of the Weddell seal, the degree of bradycardia may be correlated with the duration of the dive: in short dives the heart rate may fall slightly, but only to levels similar to that recorded during short respiratory pauses when breathing at the surface; in long dives the fall in heart rate is more dramatic and may match that seen in forced dives (Kooyman and Campbell 1972). In unrestrained muskrats,

large and very rapid falls in heart rate have been recorded which may exceed that evoked by forced dives (Drummond and Jones 1979). However, in this study, though unrestrained, the muskrats were actively provoked to dive. Porpoises, on the other hand appear to develop more intense bradycardia during trained dives than when spontaneously diving (Elsner et al. 1966).

The implications of these discrepancies in the dive characteristics between freely and forced dived animals is that higher neural centres might exert significant control over the cardiovascular responses, whether it be to attenuate or enhance them. This is further exemplified by some of the data collected from unrestrained seals which show decreases in heart rate even before submergence and increases before emergence (Jones et al. 1973; Casson and Ronald 1975). This anticipatory change in heart rate has also been claimed to occur in unrestrained diving ducks, such as pochards and tufted ducks (Butler and Woakes 1979). These data suggest that some form of associative learning may be involved (Butler and Jones 1982), and by the same token, the failure of some animals to display bradycardia during voluntary dives could mean that peripheral sensory inputs are "ignored" either by conditioning or by habituation.

Experiments have been reported in which seals have been conditioned to develop extreme bradycardia in response to an acoustic command signal (Ridgway et al. 1975). The rate of development and even the degree of bradycardia during breath-holding was enhanced by conditioning experiments. These results

suggest that the seals may be capable of some control over their heart rates. However, results from this kind of conditioning experiment are equivocal because animals may perform an activity in response to conditioning stimuli which only secondarily has the desired effect on heart rate. In other words, it is this activity which has been conditioned, and not cardiac control.

Neural Substrate

The early assumptions of cardiovascular control were modelled on negative feedback systems that operated on essentially two effector processes. Thus the "pressor" and the "depressor" reflexes seemed adequate to control blood pressure by their mutual antagonism. Furthermore, this was believed to echo the sympathetic/parasympathetic antagonism of the autonomic nervous system. Activation of the pressor reflexes, for example by lowering blood pressure, caused a sympathetically mediated vasoconstriction and an inhibition of vagal deceleratory effects on the heart, and blood pressure consequently rose. The reverse condition occurred during depressor activation. Control of fine tuning in such a system was restricted to variations in the magnitude of CNS activity (Uvnas 1960; Wang 1964).

The neural substrate for such control was envisaged to be confined within the domain of the medulla oblongata (myelencephalon) and was appropriately designated the medullary vasomotor centre. Experiments in the latter part of the last century delimited the medulla as the region most crucial for

control of normal blood pressure (Dittmar 1870; Owsjannikow 1871 : see review by Korner 1979). By sectioning the brainstem at progressively more caudal planes, a level was reached at which blood pressure fell drastically. Thus this level, just rostral to the obex in the medulla, marked the upper limit of a "centre" that maintains vasomotor tone. Further section of the medulla caused extreme hypotension and abolished vascular reflexes to both nerve stimulation and vascular occlusion of the major arteries (Uvnas 1960; Wang 1964).

Research following these experiments focussed on electrical stimulation within the medullary vasomotor centre and reports soon suggested discrete centres that were responsible for raising blood pressure (Ranson and Billingsley 1916; Chen et al. 1936). The depressor centre was then characterised and the general distribution of these areas was confirmed and mapped by Alexander (1946).

Progress continued to characterise the details of the vascular responses and it became apparent that an animal is capable of much more refined and selective control of different vascular beds. The vascular response pattern was quite different, for instance, in exercise when compared with that in arterial hypoxia, or in the defence reaction when compared with vascular changes in response to heat stress (Abrahams et al. 1960; Hilton 1965; Korner 1971; Heistad and Abboud 1980). Moreover, when examining these responses, another order of complexity should be considered: this is the coordinated integration of various somatomotor responses which are often

inextricably linked to the autonomic responses (Hess 1957). The
concept, held for so long, of a diarchical control system seemed
inadequate to account for both the complexity and flexibility of
intact systems. That the earlier model sufficed merely reflects
the limited scope of the tests of vascular viability (Wang 1964;
Peiss 1964; Hilton 1975).

Current concepts of the location of the control of the
cardiovascular response patterns shifted rostrally along the
neuraxis and, accordingly, a hierarchical system seemed more
appropriate (Peiss 1964). The neural substrate was considered
to incorporate higher regions of the brain with interaction
occurring in both "upward" and "downward" directions. In this
way, the cardiovascular response patterns evoked by, say, the
defence reaction are the product of interactions between the
brainstem, the diencephalon and relevant regions within the
limbic system (Hilton and Zbrozyna 1963; Hilton 1975). The
response includes a mobilisation of venous reserves, an increase
in cardiac output and a redistribution of blood flow.
Mesenteric vascular beds vasoconstrict and those to the skeletal
muscles vasodilatate so that blood flow is diverted in
preparation for muscular activity (Hilton 1965, 1975). These
regions of the neuraxis can be regarded as a functional unit
which must remain intact for the full expression of the defence
reaction (Abrahams et al. 1964).

Typically, response patterns demanding bidirectional
changes in different vascular beds (that is, the simultaneous
vasoconstriction in one bed with vasodilatation in another)

involve the integrated participation of levels of the brainstem other than the medulla (Manning 1965,1977). That the hypothalamus has an autonomic regulatory function is well known, and its control of widespread sympathetic and parasympathetic effects can be demonstrated by stimulation of this region (Hilton 1975). As an example, O.A. Smith in a discussion of the location of cardiovascular control, stated that "stimulation of the hypothalamus can force the cardiovascular system to do anything and everything within its limits" (Smith 1964).

It is because the hypothalamus regulates or influences many visceral functions and behaviours that it seems reasonable to suggest that regions within this area may integrate the behavioural responses and cardiovascular changes during diving. This line of reasoning led to a remarkable study in which the diencephalon in the duck was probed for areas which would elicit diving type responses upon electrical stimulation (Folkow and Rubinstein 1965). A ventral hypothalamic area was located which elicited cardio-respiratory responses which closely resembled those in the diving response. Although behavioural responses were not observed, stimulation of this area during forced submersion potentiated the cardiovascular responses. Another area in the dorsal hypothalamus , on the other hand, elicited behavioural responses but not the cardio-respiratory responses. When this area was electrically activated in the free-swimming animal, it would immediately submerge its head and swim around pecking the bottom of the pool as if searching for food. Other studies have also attempted to locate suprabulbar regions

integrating diving responses and mesencephalic sites eliciting apnoea have been found (Feigl and Folkow 1963; Kotilainen and Putkonen 1972).

The diving response can be visualised as being a compilation of complex and differential response patterns with separate action on cardiomotor and vasomotor neurons. Parasympathetic activation provokes maximal heart slowing while at the same time sympathetic activation appears capable of evoking complete shutdown in diverse systemic vascular beds while leaving open those supplying the few tissues most susceptible to oxygen deprivation. The central nervous controller must interpret a host of afferent information arising from facial, narial and glottal receptors, pulmonary receptors, chemoreceptors, baroreceptors, possibly vestibular receptors and an undefined barrage from cognitive or higher centres.

Accordingly, control of the diving response might involve distributed CNS networks for neural integration. This concept receives support from experiments carried out by Korner and his co-workers (Korner et al. 1969; Korner 1971) demonstrating the crucial need for an intact diencephalon, especially the hypothalamus, for the full expression of chemoreceptor-driven bradycardia.

In the present study, the control of the cardiovascular responses of the Pekin duck to submersion has been investigated with particular regard to the mechanisms involved with the initiation and maintenance of these responses. In the first section, the participation of the barostatic reflex toward the

development of the cardiac response is assessed. An investigation is also made into the possibility of the presence of a central chemosensitive site which may contribute to the submersion responses. Using a technique whereby the central circulation is separated, an attempt is made to evaluate the relative infuences of decreased arterial oxygen and increased arterial carbon dioxide on each receptor group. In the second section, the influence of a simple form of learning is investigated in the control of the cardiac response to forced submersion. With the consideration of chemoreceptor input, an analysis is made of the locus of this influence. The final section re-examines the role of the higher brainstem centres, particularly the hypothalamic region and a technique is developed whereby reversible transection is accomplished.

SECTION 1.

The Role of Central and Peripheral Chemoreceptors in the Diving Response.

Introduction

The chemoreceptive nature of the diving response is now well established for both mammals (Angell-James and Daly 1973; Daly et al. 1977; Huang and Peng 1976) and ducks (Jones and Purves 1970; Holm and Sorensen 1972; Jones and West 1978; Lillo and Jones 1982). Although the initiating mechanisms may differ among animals, it is clear that the responses during the later stages of prolonged submersion are strongly influenced by chemoreceptor stimulation (Daly et al. 1977; Butler and Woakes 1982). There seems little doubt that stimulus modalities are the progressive hypoxia and hypercapnia; however, it is unclear how much each of these contribute to the development of the responses. In ducks and geese, the magnitude of the diving bradycardia is determined by the level of arterial oxygen (Cohn et al. 1968; Mangalam and Jones 1984) and expression of heart rate change in ducks is dependent on the presence of the peripheral chemoreceptors (Jones and Purves 1970). The role of arterial PCO_2 has proven to be more difficult to establish. As with studies on oxygen, many investigators have altered the level of arterial PCO_2 by changing the content of inspired CO_2 (Feigl and Folkow 1963; Andersen 1963a,b); however, avian pulmonary receptors are inhibited by high levels of CO_2 in the

airways (Fedde et al. 1974a,b). Therefore, because of the linkage between ventilatory activity and heart rate (Butler and Taylor 1973; Bamford and Jones 1976), it has been difficult to distinguish between changes in HR due to arterial PCO_2 and changes due to alteration of ventilation. Controversy also surrounds the precise nature of how the autonomic response is generated. It is not certain whether the bradycardia is due to chemoreceptor stimulation alone, or whether it is due to a more indirect response as part of the barostatic reflex. Chemoreceptor-driven vasoconstriction could cause an incipient rise in blood pressure that would stimulate arterial baroreceptors (Andersen and Blix 1974, Blix et al.1974, 1975). To establish the role of the barostatic reflex, part of this investigation was designed to compare the cardiac response to submersion before and after denervation of the baroreceptors while perfusing the peripheral chemoreceptors with blood containing high or low oxygen tension.

Before attempts can be made to establish the separate contributions of hypoxia and hypercapnia to the diving response, the possibility of involvement by receptor groups other than peripheral chemoreceptors must be examined. For example, when both peripheral chemoreceptor and baroreceptor groups were denervated, a marked rise in mean arterial blood pressure occurred when the animal was submerged (Jones and West 1978; Lillo and Jones 1982). As the heart rate remained constant, stimulation of other receptor groups must have given rise to an increase in either stroke volume or vascular resistance.

Since the discovery of central chemoreceptors and their influence on respiration in both birds and mammals (Mitchell et al. 1963; Loeshcke et al. 1970; Sebert 1979; Milsom et al. 1981) a growing body of evidence suggests that these receptors may also contribute to vascular tone. In dogs, after denervation of peripheral chemoreceptors, modulation of arterial carbon dioxide is reflected in concomitant changes in vascular resistance (Lioy et al. 1978; Hanna et al. 1979). Based on the assumption that central chemoreceptors are responsive to elevated arterial PCO_2, experiments were designed to assess the magnitude, if any, of the cardiovascular adjustments which may result from their stimulation during submersion.

In order to determine the contributions from separate receptor sites it was necessary to isolate the vascular flow to each receptor site and examine the subsequent effects of altering blood gas tensions on their individual circulations. It was decided that while the systemic circulation could be perfused with the animal's own blood, isolation of the circulation to the head could be best accomplished by cross-perfusion of blood from a donor animal. In this way, the blood gas tensions in each circulation could be independently altered by varying the oxygen and carbon dioxide contents of the inspired gas in the individual animals.

METHODS

Experiments were done at room temperature (20-22°C) on 22 pairs of White Pekin ducks (Anas platyrhynchos) varying in weight from 2.5 to 3.8 kg. The ducks were acclimated to this temperature for at least one week before any experiments.

Since cardiovascular responses to diving are abolished by anaesthesia, all experiments were done on unanaesthetised animals. Only minor surgery for implantation of cannulae, under local anaesthesia (2% Xylocaine, Astra Pharmaceuticals) was done on the day of the experiment, and great care was taken to minimise stress to the animals through gentle handling and by limitation of excessive noise. One to 2 days before the experiment day, major surgery was performed under general anaesthesia (pentobarbital 30mg/kg or urethan 1g/kg i.v.). Artificial or cross-perfusion of restricted regions of the vasculature requires extensive use of heparin to prevent blood clotting in the perfusion cannulae, making it important that all incisions had time to heal before the day of the experiment. The areas of these incisions were also periodically infiltrated with a local anaesthetic during the course of the experiment.

In all experiments hyperoxic blood refers to arterial blood with partial pressures of oxygen (PaO_2) at least 200 mm Hg above normal levels; mean values were 373 ±49 mm Hg. Hypoxic blood values were at least 50 mm Hg below normal with a mean of 43.6 ±1.7 mm Hg. Hypercapnic blood refers to arterial blood with partial pressures of carbon dioxide ($PaCO_2$) at least 10 mm Hg above normal values and hypocapnic blood values were at least 10

mm Hg below normal; mean values of $PaCO_2$ were respectively 62.9 ±2.5 and 21.4 ±1.9 mm Hg. When referring to perfusion with blood from a donor animal, the term "cross-perfusion" will be used, and when all regions of the circulation are perfused with the animal's own blood, the term "auto-perfusion" will be used. The adjective "normal" refers to ducks which were autoperfused and "intact" refers to ducks before baroreceptor denervation.

Effect of Unilateral Vagotomy on Diving Bradycardia

Carotid body chemoreceptors in birds are located low in the neck and are innervated by branches arising from each nodose ganglion of the vagus (Jones and Purves 1970). Systemic arterial baroreceptors are located at the root of the aorta and are similarly innervated by a different branch of the vagus nerve (Jones 1973).

In order to assess the role of peripheral chemoreceptors, experiments were designed to isolate and artificially perfuse one carotid body with blood of various blood gas tensions. The contralateral carotid body was denervated by section of the vagus nerve. Isolation of the circulation to one carotid body entailed disruption of blood flow in the ipsilateral carotid artery. Richards and Sykes (1967) showed that, in birds, normal flow was provided to the head when all but one artery were occluded. This maintenance of flow was attributed to extensive intercarotid and extracranial anastamoses (Richards and Sykes 1967; Baumel and Gerchman 1968; West et al. 1981). Only the

afferent fibres from the artificially perfused carotid body were left intact. For this reason, an initial series of experiments was performed to assess the effect of unilateral vagotomy on the cardiac response to diving by comparing the response before and up to 1 or 2 days after nerve section.

Surgical preparations and experimental protocol

Six ducks were used in this experiment. Heart rate was determined from the electrocardiogram (ECG) obtained from subcutaneous electrodes placed on each side of the chest. Each animal was lightly restrained in a supine position with its head positioned in the wide mouth of a large plastic funnel. For diving, the funnel was filled with sufficient tap water (10 - 14°C) to completely submerge the head. Termination of the dive was achieved after 2 minutes by draining the funnel through the spout.

Following dives with intact animals, the right vagus was exposed under local anaesthesia high in the neck and a short segment of the nerve cooled with a thermode. The nerve was then cut just distal to the cooled section and the incision was promptly closed with sutures and the animal allowed to recover. Two-minute test dives were carried out later that day or within the next day or two.

Effect of Artificial Perfusion of Carotid Body Chemoreceptors

Surgical preparations

The cardiovascular area of the carotid body was exposed through a ventral incision in the skin and through the wall of the interclavicular air sac. To prepare for carotid body perfusion all arteries in the region of the carotid body on the left side were ligated except for the common carotid artery. The thyroid artery was ligated distal to the thyroid gland on the dorsal suface of the cervical air sac. This air sac was punctured to allow access to the vertebral artery and other arteries in the region dorsal to the carotid body. These arteries, usually two in number, were ligated close to their junction with the common carotid artery. The common carotid artery was freed from connective tissue on both sides of the carotid body region. A pneumatic or "snare-type" blood vessel occluder was placed around this artery proximal to the carotid body (Figure 2), while the artery on the distal side was wrapped in rubber dental dam to isolate it from tissue regrowth and to aid identification for cannulation on the day of the experiment. In some animals a stainless steel snare was placed around the left baroreceptor nerve to provide a convenient means of nerve section on the day of the experiment. The cervical air sac and the overlying tissue and the skin was sutured closed.

The right vagus was exposed, high in the neck, and either sectioned or freed from surrounding tissues and wrapped around with dental dam. In the latter case the vagus was re-exposed on

Figure 2.   Schematic diagram illustrating the experimental
      arrangement for artificial perfusion of one carotid
      body.   Left vagus nerve intact; left carotid body
      perfused from infusion syringe.   Right vagus nerve
      sectioned; right carotid artery intact.

gas flow

right vagus
sectioned

41° C heating
coil

carotid artery

carotid body

infusion
syringe

pneumatic
occluder
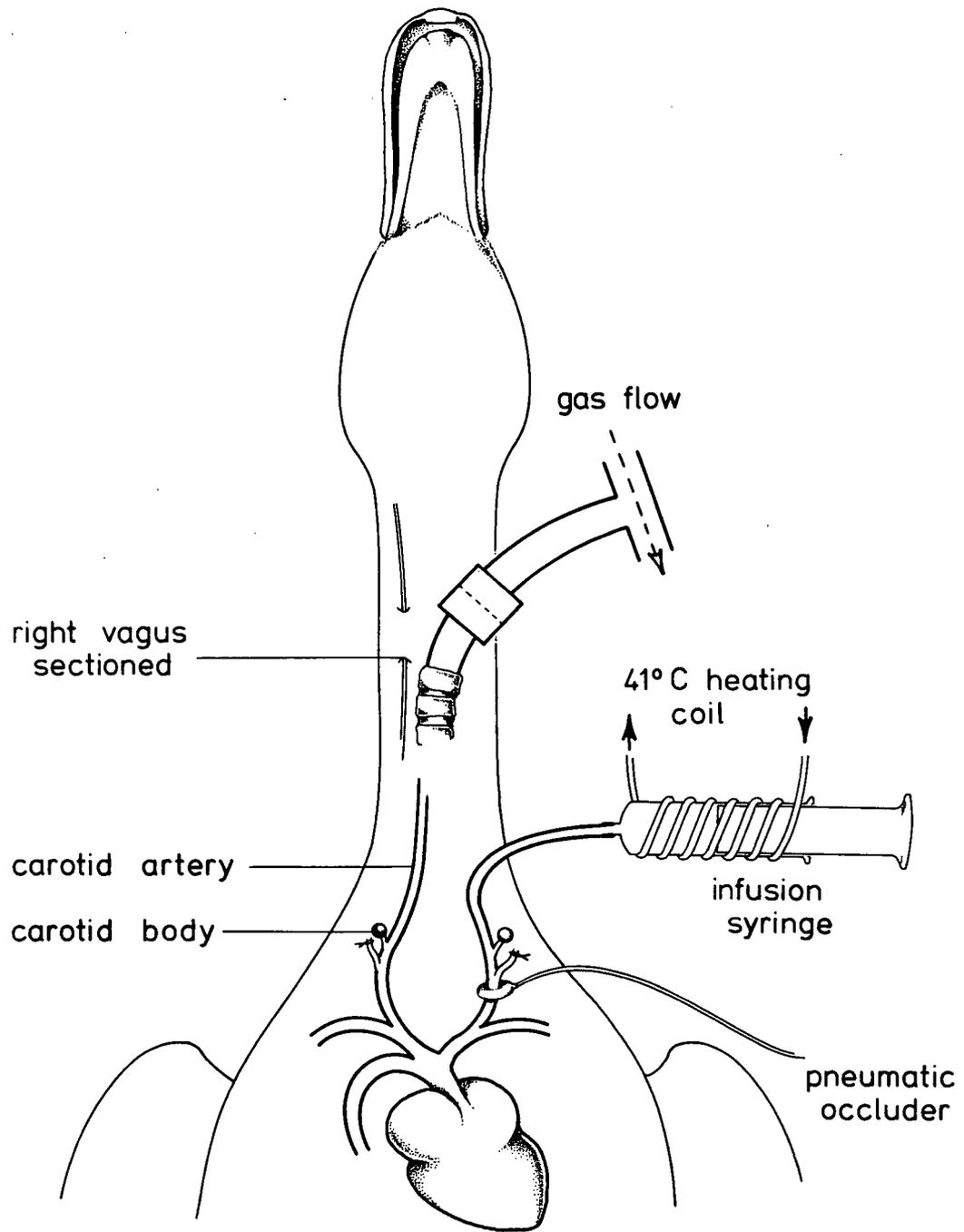
the day of the experiment, under local anaesthesia, and then cooled, as described above, before sectioning just distal to this region. This type of unilateral vagal section denervates the carotid body and baroreceptors, on that side, in ducks. One ischiatic artery was exposed in the leg, for future blood pressure recordings on the day of the experiment, and wrapped around with dental dam. The skin over all incisions was closed using stainless steel wound clips.

On the day of the experiment, the ischiatic artery was carefully re-exposed, under local anaesthesia, and cannulated in both upstream and downstream directions with P.E. 240 (Intramedic Polyethylene tubing, Clay Adams) tubing which was pulled out over a flame to reduce its diameter to fit the vessel. The cannulae were inserted into the ends of a 25 cm length of Tygon tubing (i.d. 3mm; Norton, Ohio) which had T-pieces close to each end. The side arms of the T-pieces were connected to blood pressure transducers. A variable flow infusion pump was positioned to act, on the Tygon tubing, between the T-pieces. To inhibit adhesion of blood cells to the walls of the cannulae, all cannulae and tubing were pre-treated with a silicon solution (Prosil, PCR Research Chemicals Inc.).

The anterior tip of the interclavicular air sac was re-opened and the left common carotid artery was cannulated with P.E. 160 tubing. The tip of the cannula was advanced until it was positioned close to the distal end of the thyroid gland. This cannula was connected to a syringe infusion/withdrawal pump (Harvard Apparatus Inc., Millis, Massachusetts. Figure 2). The

pump's syringe was surrounded by a copper heating coil which maintained blood temperature in the syringe at 41°C over a 30 minute test period (Figure 2). One brachial vein was cannulated for injecting adrenaline to test for baroreceptor denervation.

Measurements

Arterial blood pressure was monitored in the ischiatic artery cannula upstream of the pump used for hind limb perfusion while the downstream ischiatic cannula was used to monitor the hind limb perfusion pressure. When the carotid body was perfused, flow was adjusted so that the perfusion pressure, monitored in the carotid artery cannula, was equal to the mean arterial blood pressure. Biotec BT-70 pressure transducers were used for all measurements. Between dives the hind limb was perfused at a flow rate which yielded a perfusion pressure similar to mean arterial pressure (MAP). Just before a dive, flow was reduced and perfusion pressure fell to about 50 mm Hg. This flow was maintained throughout the dive and recovery. In a series of preliminary experiments it was established that at all flows above 9 ml/min, hind limb vascular resistance (HLVR) was independent of flow rate. Below 9 ml/min HLVR rose along with the reduction in flow. Consequently, hind limb flow was always maintained greater than 9 ml/min to avoid passive increases in HLVR being added to the values for HLVR obtained pre-dive. ECG was obtained from subcutaneous electrodes and heart rate (HR) from an instantaneous rate meter triggered by the QRS wave of

the ECG.

The trachea was cannulated in the neck, just rostral to its bifurcation. The distal end of the cannula was attached to a T connection, one arm of which was open to the atmosphere and the other to a gas supply (Figure 2). With a system of gas flow meters the composition of the gas flowing past the end of the cannula could be altered; thus, the composition of the inspiratory gas could be controlled.

Breathing was monitored with a pneumotachograph attached to the tracheal cannula (Hewlett-Packard pneumotach A547. Figure 2). The pressure drop across the pneumotachograph during tracheal air flow from breathing was recorded with a Hewlett-Packard Model 270 differential pressure transducer and the air flow signal was fed through a Hewlett-Packard 350-3700A integrating preamplifier to give tidal volume. The gas composition of the respiratory gases supplied via the T-piece was monitored with a Centronic 200 MGA clinical mass spectrometer.

Arterial blood samples were taken from the animals before diving and after 55 or 90 seconds of diving. The samples were measured using an Instrumentation Laboratories IL micro 13 blood gas analyser which was calibrated with precision gas mixtures before each analysis.

All signals were amplified using conventional means and the blood pressure, tracheal air flow, ECG, and respiratory O_2 and CO_2 compositions were displayed on a Technirite 8-channel thermal pen recorder writing on rectilinear coordinates and

stored on an 8-channel FM tape system for later analysis by computer. The stored data were analysed using a specially prepared computer programme for a Digital PDP Lab 8e computer.

Experimental Protocol

On the day of the experiment, unanaesthetised ducks were lightly restrained on an operating table in the supine position. The head was positioned firmly in the mouth of a funnel and diving was accomplished as described previously. The body temperature of all birds used was continuously monitored during experiments and maintained at 41±1°C by infra-red lamps mounted above the birds.

In normal dives, the carotid body was autoperfused and HR, MAP and HLVR were recorded. After 1 to 1.5 minutes of submersion, a blood sample was taken for analysis. To fill the infusion pump, the dives were extended beyond 1 minute before blood was withdrawn from the carotid artery into the syringe (Figure 2). A sub-sample of this blood was taken for gas analysis. Some time later (5 - 10 minutes), the common carotid artery was occluded on the side with the intact carotid body, proximal to the carotid body, and perfusion from the pump was started. When stimulation of carotid chemoreceptors by this hypoxic, hypercapnic end-dive blood was stable, as judged from the breathing trace, the animal was submerged for 1 to 1.5 minutes. Carotid body perfusion was maintained for 2 minutes into the recovery period after the dive. The perfusion rate was

set to yield a perfusion pressure which was equal to arterial blood pressure. Hyperoxic blood for perfusion was obtained by giving the duck a few breaths of 100% O_2. Blood was then withdrawn to fill the pump reservoir and the animal returned to breathing air for 5 - 10 minutes. The same procedure for perfusion of the carotid bodies with hypoxic-hypercapnic blood during diving was used for perfusion with hyperoxic blood from the pump.

Baroreceptors were denervated by pulling the snare, which cuts through the left depressor nerve (baroreceptors on the right side were previously denervated by vagotomy) and after 30 - 60 minutes recovery the above protocol was repeated. Complete baroreceptor denervation was confirmed by the lack of any cardiac chronotropic response during hypertension evoked by injection of 5 μg/kg adrenaline into the brachial vein.

Cross-Perfusion Between Ducks

To enable investigation of the relative contribution of peripheral and central chemoreceptors, it was necessary to separate the blood flow to these sites. This was achieved by preventing all blood flow to the head except for flow through previously cannulated carotid arteries. The carotid arteries were cannulated in both upstream and downstream directions with connections to the vascular system of a donor animal. By this arrangement peripheral chemoreceptors could be either auto-perfused or cross-perfused with blood from the donor animal.

Surgical Preparations

In one animal, designated hereafter as the recipient, it was necessary to completely disrupt blood flow between the head and the body except for the common carotid arteries and the jugular veins. To accomplish this the jugular veins and the vagi were exposed high in the neck and carefully separated from surrounding tissue. The skin and superficial muscle in this region were infiltrated with local anaesthetic and clamped. A cut was carefully made to completely encircle the neck and the edges were then sewn back together. In this way all flow in these tissues was disrupted. A cord was passed beneath the exposed jugular veins, vagi and trachea, encircling the oesophagus, vertebral column and associated muscles and the ends were led to the surface at the back of the neck. On the day of the experiment this cord could be tightened to occlude any blood flow to the head through these tissues. The common carotid arteries were exposed low in the neck by opening the interclavicular air sac and again, high in the neck, by dividing skin and muscle just behind the articulation of the lower jaw. The common carotid arteries were wrapped in rubber dental dam. The vertebral, cervical and thyroidean arteries were exposed and ligated bilaterally. The ischiatic artery in the leg was also exposed and wrapped with dental dam. The walls of the air sac were carefully sewn back together and the skin sutured over this and all other incisions.

In a second animal, hereafter designated as the donor, one ischiatic artery was exposed in the leg and wrapped with dental

dam. A sealed large bore cannula (1.0 cm i.d.) was inserted and sewn into the interclavicular air sac.

On the day of the experiment, the donor duck was intubated, the interclavicular cannula opened and the animal placed on unidirectional ventilation (UDV). The ischiatic artery was carefully re-exposed, under local anaesthesia, and cannulated in both upstream and downstream directions. In the recipient duck, also under local anaesthesia, the neck was carefully re-opened and the right carotid artery cannulated low and high in the neck with P.E. 240 tubing (Figures 3 and 4). Tygon tubing (i.d. 3 mm) was used to connect the carotid artery of the recipient to the ischiatic artery of the donor duck. The tube indicated as carrying flow from the donor was connected to the upstream ischiatic cannula of the donor (flow from carotid artery of recipient to hind limb of donor. Figures 3 and 4). The ischiatic artery of the recipient was prepared for pump driven perfusion as described above to measure HLVR. All ducks used in cross perfusion experiments had intact vagi and baroreceptor innervation.

Measurements

Arterial blood pressure was monitored in the upstream cannulae of the carotid and ischiatic arteries in the recipient and donor ducks respectively and perfusion pressure was measured in the cross-perfusion cannulae supplying the recipient (using Biotec BT-70 pressure transducers). When perfusion was

Figure 3.   Schematic diagram of the recipient duck in cross-
        perfusion experiments.

carotid artery

from DONOR

pneumotachograph

to DONOR

gas flow

to mass
spectrometer

carotid body

ligature

aorta

38
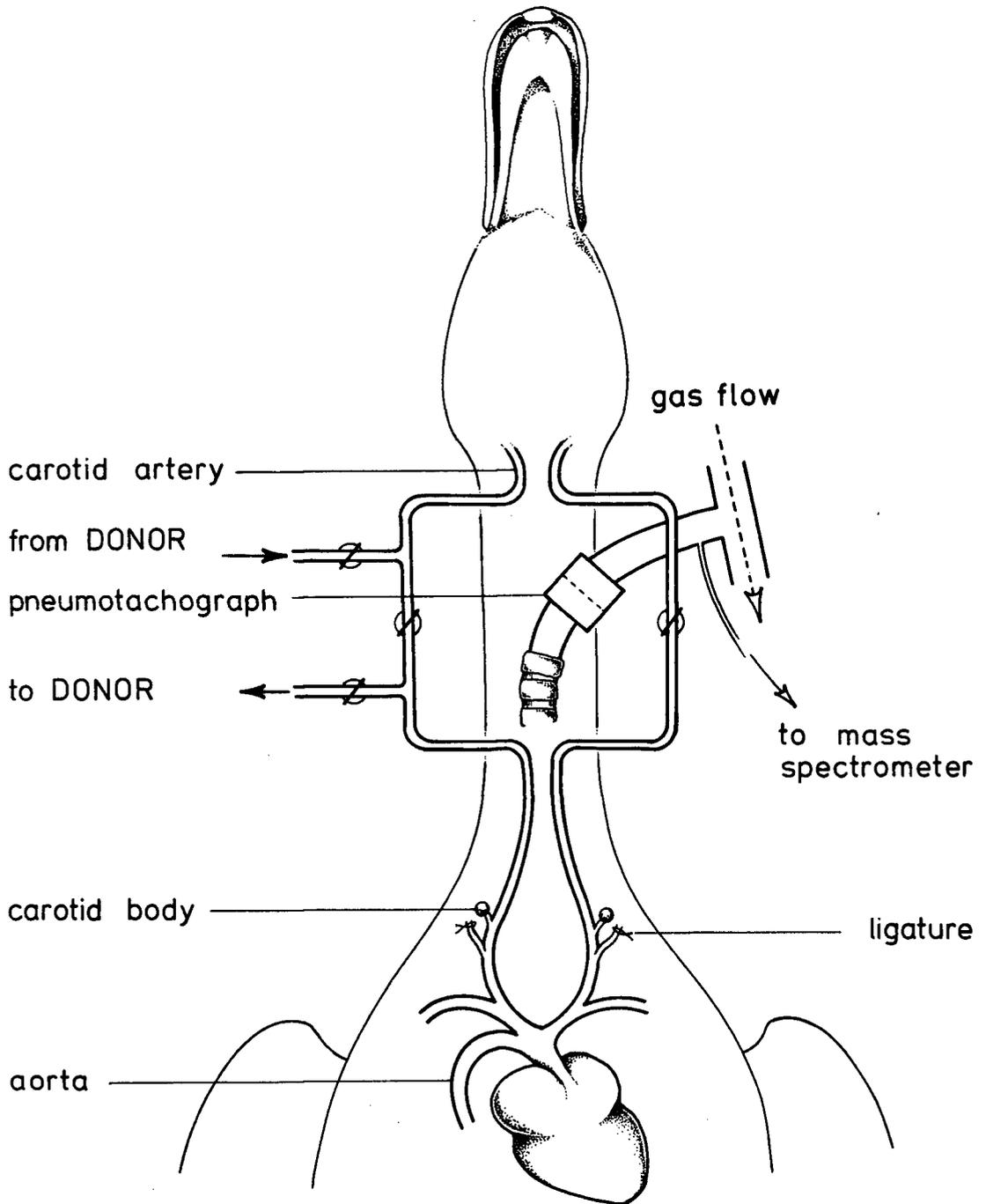
Figure 4.   Schematic diagram illustrating the experimental
        set-up for cross-perfusion between recipient and donor
        ducks.

RECIPIENT

hind limb perfusion
pressure

blood pressure

heart rate

occluder on right
carotid artery

spontaneously breathing
monitored gas
mixtures

DIVE

monitored gas
mixtures (UDV)

DONOR

blood pressure and blood gas
composition monitored
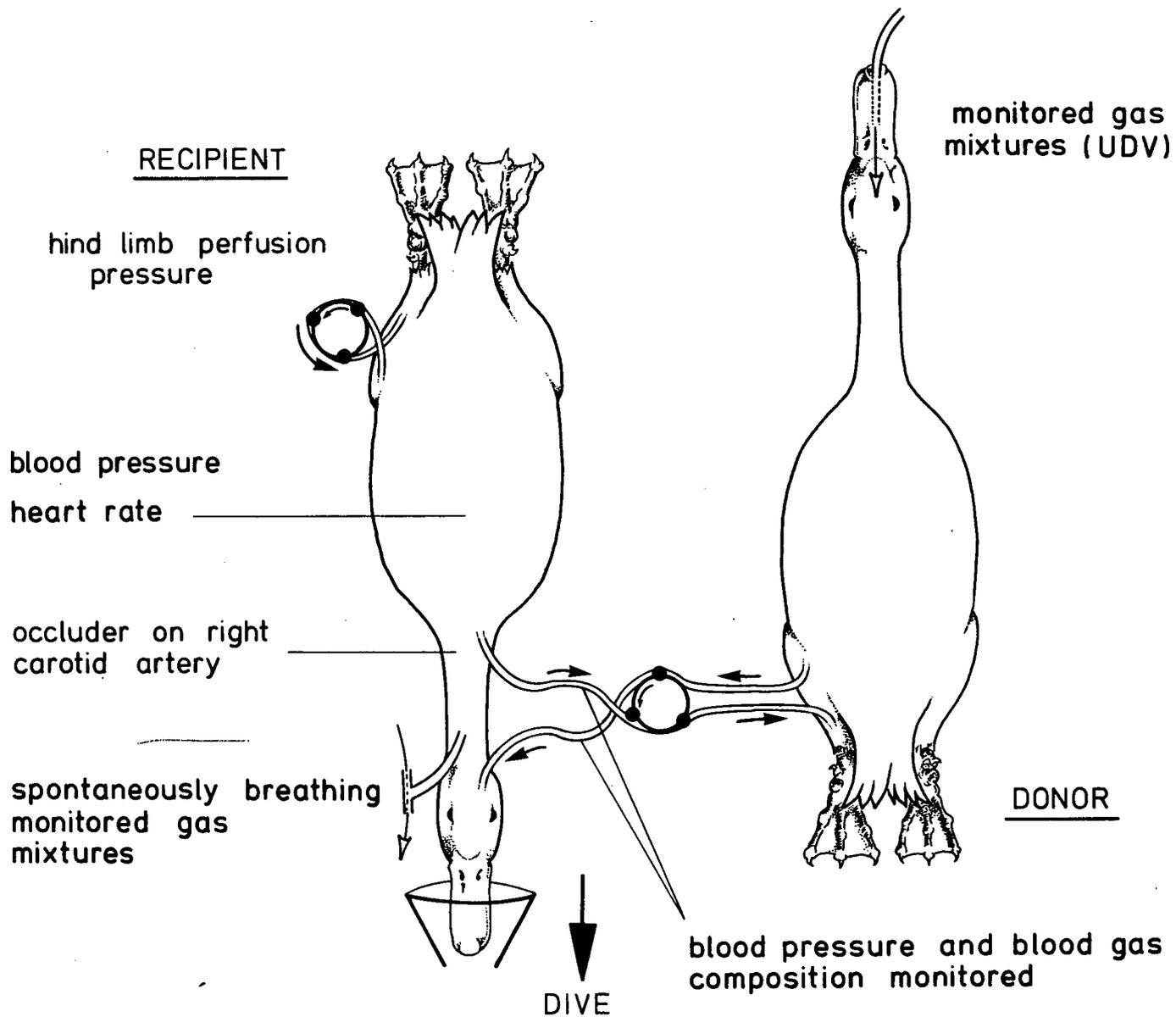
established, flow was adjusted so that the cross-perfusion pressure to the head of the recipient duck matched the carotid arterial blood pressure of the recipient. ECG and hind limb perfusion pressure was measured as in carotid body perfusion experiments.

In the recipient one common carotid artery was intact and carried flow to the head during auto-perfusion. As mentioned before, normal flow was provided to the head despite occlusion of all but one artery (Richards and Sykes 1967; Baumel and Gerchman 1968; West et al. 1981). Soon after cross-perfusion was established this vessel was closed off with haemostatic forceps. The cord previously placed around the vertebral column and associated musculature was tightened, after first carefully infiltrating the area under the ligature with a local anaesthetic. Flow through the cross-perfusion tubes was driven by a Harvard Model 1210 peristaltic pump. Breathing and inspiratory gas composition in the recipient were monitored as in the carotid body perfusion experiments. Composition of the respiratory gases supplied to the donor duck via UDV was monitored with the mass spectrometer.

Arterial blood samples were taken from the animals for analysis before diving and after 55 or 90 seconds of diving as in the carotid body perfusion experiments. It should be noted that blood cross-perfused to the recipient would have been drained back into the systemic circulation by the jugular veins and thus would affect PVO_2 and $PVCO_2$ of the recipient. The extent of this contamination as it affected systemic PaO_2 of the

recipient in dives, was assessed by using an indwelling oxygen electrode in the hind limb perfusion circuit. The decrement in PaO_2 in a dive with cross-perfusion was compared to that occurring when the recipient was auto-perfused. As there was no significant difference between PaO_2 in both types of experiment it was judged that the extent of any contamination was small.

Experimental protocol

The donor duck was placed on unidirectional ventilation. Gas was administered via the trachea and vented via the interclavicular air sac. Changing the gas composition allowed rapid alteration of the oxygen and carbon dioxide content of the arterial blood for cross-perfusion to the recipient duck. The recipient was allowed to breathe spontaneously.

Auto-perfusion represents the normal condition where the recipient animal is in total control of its own blood flow. Under this condition, a series of 2 minute dives was done after the animal had been breathing either air or pure oxygen for at least 5 minutes pre-dive. These dives were done randomly, interspersed with dives in which the head was cross-perfused.

Cross-perfusion was established by occluding the remaining intact common carotid artery after first switching on the perfusion pump. Once cross-perfusion was established, 1000 i.u. Heparin was administered every two hours throughout the remainder of the experiments. Recipient ducks were dived for 2 minutes, either first having breathed air or pure oxygen, while

their heads were perfused with blood from donors which had been ventilated with air, pure oxygen, high oxygen and high CO_2, or high CO_2 and low oxygen gas mixtures. An arterial blood sample was taken from the donor before, during and at the end of a dive to ensure that blood gases remained stable. Carotid arterial blood samples were taken from the recipient before and after 1.5 minutes of the dive, in addition to continuously recording PaO_2 in the hind limb perfusion line. Recipients were allowed at least 30 minutes between dives for full recovery as indicated by HR, HLVR, MAP and arterial blood gas tensions.

At the end of the day's experiments the recipient animal was deeply anaesthetised, placed on cross-perfusion, and then the perfusion pump was turned off. Only if the recipient animal died within 1 minute (as judged by respiratory failure) was cross-perfusion considered to be supplying all of the recipient's cerebral blood flow and the experimental results acceptable.

Statistics and the Analysis of Data

In the text and figures, numerical values, when referring to determinations of variables in a group of animals of number N, are given as means ±S.E. of the mean of n determinations. Only in the carotid body perfusion series of experiments were replicates carried out. Data from the various groups, in each series of experiments, were compared at each sampling time using a one-way analysis of variance (ONEWAY, SPSS; Nie et al. 1975).

In the case of significant F values (P<0.05), paired comparisons of means were done with Scheffe's method (Scheffe 1959).

RESULTS

Effect of Unilateral Vagotomy on Diving Bradycardia

Pre-dive heart rates of Pekin ducks were elevated by section of one (right) vagus but the increase was not significant (P>0.05). However, in two ducks unilateral vagotomy did cause a substantial elevation of the resting rate and completely eliminated diving bradycardia. This phenomenom where one vagus carries all the cardiac chronotropic inhibitory fibres has been observed previously in ducks (Johansen and Reite 1964; Butler and Jones 1968). These ducks were not included in the study. In the remaining ducks unilateral vagotomy diminished diving bradycardia, heart rate being significantly above that in intact ducks after only 15 seconds of submersion (Figure 5). After 2 minutes submergence heart rate had fallen to only 26% of the pre-dive value (60 ±4 beats/min compared to the pre-dive value of 203 ±12 beats/min; n=22; N=4) in unilaterally vagotomised ducks compared with a decline to 17.6% of pre-dive in the control (38 ±4 beats/min, pre-dive 187 ±13 beats/min; n=10; N=4).

Effect of Perfusion of the Carotid Body Chemoreceptors

The mean pre-dive HR in intact ducks with autoperfused carotid bodies was 328 ±28 beats/min. During the first 3 seconds of submergence, HR fell rapidly at a mean rate of -

Figure 5.   Effect of unilateral vagotomy on diving
      bradycardia.   Time= 0 seconds represents the start of a
      2 minute dive.   Values for the intact animal are
      represented by closed circles (n=22), those for
      vagotomised animals by open circles (n=10).(bars ±S.E.)
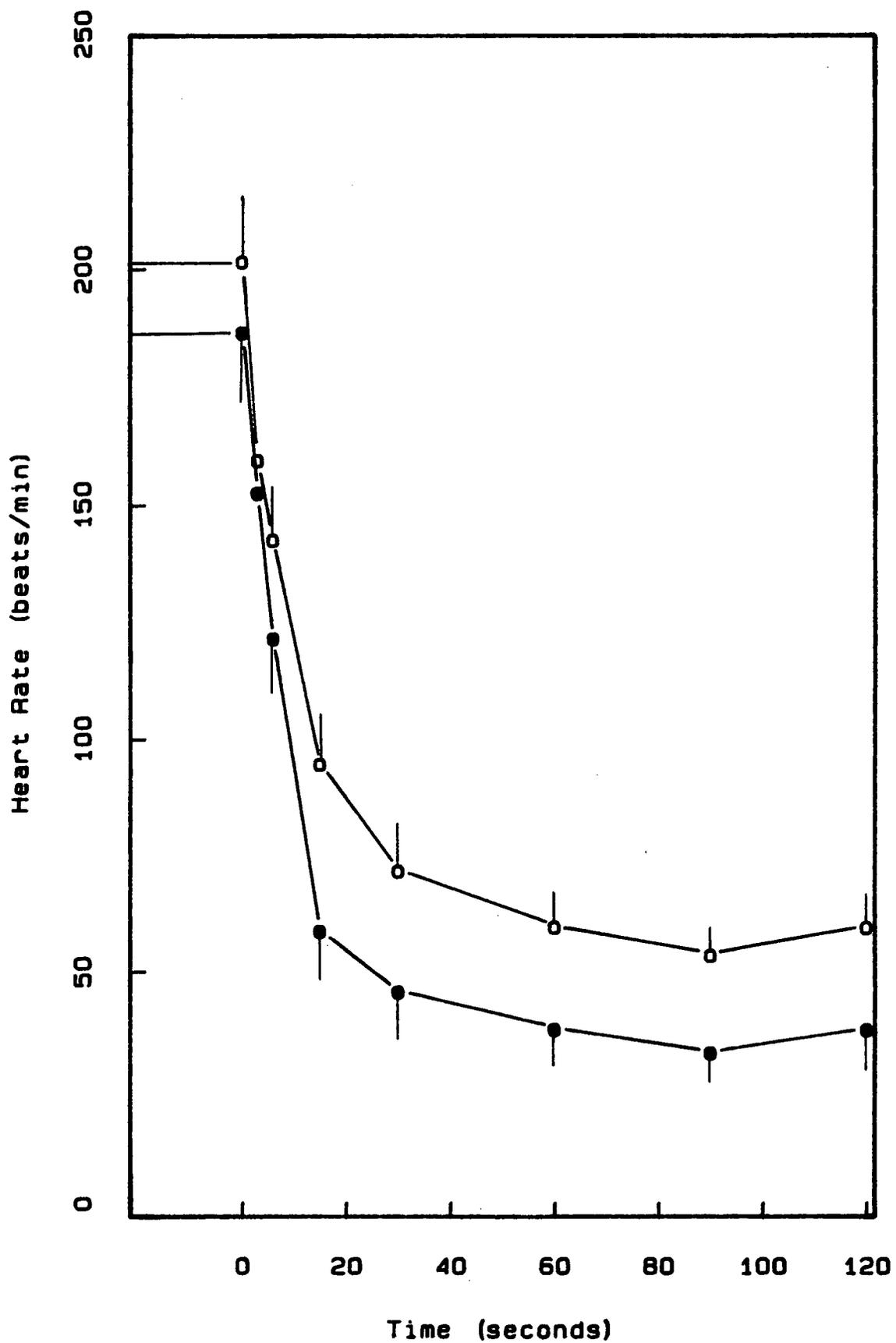
24.7±6 beats/s. The rate of fall then declined from -10.9 ±2.5 beats/s in the period from 3 to 6s, to -1.53 beats/s for the period from 0.5 to 1.0 minute, reaching a level of 91.3 ±10 beats/min (Table 1). HR was not yet stable, continuing to decline to 65±7 beats/min after 1.5 minutes underwater. HLVR rose slowly throughout the dive increasing significantly from the pre-dive value after 1 minute of submergence. MAP also rose during a dive but at no time was the increase significantly above the pre-dive value (Table 1).

Perfusion of the one intact carotid body with hyperoxic blood (PaO_2 = 272 mm Hg; $PaCO_2$ = 27.2 mm Hg; pHa = 7.67) significantly reduced both diving bradycardia ($P< 0.05$) and the increase in HLVR ($P< 0.05$) after a 1 minute dive, compared with an autoperfused dive (Table 1). In fact in these dives, although HR declined significantly from the pre-dive value there was no significant increase in HLVR. There was little effect of carotid body perfusion on cardiovascular variables in breathing ducks (Table 1).

Perfusing carotid bodies with hypoxic blood (PaO_2 = 35.4 mm Hg; $PaCO_2$ = 44.4 mm Hg; pHa = 7.36) throughout 1 minute dives gave mean values for HR and HLVR intermediate between those obtained from auto-perfused and from perfusion of carotid bodies with hyperoxic blood (Table 1). After 1 minute underwater HR was significantly below that in ducks in which the carotid body was perfused with hyperoxic blood but not significantly different from values in ducks with auto-perfused carotid bodies (Table 1). HR fell rapidly in the first 3 seconds of the dive

Table I.   Effect of Carotid Body Stimulation and Withdrawal
        on Cardiovascular Responses to Submergence in intact and
        Barodenervated Ducks.
        Values are means ±S.E..   HR denotes heart rate
        (beats/min); MAP denotes mean arterial pressure (mm Hg);
        HLVR denotes hind limb vascular resistance (PRU's);
        N=animals, n=total dives.

Table I. Effect of Carotid Body Stimulation and Withdrawal on
Cardiovascular Responses to Submergence in intact
and Barodenervated Ducks

		Surface	Dive	
			30 s	60 s
Intact Duck, Normal Dive N=8 n=15	HR	328.0±27.7	132.9±11.6	91.3±10.2
	MAP	142.9±7.6	165.1±5.3	168.3±9.8
	HLVR	8.93±1.81	10.01±1.66	16.71±2.37
Baroreceptors Denervated Normal Dive N=5 n=10	HR	383.0±22.1	247.0±25.1	137.8±15.8
	MAP	167.8±19.2	206.5±13.8	205.4±16.7
	HLVR	7.39±1.03	13.19±2.53	18.44±4.41
Intact Duck. Hypoxic-Hypercapnic Perfusion N=7 n=10	HR	313.0±23.2	150.0±15.6	126.0±13.7
	MAP	144.8±6.7	153.1±3.9	146.8±6.7
	HLVR	8.30±1.18	10.27±1.18	12.72±3.00
Baroreceptors Denervated Hypoxic-Hypercapnic Perfusion N=5 n=8	HR	408.8±21.3	227.5±25.6	197.5±22.5
	MAP	194.0±19.2	211.7±16.8	207.6±9.4
	HLVR	8.99±1.33	13.01±1.43	16.26±3.55
Intact Duck, Hyperoxic Perfusion N=7 n=12	HR	330.8±25.0	220.8±2.1	179.2±14.3
	MAP	139.1±9.4	144.9±7.2	158.6±8.4
	HLVR	6.88±1.05	8.14±1.11	10.75±2.17
Baroreceptors Denervated Hyperoxic Perfusion N=4 n=5	HR	400.0±10.9	297.6±23.7	280.0±33.5
	MAP	153.3±8.5	157.5±23.7	160.8±14.3
	HLVR	7.30±1.99	10.23±2.30	12.60±4.99

at a rate of -19.8±2.5 beats/s which persisted for the next 3 seconds of the dive unlike the situation in auto-perfused or hyperoxic blood perfused dives in which the rate of fall in HR was usually halved from the initial 3 second period. Before the dive, perfusion of the carotid body with hypoxic blood had little effect on cardiovascular variables in spite of the fact that minute ventilation increased by at least 2 times. HLVR during the dive did not increase significantly from pre-dive values although it increased sufficiently so that HLVR was not significantly different from that in auto-perfused ducks (Table 1).

Denervation of systemic arterial baroreceptors generally caused increases in HR, MAP and HLVR before and during diving when the carotid body was auto-perfused or when one intact carotid body was perfused with hypoxic or hyperoxic blood. However, in no cases were the increases significant before diving (Table 1). After 0.5 minute underwater all groups of baro-denervated ducks had HR significantly above those in intact auto-perfused ducks. MAP in denervates was also significantly above that in their respective controls except in the case of perfusion of the carotid body with hyperoxic blood (Table 1). Nevertheless, after 1 minute, the only significant difference in HR existing between a baro-denervate and its control group was for hyperoxic blood perfusion, while for MAP the only significant difference existed in the hypoxic blood perfused group (Table 1). HLVR in baro-denervates, before submergence, was above that in their respective control group except in auto-

perfused animals. However, after 1 minute underwater, HLVR in all denervated animals was above that in controls although in no cases were the differences significant. Denervation had no significant effect on blood gas tensions or pHa, before or during diving, in auto-perfused ducks. Expressing HR or HLVR in dives as a proportion of the pre-dive rate confirmed the trends noted above. In all three groups, the proportionate fall in HR was reduced while the proportionate rise in HLVR increased.

Effect of Changing Blood Gas Tension in the Cerebral Circulation

The early period in all dives in which both ducks breathed air or 100% oxygen pre-dive and the recipient's head was either auto- or cross-perfused were characterised by a very rapid fall in heart rate. The fall was particularly noticeable when pre-dive HR was in excess of 250 beats/min. Pre-dive HR (y) was strongly correlated to the change in HR (x) which occurred in the first 12 seconds of the dive (r=0.82). The regression equation was y = mx - 188. In other words, if the pre-dive HR was around 188 beats/min there was no change in the HR early in the dive. There was no correlation between pre-dive HLVR and any change in HLVR that occurred in the first 12 seconds of the dive. In fact, the same mean value of HLVR (n=112) was obtained after 12 seconds of submergence as existed before the dive and yet, MAP rose significantly by, on average, 8% of pre-dive.

The aim of these experiments was to isolate and identify the effects of hypoxia and hypercapnia at the central and

peripheral chemoreceptors, alone or together. This was not achieved since all combinations of auto- and cross-perfusion subjected the peripheral chemoreceptors to hypercapnia. Eleven different combinations of gas tensions were applied to central and peripheral receptors but, based on measurements of blood gas tensions perfusing the receptors, it was decided to merge some of these combinations, along with the data from autoperfused ducks breathing air or oxygen pre-dive, to give 5 experimental groups (Table 2).

During dives in which the head was auto-perfused both peripheral and central receptor sites were exposed to high $PaCO_2$ in combination with low PaO_2. In these dives the greatest bradycardia and largest increase in HLVR developed (Table 2). HR fell rapidly from the pre-dive rate of 342±22 beats/min to stabilise after 1 minute underwater at 43±4 beats/min. MAP fell significantly by 22 mm Hg and HLVR increased significantly by 3 times. Continuously measured PaO_2 showed the features noted previously by Jones and Purves (1970); PaO_2 fell rapidly after an initial latent period of 5 - 10 seconds but once bradycardia was fully developed, only fell slowly (<5 mm Hg/min) for the rest of the dive. In one group of cross-perfusion experiments both receptor sites were stimulated with similar blood gas tensions, as in diving auto-perfused ducks, by allowing the recipient to breath air before the dive while cross-perfusing the head with low PaO_2 and high $PaCO_2$. There was no significant difference between the cardiovascular variables monitored in the cross-perfused and auto-perfused animals during dives, so the

Table II.   Effect of Change in Blood Gas Tensions at Carotid
        Body and Central Chemoreceptors on Cardiovascular
        Responses to Forced Diving.
        Values are means ±S.E..   Animals were divided into 5
        groups according to which receptors were stimulated (*)
        during the dive.   In all groups the carotid bodies were
        autoperfused.   In group 1 the head was either auto- or
        cross-perfused; in all other groups the head was cross-
        perfused.   Abbreviations are the same as in Table 1.

Table II. Effect of Change in Blood Gas Tensions at Carotid Body and Central Chemoreceptors on Cardiovascular Responses to Forced Diving

|		Group				
		1	2	3	4	5
Pre-Dive	HR	304.7±25.7	371.1±38.0	338.1±20.5	356.0±64.9	255.6±25.1
	MAP	131.1±4.1	130.7±7.7	122.3±5.0	133.8±11.8	119.3±11.2
	HLVR	4.49±0.45	3.76±0.53	4.65±0.40	3.94±0.76	5.03±0.68
Dive,30s	HR	94.0±16.1	92.2±10.4	207.4±16.8	256.0±59.5	178.9±59.5
	MAP	129.9±5.9	127.3±7.7	135.6±6.3	136.2±13.4	126.4±12.1
	HLVR	8.47±0.82	7.54±0.87	6.96±0.70	4.64±0.77	6.76±0.94
Dive,60s	HR	41.3±8.6	65.6±6.0	170.6±14.9	220.0±49.7	151.2±20.6
	MAP	114.5±5.5	110.0±8.1	131.4±6.4	133.4±13.0	127.3±10.6
	HLVR	13.09±1.31	9.82±0.76	7.79±0.73	5.22±0.69	6.55±0.69
Dive,90s	HR	43.3±7.9	53.3±6.5	157.1±15.0	214.0±53.8	150.0±17.4
	MAP	109.6±5.8	96.5±5.2	124.9±6.1	124.4±10.6	122.9±11.8
	HLVR	13.29±1.13	11.63±0.86	8.62±0.74	6.38±1.68	6.32±0.65
Dive,120s	HR	52.0±9.2	70.0±6.8	161.3±15.6	180.0±42.4	157.7±13.6
	MAP	111.6±5.9	93.4±6.7	126.0±5.6	119.8±10.1	121.6±11.7
	HLVR	13.40±1.17	9.76±1.07	8.32±0.80	5.75±1.47	6.03±0.53
n		15	8-9	28-31	4-5	9
Low O2 Peripherally		✱	✱			
High CO2 Peripherally		✱	✱	✱	✱	✱
Low O2 Centrally		✱			✱	
High CO2 Centrally		✱		✱		

groups were combined to form Group 1 in Table 2. Consequently, these experiments confirmed that the cross-perfusion per se had no effect on the cardiovascular responses to diving. Furthermore, since diving heart rate in auto-perfused ducks was not significantly different from that in intact ducks (described in the previous experiment), the extensive surgical procedure associated with cross-perfusion experiments had no effect on the diving response.

Although the pre-dive values were not significantly different from each other in Groups 1 to 5, after only 0.5 minutes of submergence, HR of Group 1 ducks was significantly below the HR's of Groups 3 to 5. After 1 minute, HR and HLVR of Group 1 were significantly below and above, respectively, all other groups except Group 2. These significant differences were maintained for the rest of the dive. At no time did significant differences exist in MAP among the groups. In group 2 ducks only the peripheral chemoreceptor zone was excited by hypoxic and hypercapnic blood yet there was never any significant difference in HR and HLVR during dives by Groups 1 or 2 ducks (Table 2). On the other hand, HLVR in Group 2 ducks was significantly above that in Group 5 ducks after 1 minute of submergence. In Group 5 animals, peripheral chemoreceptors were excited by hypercapnic blood alone (Table 2; Figure 6).

Even though no significant differences existed among groups 3, 4 and 5 in terms of HR and HLVR before and during diving (Table 2), normalisation of the data by comparing the changes in HR and HLVR in dives revealed one interesting difference between

Figure 6.   Effects of varying $PaCO_2$ at central chemoreceptors
in the recipient duck during 2 minutes submersion.
Peripheral chemoreceptors were subjected to progressive
hypercapnia in both A and B.   A: donor breathing high
O_2 and low CO_2 gas.   B: donor breathing high O_2 and
high CO_2 gas.

A. DONOR breathing hyperoxic-hypocapnic gas
RECIPIENT breathing oxygen
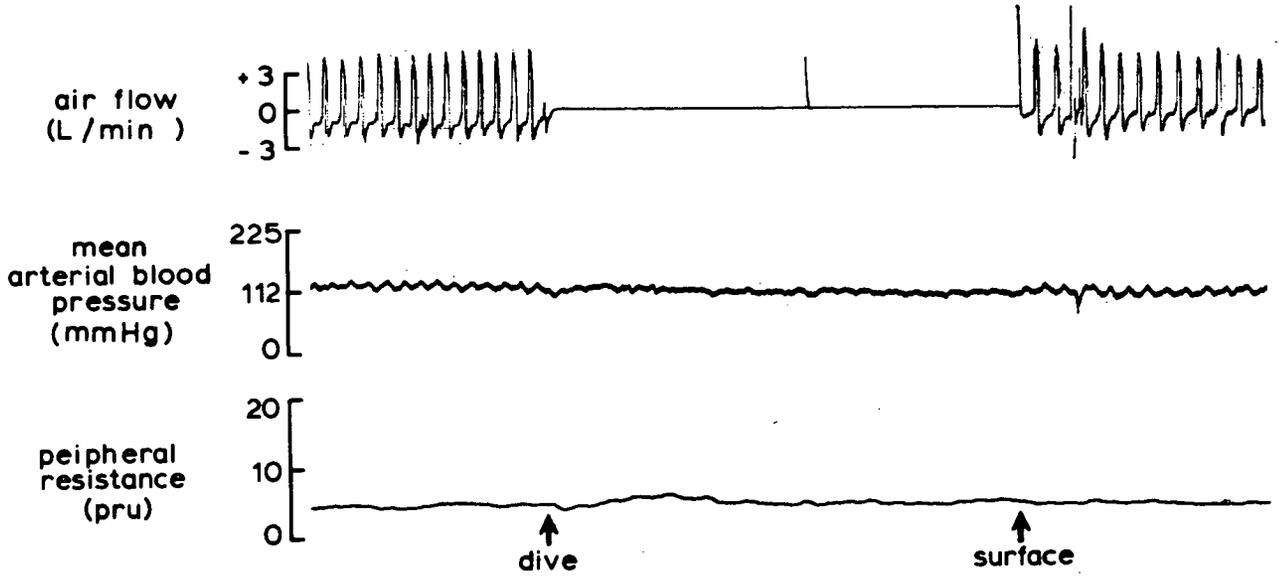
B. DONOR breathing hyperoxic - hypercapnic gas
RECIPIENT breathing oxygen
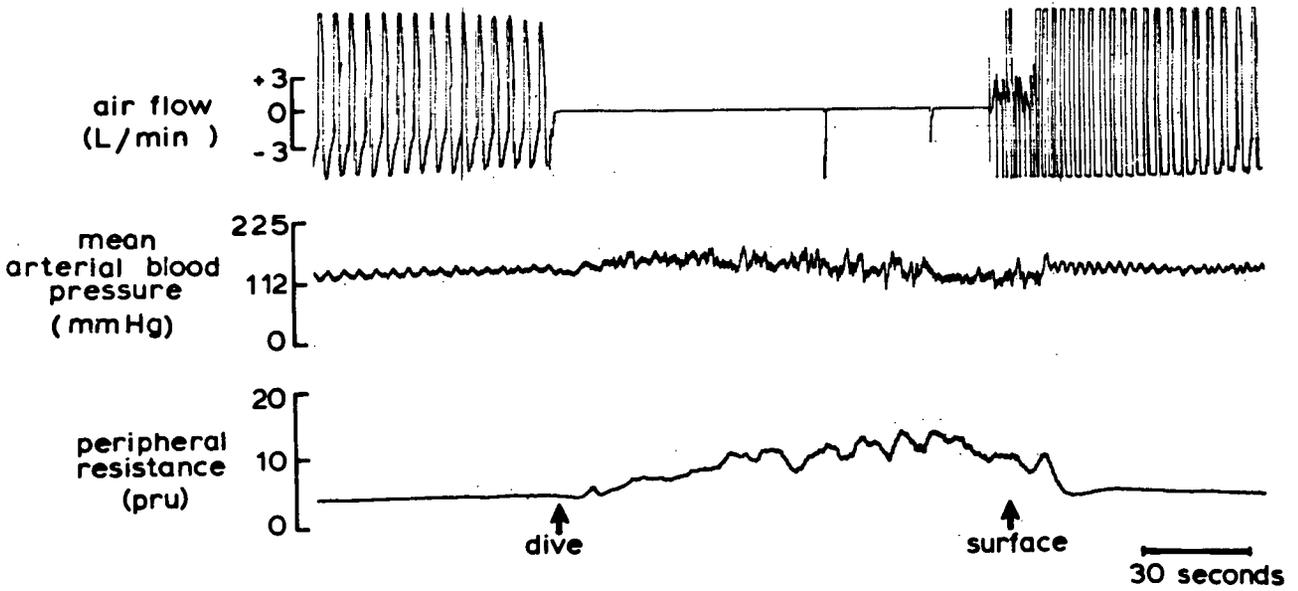
Groups 3 and 5. The increase in HLVR when both central and peripheral chemoreceptors were stimulated with high $PaCO_2$ (Figure 7a and b) was significantly greater than when only peripheral receptors were stimulated (Table 2). In a further attempt to look at the effect of chemoreceptors on HR, the difference between mean HR at 12 seconds of submergence (188 beats/min) and HR at other times in the dive was tested. This confirmed that excitation of peripheral chemoreceptors with blood low in oxygen gave the only significant reductions in HR in the later period of the dive.

DISCUSSION

The chemoreceptive nature of the diving response in the Pekin duck has been confirmed in this investigation. Moreover, in addition to the peripheral chemoreceptors, located in the carotid bodies, a central chemoreceptor group with an important role in establishing the full cardiovascular response to submersion has been revealed. The collective response from both these groups essentially accounts for the major part of the cardiovascular changes.

From the performances of animals during submersion before and after baroreceptor denervation it was inferred that these receptors serve to mitigate the responses evoked by submersion. The development of bradycardia and the increase in peripheral resistance occur in spite of their absence and this is in agreement with previous reports indicating their limited role during diving (Kobinger and Oda 1969; Jones 1973; Jones et al. 1983). Though they may not contribute to the cardiovascular changes of the diving response itself, they may retain a significant role in the regulation of MAP during submersion. When cardiac output is greatly reduced in the dive, even small adjustments in output in the face of the increased total peripheral resistance (TPR) will have marked a effect on MAP.

In regard to the cardiac chronotropic response, there are important contributions from receptor groups not yet identified in these experiments. If the pre-dive HR was high it was not unusual for HR to decline as much in the first 12 seconds of submergence as it did in the rest of the 2 minute dive. A rapid

early fall in HR, when pre-dive HR was high, has been noted by others (Feigl and Folkow 1963) and attributed to a "narial-type" reflex, although the sudden withdrawal of a stimulatory interaction between feedback from pulmonary afferents or central respiratory neuron activity and medullary cardiovascular controlling neurons, consequent upon apnoea, could also be involved (Bamford and Jones 1976). What was most interesting, however, was that MAP rose significantly in the first 12 seconds of submergence, yet HLVR did not change. Consequently, either HLVR, as a measure of total peripheral resistance (TPR), lags behind the resistance changes in a substantial number of other vascular beds, or stroke volume must have increased. Although most authors find stroke volume is unchanged during diving in ducks (Folkow et al. 1966; Jones and Holeton 1972), an increase in stroke volume, early in the dive, has been reported by Jones and co-workers (Jones et al. 1979). For this reason, only in the later stages of the dive is the change in HR a good indication of the change in cardiac output. The cardiac chronotropic response from 12 to 120 seconds submergence, in ducks in which both peripheral and central receptor zones were stimulated with both low PaO_2 and high $PaCO_2$, was matched by a similar proportionate increase in HLVR, and MAP was not significantly different from pre-dive MAP throughout the dive (Figure 7,A and B).

From comparisons between the baroreceptor denervated groups and their respective control groups (Table 1) it was deduced that baroreceptors are capable of enhancing submersion

Figure 7.   Assessment of the contribution of central (c) and
      peripheral (p) chemoreceptors to bradycardia and changes
      in hind limb vascular resistance (HLVR) in ducks during
      the period from 12 to 120 seconds of submersion.
      (baros.) denotes the presumed contribution of the
      barostatic reflex.   (PRU's) = peripheral resistance
      units.
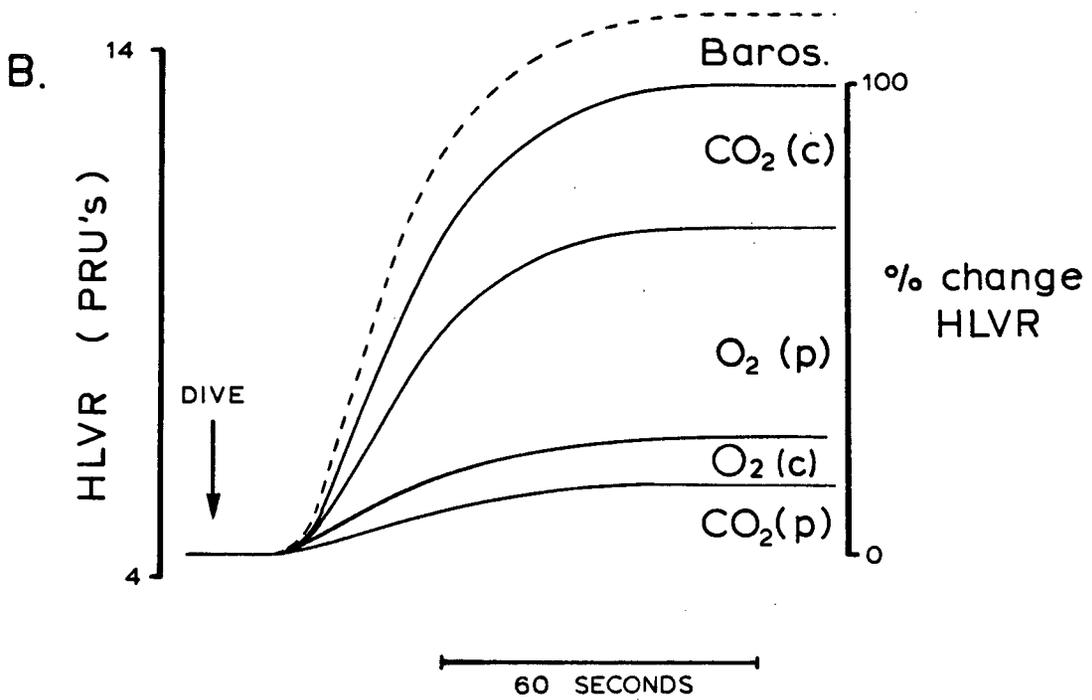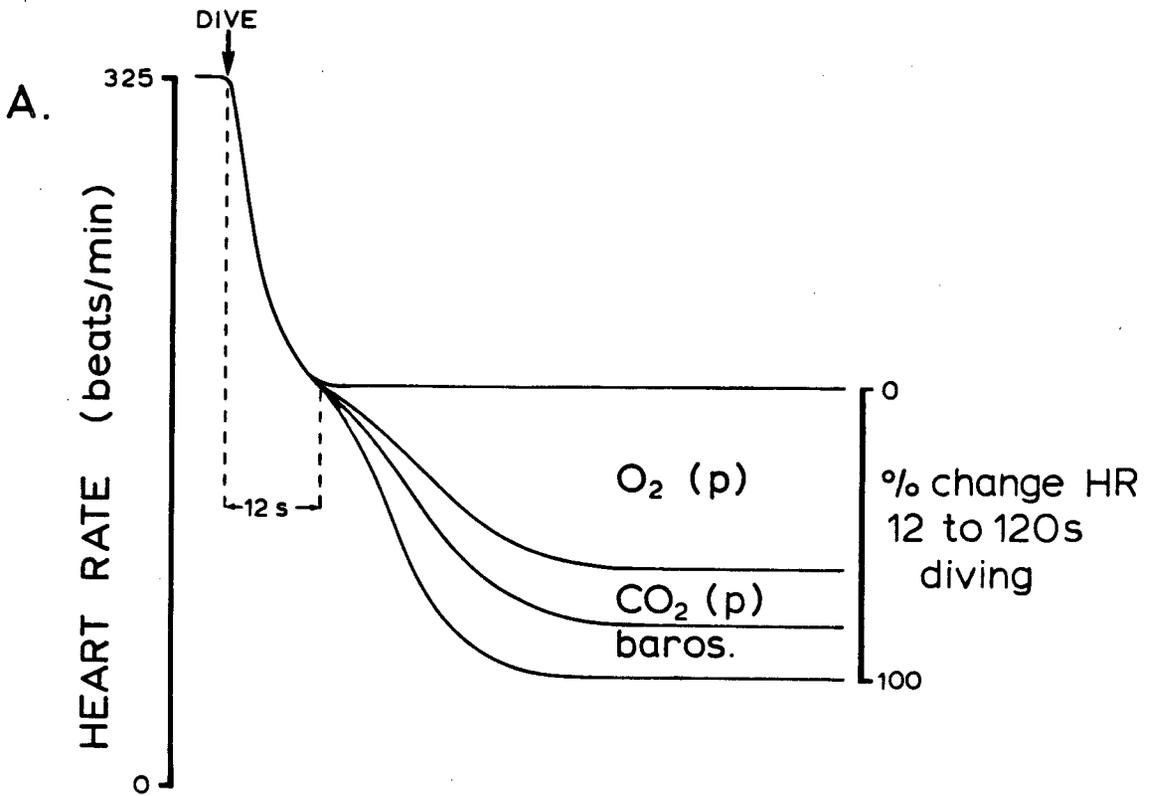
bradycardia by about 12%. Resting HR, MAP and HLVR were all increased following barodenervation, but these changes were not significantly different from the intact condition (P>0.05). After 30 seconds of submersion, HR was significantly above the intact controls (P>0.05) but after 60 seconds the only significant difference remained in the group exposed to hyperoxia during submersion.

The present study shows that a central chemoreceptor zone in the cephalic circulation makes a significant contribution to the increase in HLVR during diving. Although both blood gases prove stimulatory, the rising $PaCO_2$ elicits the more potent effect on resistance changes. It has previously been shown that the change in HLVR in dives reflects the change in TPR (Jones et al. 1983); it seems reasonable to assume, therefore, that central chemoreceptors also contribute to the increase in TPR. Nevertheless, a greater portion of the increase is attributable to the peripheral chemoreceptors.

By 30 seconds after immersion the greatest part of the cardiovascular changes have occurred but the responses continue to develop and stabilise only after 60 seconds of submersion. Consequently, assessments of the relative contributions of receptor groups in response to the stimulatory blood gases were made primarily after this point in the dive.

Cardiac chronotropic responses were identical when only peripheral chemoreceptors were stimulated, and when both central and peripheral chemoreceptors were stimulated (Groups 1 and 2, Table 2). Clearly, peripheral chemoreceptors are mainly

responsible for submersion bradycardia and this is further emphasized by the lack of difference between auto-perfused ducks and Group 2 ducks (Table 2). By comparing responses of animals stimulated only by hypercapnia peripherally and animals stimulated by hypoxic hypercapnic blood at both sites it is possible to discern the changes due to the peripheral action of $PaCO_2$. Similar comparisons between other groups representing four permutations of receptor stimulation evince the action of individual stimulus-receptor pairing. These estimates were assessed with due consideration of the baroreceptor contribution and are summarised in Figure 7A.

In ducks in which both peripheral and central receptor sites were stimulated simultaneously with high $PaCO_2$ and low PaO_2, there was a change in HLVR, from pre-dive to end-dive of some nine peripheral resistance units (PRU)(Table 2). From data in Table 2 the changes in HLVR apportioned among the receptors and stimulatory blood gases are represented in Figure 7B. The greatest contribution to the total change was attributed to the stimulation of peripheral receptors by blood low in oxygen. Together with an estimated 11% of the change due to the peripheral action of hypercapnia, the peripheral receptor group is responsible for two-thirds (67%) of the total change. A little less than a third of the change could be attributed to the stimulation of central receptors by hypercapnia while the remaining 9% or so is caused by the central action of hypoxia. Carotid body perfusion in barodenervated ducks caused HLVR to increase much more than in ducks with an intact barostatic

reflex (Table 1). This is unlike the situation in chronic barodenervates (Jones 1973; Jones et al. 1982) in which HLVR does not rise anywhere near as much as in intact ducks. The portion of change due to baroreceptors in each group was taken into account and is represented in Figure 7B as the overall increase in HLVR which would occur in their absence.

When brought into the laboratory without any preparative operations, ducks have HR's usually in the range of 150 to 200 beats/min, which is below the level where non-chemoreceptor inputs contribute to bradycardia. When these animals dive, 85 to 88% of the HR and HLVR responses will be caused by the stimulation of central and peripheral chemoreceptors by the advancing hypoxic hypercapnia. Hence, diving bradycardia in ducks is not an expression of a barostatic reflex set in train by an incipient rise in MAP caused by a chemoreceptor-driven increase in TPR (Andersen and Blix 1974; Blix el al. 1974). Instead, baroreceptors serve to modulate the cardiovascular responses provoked by submersion and may help to regulate MAP via the barostatic reflex.

These results have confirmed the importance of chemoreceptors in activating the cardiovascular responses to forced submersion. However, as was reported by Irving, Scholander and Grinnel (1941) for seals and later by Folkow, Nilsson and Yonce (1967) for ducks, animals may be able to exert a considerable degree of influence over the full development of these responses. In the following section, an attempt is made to assess the scope of this control.

SECTION 2.

The Effect of a Simple Form of Learning on the Diving Response

Introduction

The purely reflexogenic aspects of the response to submersion can be readily examined in the laboratory. However, interpretation of the data must include the influence of higher levels of the CNS. The most evanescent interference, according to the literature, arises from the animal's state of arousal when restrained. It appears that the animal's level of CNS arousal is capable of either maximising the development of the diving response or completely abolishing some aspects of it (Irving et al. 1941; Folkow et al. 1967). The discrepancy between animals forcibly submerged in the laboratory and animals voluntarily diving in the wild, as well as the variability between successive voluntary dives may be accounted for by this CNS arousal factor (Jones et al. 1973; Butler and Jones 1982; Blix 1984; Kanwisher and Gabrielsen 1984).

Simple forms of vertebrate learning have recently been considered as crucial influences on higher nervous centres controlling cardiovascular function (Galosy et al. 1981; Cohen and Randall 1984; Engel and Schneiderman 1984). With regard to diving, it has been demonstrated in seals that both the rate and degree of bradycardia can be increased by classical and operant conditioning (Ridgway et al. 1975) and this may account for anticipation or enhancement of some of the responses.

The CNS attenuation of the diving response has, however, never been demonstrated. Habituation, or the relatively permanent waning of responses, has been described as one of the simplest forms of learning (Thorpe 1956) and it has been proposed that habituation may play an important role in the general adaptation of an organism to its environment, especially in innocuous or unimportant situations (Glaser 1966). Studies of habituation have dealt primarily with defence-related responses ranging from the gill withdrawal reflex of the invertebrate, Aplysia (Kandel 1976), to complex behaviour such as the mobbing responses of chaffinches to predators (Hinde 1970). However, several simple reflexes, such as hind limb flexion in the cat have also been examined (Thompson and Spencer 1966).

Various cardiovascular responses have been investigated and found amenable to habituation. These have included the heart rate response to noise (Raskin et al. 1969) or sudden change in temperature (Glaser and Griffin 1968); and blood pressure responses in man, such as the "cold pressor response" (Krebbel and Zbrozyna 1982; Zbrozyna 1982). Although habituation appears to be a ubiquitous phenomenon, certain responses appear resistant to habituation. For instance, Furedy (1969,1971) was unable to observe habituation of the digital vasomotor component of the human orienting reflex.

Long term habituation of the defence reaction in the cat has been studied and independence of the response components was also demonstrated. It was discovered that the vasodilatory

response habituated more readily than the behavioural components, such as posture (Sutherland and Zbrozyna 1974; Martin et al. 1976). Renal vasoconstriction evoked in dogs by noise and in baboons by fear has also been shown to habituate (Zbrozyna 1976; Seal and Zbrozyna 1978). In addition, marked variability in the habituation of these responses was noted in both these studies.

To date, habituation of the responses to forced submersion has not been adequately investigated, although a study was carried out by Rey (1971) in which ducklings were dived repeatedly for many trials without observing any change in the diving bradycardia. This study was not undertaken to directly examine habituation, but the protocol could have resulted in response decrement. The present study was designed to examine whether the cardiac chronotropic response to submersion would habituate and, if so, to characterise some of the physiological features. Although dabbling ducks were the main subjects of this investigation, a parallel set of experiments was conducted to establish if habituation would occur in a species of "true" diving ducks (Aythya americana) known to possess rapid onset of bradycardia: in other words, ducks that respond to immersion per se with an immediate change in heart rate.

METHODS

Fifteen adult domestic Pekin ducks (<u>Anas</u> <u>platyrhynchos</u>; 8 female, 7 male) were used in the first four sets of experiments, and three adult Redheads (<u>Aythya</u> <u>americana</u>; 2 female, 1 male) were used in the last set of experiments.

Five sets of experiments were carried out:

a) Habituation of the Cardiac Response to Diving;

b) Effect of Habituation on Blood Variables;

c) i. Effect of Breathing Pure Oxygen on Diving Bradycardia;

   ii. Effect of Hypoxia on the Habituated Response;

d) Effect of Habituation on the Oxygen Breathing Test

e) Habituation of the Cardiac Response in "True" Diving Ducks

General Protocol

The animal's body was secured firmly, without restriction of breathing movements, to a padded platform. The head was positioned in a padded brace allowing no movement. In order to prevent cranial oedema, the platform was inclined at 25° to keep the animal's head level with the rest of the body (Figure 8). The beak was kept partially open by means of a 2 cm section of soft large bore tubing taped in position to allow water to drain completely out of the mouth. The entire apparatus was situated within a light-tight chamber which prevented the animal from seeing the trainer.(Figure 9) The diving bucket was operated

remotely by a lever. Raising the bucket immersed the duck's head in water to a level above its eyes; at the end of the dive the bucket was lowered. A large diameter hose was attached to a drain in the bottom of the bucket so that it could be emptied and filled without disturbing the animal. Sham dives were performed by raising and lowering an empty bucket.

Heart rate was determined from the electrocardiogram (ECG) which was obtained from three wire electrodes: one inserted subcutaneously in the abdominal wall adjacent to the left leg; a second inserted subcutaneously in the right side of the chest; and a third "grounding" electrode attached to the web of the right foot by means of an alligator clip. These ECG leads were amalgamated into a cable which led out of the chamber through a small opening in the wall.

A) Habituation of the Cardiac Response to Diving

The daily training schedule consisted of 15 trials of 40-second head immersions with 5 to 6 minute intervals between immersions. Each session was preceded by a 20 to 30 minute quiet period to allow the animal to settle down.

In the case of two animals (D-1, D-2), the immersion time for the first 9 days of training was 60 seconds, after which the usual 40 second trials commenced. In the case of one animal (J-22) an initial 7-day training schedule of 40 second trials was followed by a further 3 days of 60-second trials.

ECG was recorded on a Physiograph Six chart recorder (E & M

Figure 8. Diagram illustrating the body position of the duck during training.

water level

diving bucket

padded platform

25°
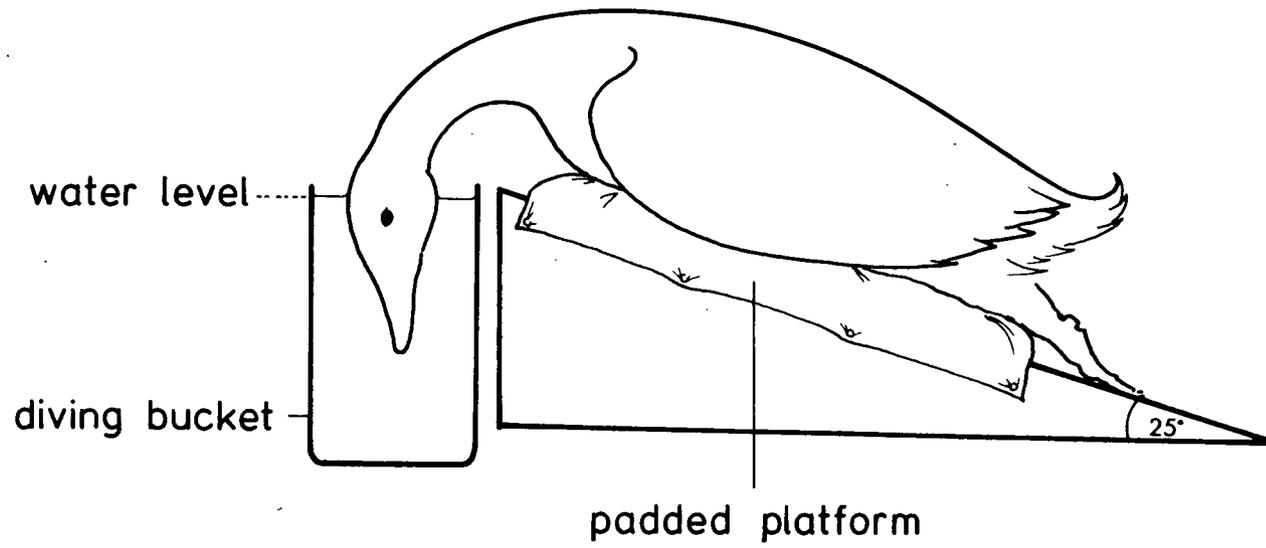
Figure 9.   Diagram illustrating the remote diving system used
        for training.
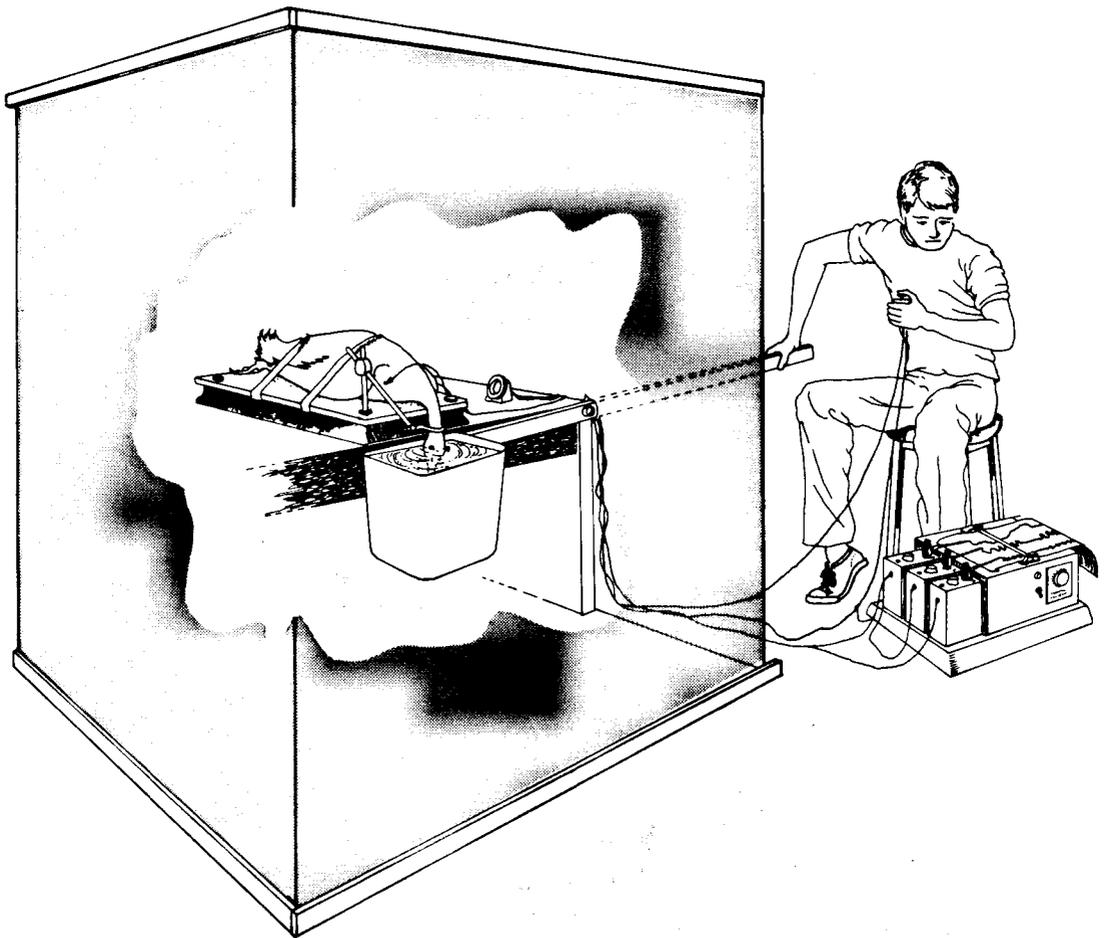
Instrument Co., Houston, Texas) and a 4-channel tape recorder (GX-280D-SS, Akai) through an FM converter (A.R. Vetter Co., Rebersburg, Pa.). Pre-dive heart rate (HR) was determined over the 10 second period just before immersion and dive HR from the final 10 seconds just before the diving bucket was lowered.

B) Effect of Habituation on Blood Variables

To obtain blood samples and monitor blood pressure, either the right or left brachiocephalic artery was cannulated. Under local anaesthesia (2% Xylocaine, Astra Pharmaceuticals) a 2cm section of the artery was exposed in the region adjacent to the femur and the flared end of a 50cm length of PE 90 tubing was inserted 7.5 to 8.0cm into the artery so that the tip lay within the ascending aorta. After the cannula was well secured, the skin was sutured closed. Animals were allowed at least one day to recover from the effects of surgery.

During experiments the trailing length of cannula extended through the chamber wall and was connected to a port on a three-way stopcock. A BT-70 pressure transducer (Bio-Tec Instruments, Pasadena, California) was connected to a second port and the remaining port was used for withdrawing blood samples. Pre-dive blood samples were taken a few seconds before immersion and end-dive blood samples were taken within the last 7 seconds of the dive. Each sample was then immediately analysed on an IL-813 blood gas analyser (Instrumention Laboratories, Lexington, Massachusetts). Blood gas analyses were done on a maximum of

six 0.5ml samples from each animal each day and hematocrit was closely monitored over the training period. In an earlier series of experiments two animals were subjected to more frequent blood gas sampling throughout the training session and hematocrit was slightly reduced. Between sessions, the cannula was flushed with heparinised saline (100 i.u./ml), sealed off and coiled into a small loop that could be tucked under the bird's wing which was then taped down to protect the cannula.

C) i. Effect of Breathing Pure Oxygen on Diving Bradycardia

A clear polythene bag was tightly attached to the top of the diving bucket. The duck's head was inserted into the bag through a hole in the base which was then loosely secured around the animal's neck. Gases of various oxygen contents entered at water level and exited via the loose fitting collar around the neck. In this way, at flow rates of 10 litres/min, it was impossible for the animal to breathe anything but the inflowing gases (Figure 10). Air of various oxygen content was obtained by mixing flows of pure oxygen and nitrogen gas using flowmeters and the percentage composition was checked with a Centronic 200 MGA gas analyser (T.C. Centronic Ltd., England). To reduce the possibility that the noise associated with switching gases might have served as a conditioned stimulus (CS) to the animal, a valve system was devised which reduced noise due to flow variations when gas mixtures were altered.

A preliminary series of experiments was done to examine the

77

Figure 10.   Diagram illustrating the system for controlling
     the air breathed by the duck before dives.

gas outflow

polyethylene
bag

water level

air tight seal

gas inflow
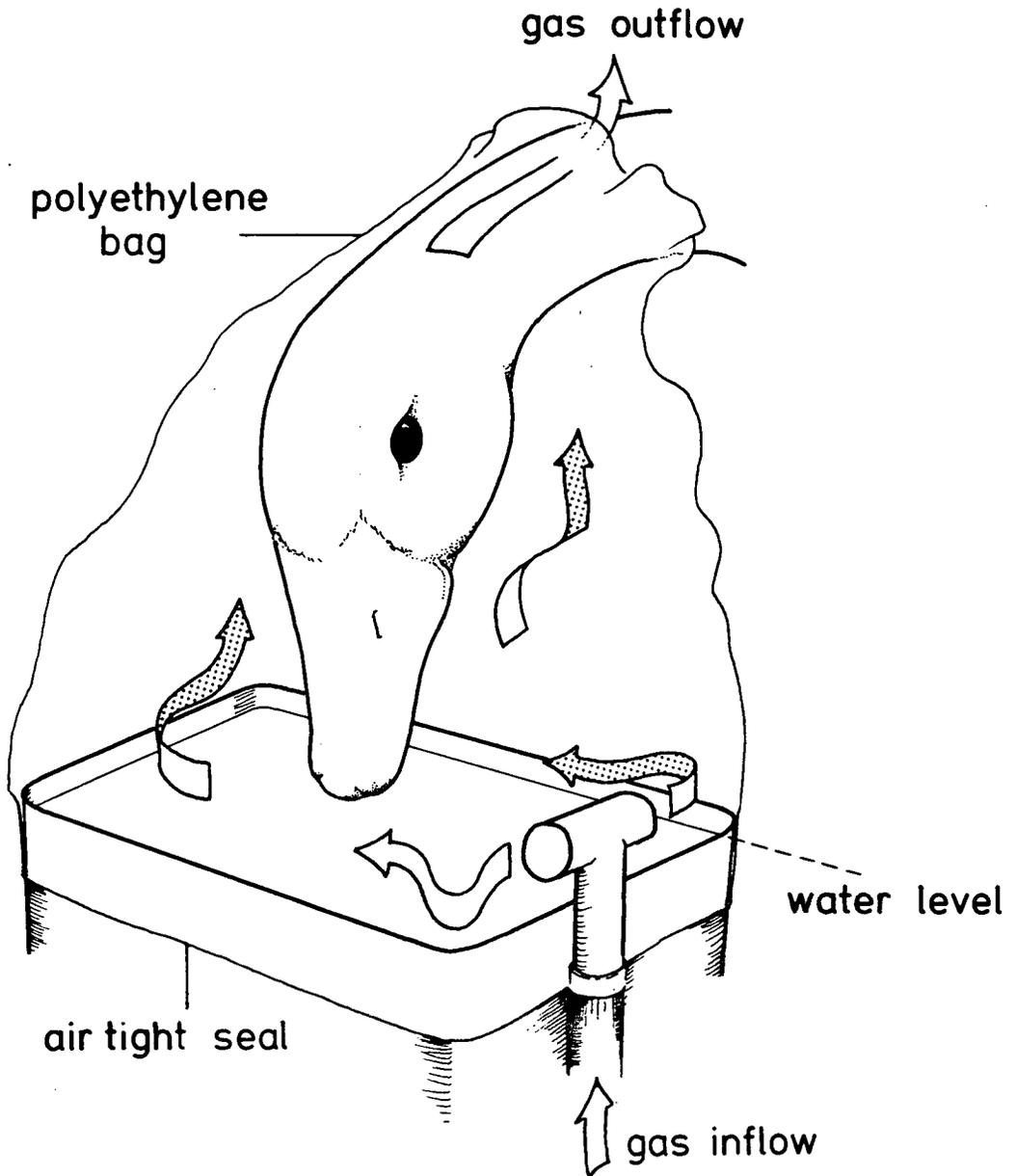
effect of breathing pure oxygen on diving bradycardia. Pre-dive HR and end-dive HR (40 second dive) was recorded for each dive. Ducks were dived twice each day: once after breathing pure oxygen and once after breathing room air. On the following day the sequence of dives was reversed. To assess the effect of the diving procedure, "sham" dives were done after the test sequence for each day. This entailed quietly emptying the bucket of water before raising it for the dive. 6 ducks were tested: 3 of which began with oxygen dives on the first day, the other 3 began with air dives.

ii. Effect of Hypoxia on the Habituated Response

To examine the effect of breathing hypoxic gas on the habituated diving response, five ducks, prior to testing, underwent the training procedure for a sufficient number of trials until a substantial level of habituation was achieved. The oxygen content of the air was then altered so that the ducks were breathing air of 10, 15, 20 or 100% oxygen for at least 3 minutes before immersion.

D) Effect of Habituation on the Oxygen Breathing Test

To test for chemosensitivity, the ventilatory response to low and high oxygen was measured before and after habituation in three animals. To accomplish these tests, the animals were placed within a temperature-controlled body plethysmograph.

Figure 11.   Schematic diagram illustrating the body
        plethysmograph for measuring ventilation.   Detachable
        face mask in position for the oxygen breathing test.

blood pressure
cannula

ECG

pneumotachograph

air flow

dental dam
collar

rectal thermometer
(Tb)

water flow
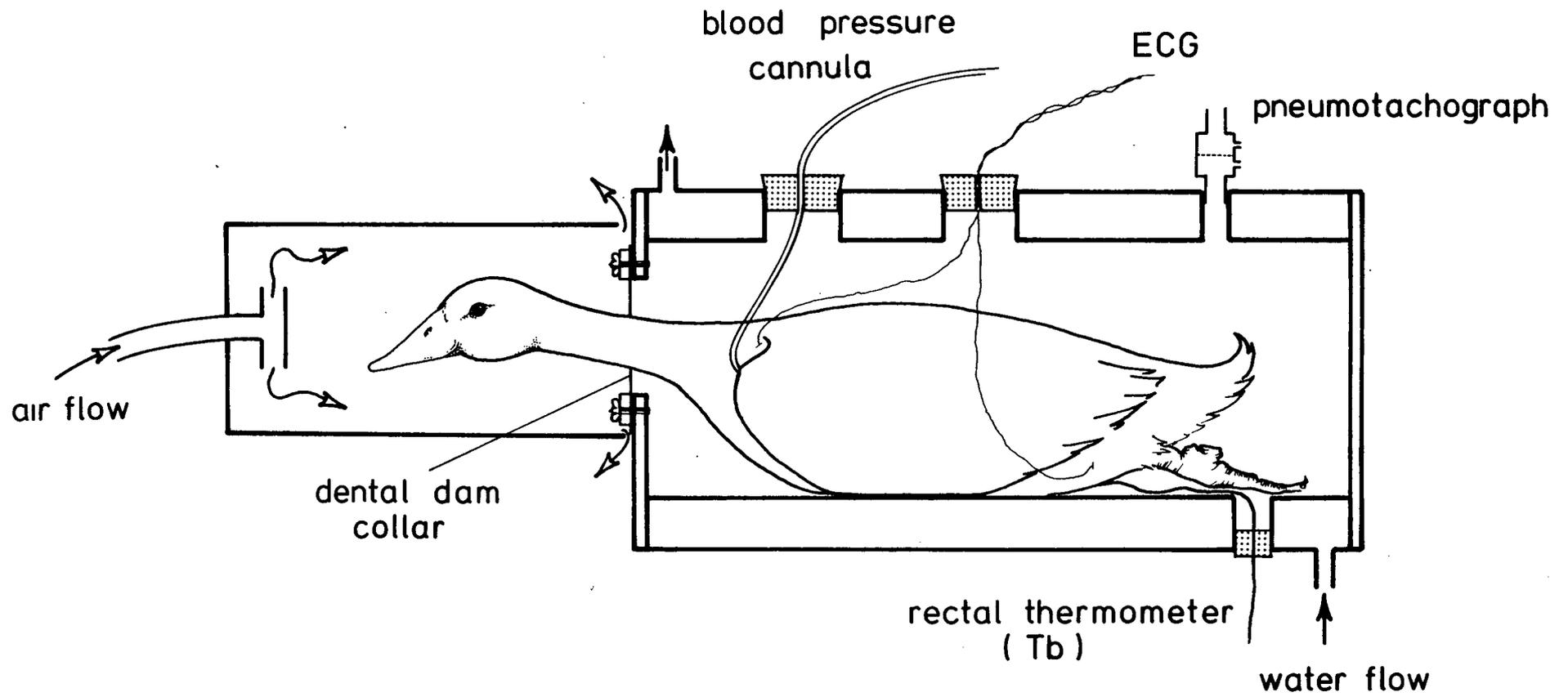
Pure oxygen, air or hypoxic air (10% O_2; balance N_2) was administered at flow rates of 6 litres/min through a face mask attached to the front of the plethysmograph (Figure 11). Breathing was monitored with a pneumotachograph (Fleisch, Switzerland) attached to a port in the plethysmograph. The pressure drop across the pneumotachograph during breathing was recorded with a pressure transducer (Validyne DP103, Northridge) and the airflow signal was fed into a Gould Integrator to provide tidal volume (Vt). Minute ventilation was calculated, two times, from the change in Vt and respiratory frequency (f) during air breathing and after 30 seconds of breathing 100% O_2 or 10% O_2. In order to dive the animal, the face mask was removed and the animal's head immersed into a beaker of water from this position. Two tests of the breathing response were run on each animal before and after a sufficient period of diving trials to establish a habituated response.

E) Habituation of the Cardiac Response in "True" Diving Ducks

The redhead ducks were secured to a level platform in a manner similar to that used for the Pekin ducks (experimental protocol A). However, their heads were left unrestrained. The trials were accomplished by firmly but gently forcing their heads into a beaker of cold water. The training protocol involved 20-second trials with 5 minutes between each trial and an average of 30 trials a day. ECG was recorded as described for the Pekin ducks. Pre-dive HR and end-dive HR were also

determined.

RESULTS

A) Habituation of the Cardiac Response to Diving

Naive or non-habituated ducks exhibited a fall in heart rate to about 70% of pre-dive levels by the end of a 40 second dive. This response gradually diminished with an increase in the number of trials. The rate and amount of habituation varied among animals and all showed some degree of spontaneous recovery overnight. Recovery was never complete and the effect of this over successive blocks of trials was to produce a rising "saw-tooth" curve of the diminishing cardiac response with increasing trials (Figure 12 and 14). With repeated training sessions a potentiation of habituation resulted in the virtual elimination of the cardiac response. In five animals habituation was so pronounced that the HR response to immersion was transformed to a sustained submersion tachycardia (Figure 13).

One animal (D-4) allowed to rest for 48 hours after sufficient training to abolish submersion bradycardia showed good retention and rapid habituation in subsequent sessions (Figure 14). Recovery to naive levels of diving bradycardia was complete, however, in two ducks tested after one month of rest from training since test dives were indistinguishable from pre-habituation dives.

Although the pre-dive HR in most ducks decreased as training progressed, the difference in mean values from all animals before and after habituation was not significant. The mean pre-dive rate for the first trial of all animals was

85

Figure 12.   Effect of habituation training on diving
     bradycardia.   End-dive heart rate is expressed as a
     percentage of the Pre-dive heart rate.(O) represents
     end-dive values; (◉) represents end-dive values obtained
     for the first trial of each training session; (●)
     represents end-dive values obtained when the animal
     breathed 15% oxygen before the dive.
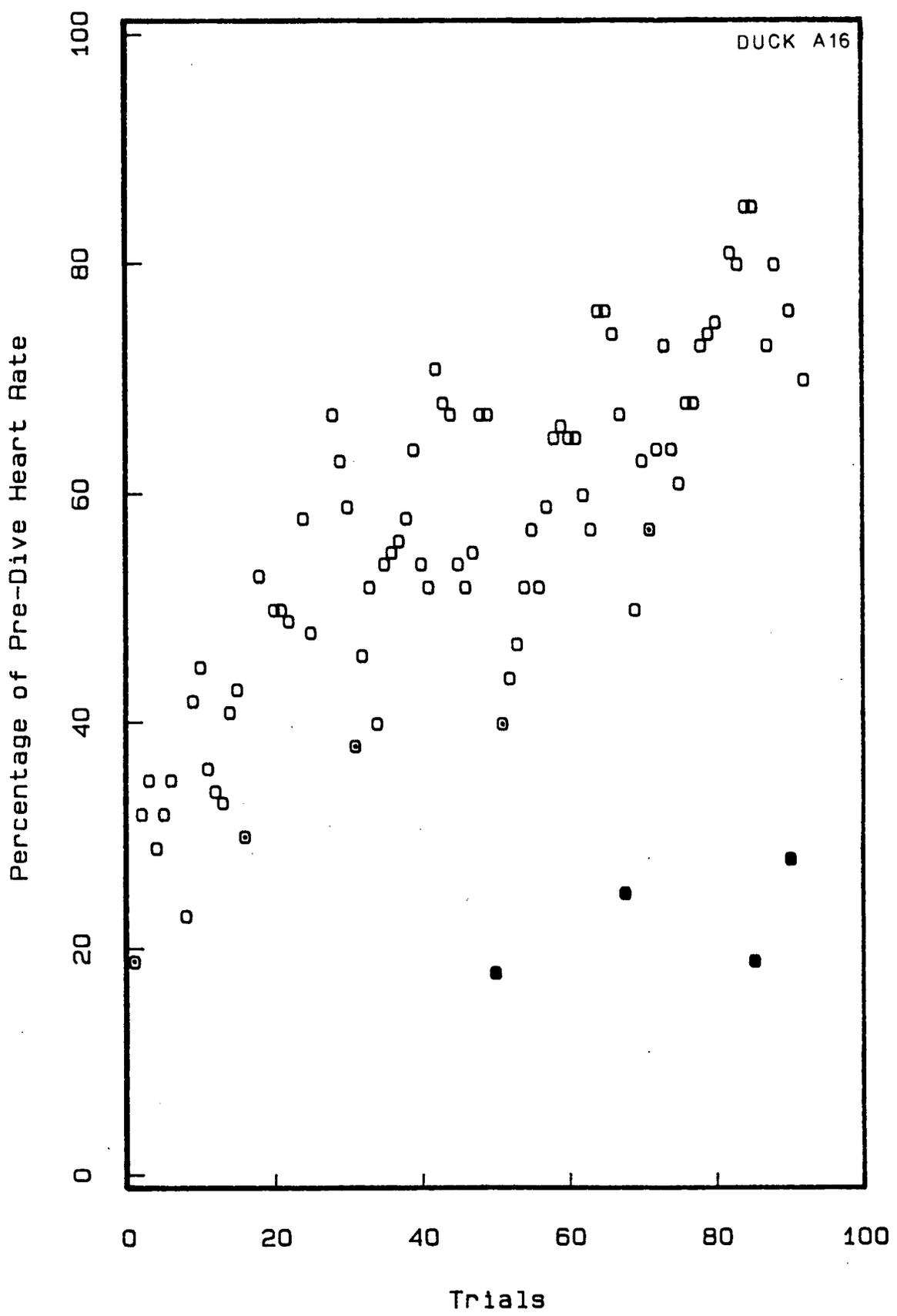
Figure 13.   Effect of training on diving bradycardia for 13
        ducks.   The solid lines represent interpolation between
        the means of the last 3 trials of each training session.
        (The lines begin with the mean of the first 3 trials of
   the first training session.)

Figure 14.   Effect of interruption of training on the
      habituation of bradycardia.   Circled points represent
      the first trial of each training session.

Percentage of Pre-Dive Heart Rate

DUCK D4

06
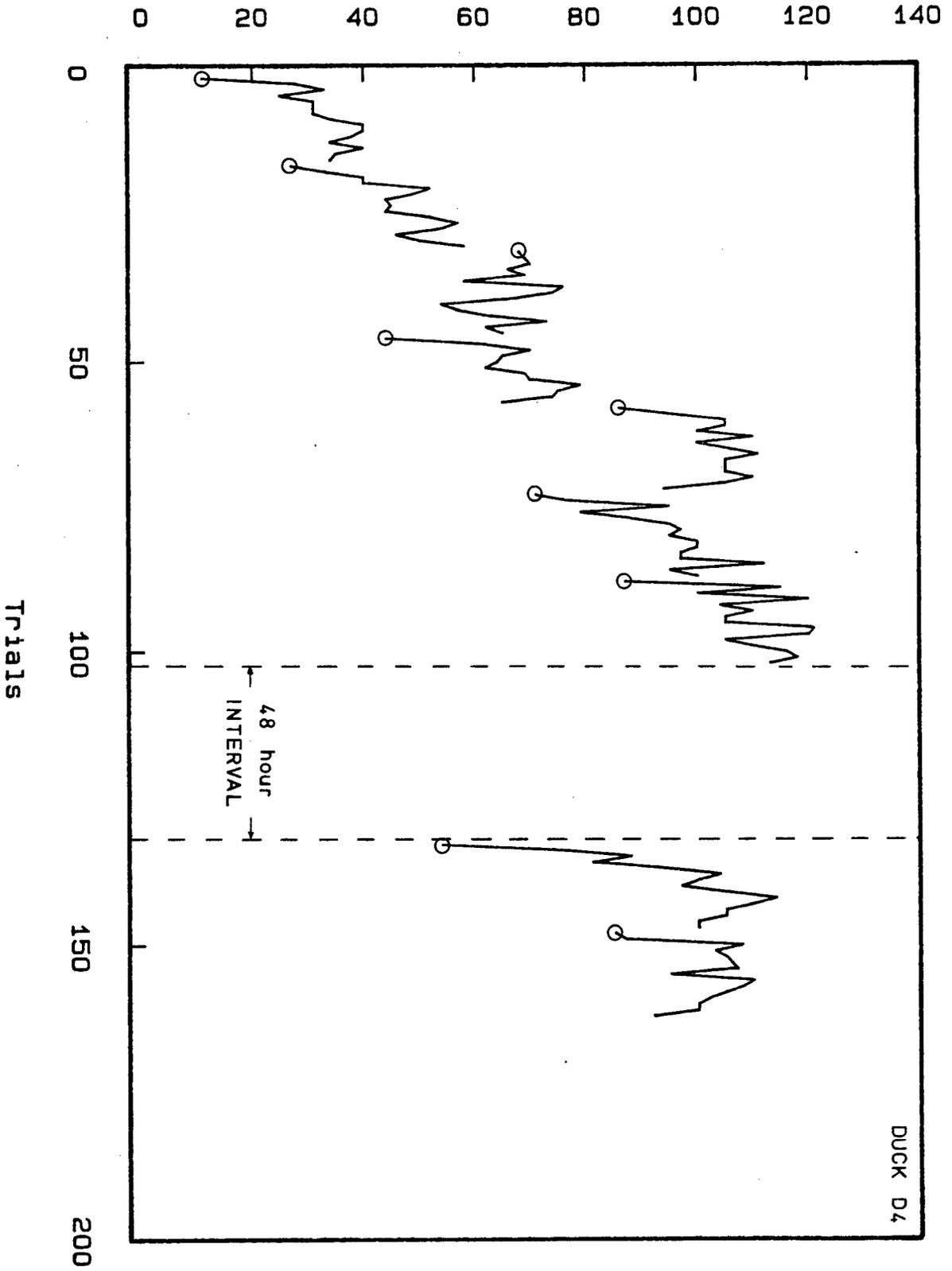
176.9±40 beats/min and the mean pre-dive value after training was 142.7±36 beats/min (N=9). The latter value was calculated from the pre-dive HR of the first trial which demonstrated significant habituation (this was arbitrarily defined as a fall in HR of no more than 10%). Of the 9 ducks used in this part of the study, 6 showed a decreased pre-dive HR with training, 2 showed an increase and 1 remained unchanged.

Habituation proceeded very slowly if the immersion period was longer than 40 seconds. The two animals (D-1, D-2) that commenced their training schedule, for several days, with 60-second immersions showed very little attenuation of the cardiac response. As soon as the immersion period was shortened to 40 seconds, habituation proceeded rapidly (Figure 15). Conversely, when the normal training schedule was altered to 80-second immersion periods (J-22) the habituated response was abolished. However, as the training progressed with the extended trials, the cardiac response once again showed some reduction (Figure 16).

B) Effect of Habituation on some Blood Variables

To assess the effects of habituation, comparisons were made between measurements obtained from the first dive of the first training session and the first dive to exhibit a fall in HR less than or equal to 20% of the pre-dive HR. From this criterion, all the animals achieved this level of habituation within 4 to 5 days.

Figure 15.   Effect of prolongation of dive time on
      habituation.   The solid line represents interpolation
      between the means of the last 3 trials of each training
      session.   The vertical broken line indicates the change
      from 60 to 40 second dives.

Percentage of Pre-Dive Heart Rate

DUCKS D1 D2

Trials
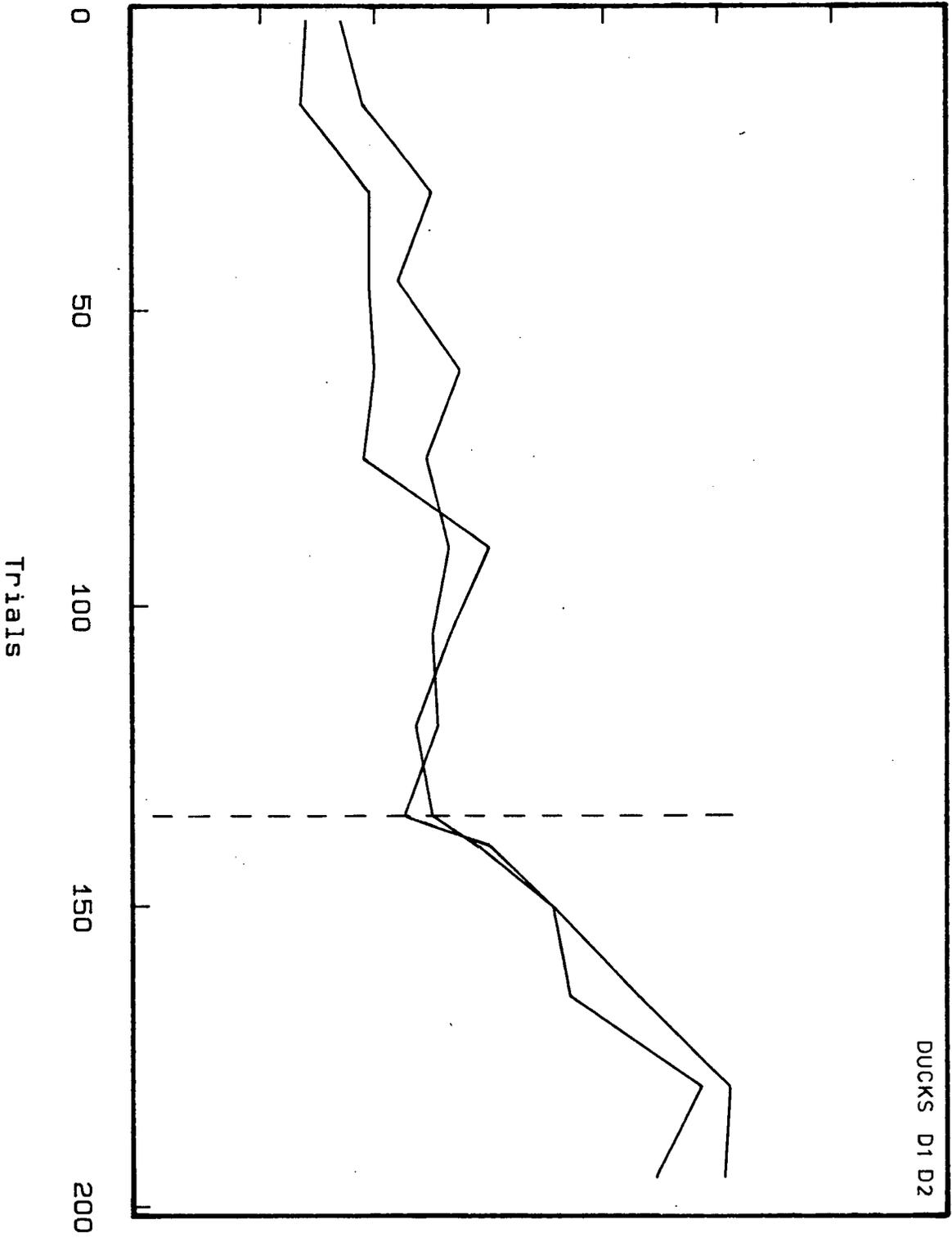
Figure 16.   Effect of reduction of dive time on habituation.
        The vertical broken line indicates the change from 40 to
        60 second dives.

Percentage of Pre-Dive Heart Rate

DUCK J22

Trials
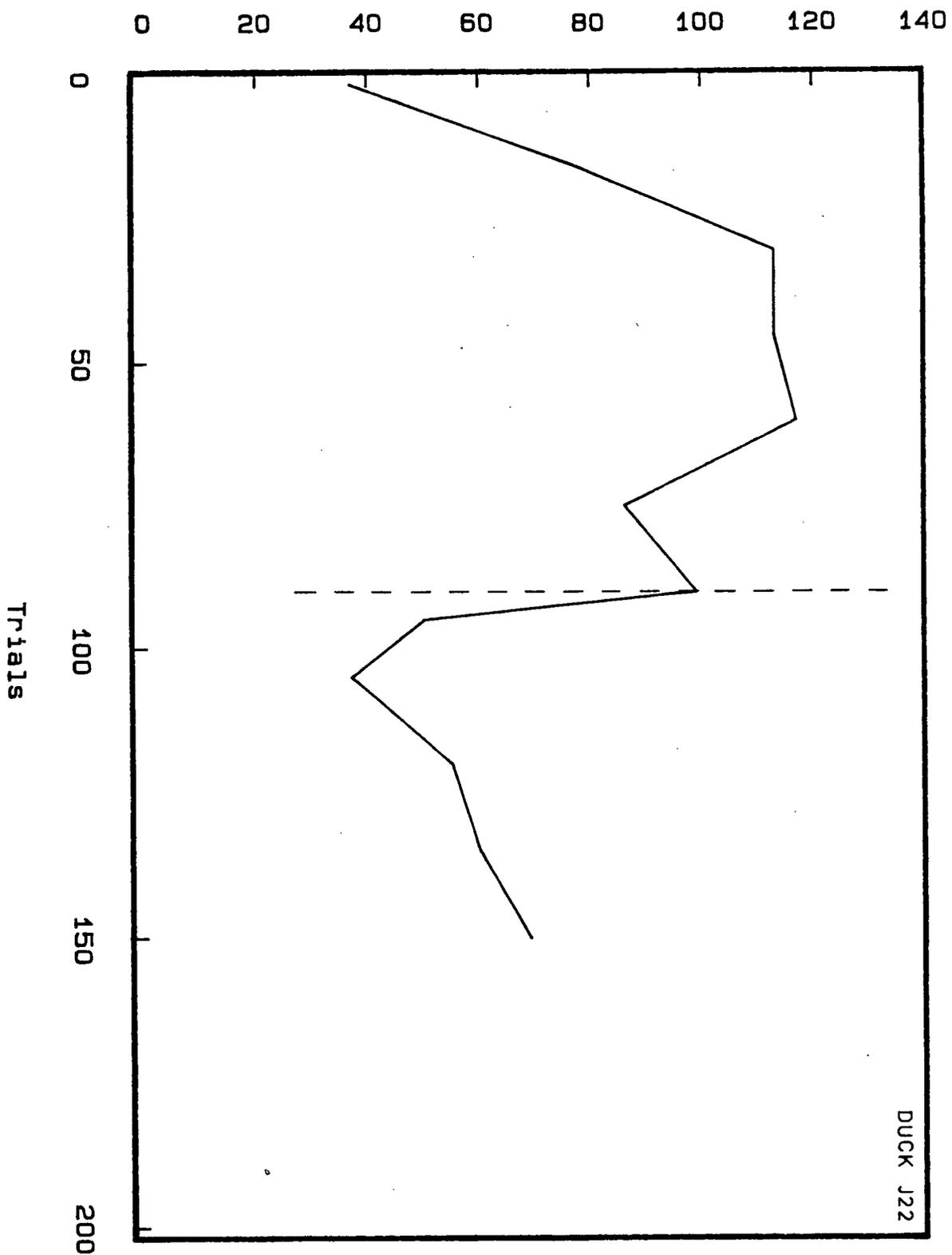
Despite the considerable bradycardia which developed after 40 seconds submersion in the naive animal, MAP remained unchanged from pre-dive levels. As training progressed and the bradycardia diminished, MAP continued to match pre-dive levels (Figure 17; Table 3). The mean end-dive MAP of five ducks before habituation was 158±7mm Hg and after habituation was 157±12mm Hg (Figure 17).

The direction of change in blood gas levels was the same before and after habituation; however, only in the case of PaO_2 was the magnitude of the change between conditions insignificantly different (Table 3). Naive ducks exhibited a mean end-dive PaO_2 of 55.7±3.0mm Hg and this value after habituation was 56.5±2.7mm Hg. The training session had no effect on pre-dive PaO_2 and end-dive PaO_2 was not significantly different between naive and habituated animals (P<0.05).

During submersion before habituation, $PaCO_2$ rose from 29.2±2.4mm Hg to 38.5±2.2mm Hg. After habituation, however, the amount of rise in the dive was slightly reduced: $PaCO_2$ rose from 27.8±5.0mm Hg to 35.3±5.8mm Hg. The increase in end-dive pHa amounted to only some 0.04 pH units both before and after habituation (Table 3). The differences in the change of both these variables were not significant at P=0.05 levels.

The hematocrits of the four ducks used in this study did not change over the training sessions and remained at 41%. However, in two ducks sampled for blood gas analysis more frequently during training, hematocrit fell by 1-3% over six days. The blood gas values for these two animals were not

Figure 17.   Effect of habituation training on arterial blood
       pressure.   A: Recordings from the first dive of the
       first day of training.   B: Recordings from a dive on
       the fifth day of training.   Top trace of each recording
   is blood pressure, bottom trace is ECG.

A. FIRST DIVE

(mm Hg)

200
150
100

ECG

DIVE

SURFACE

B. HABITUATED DIVE

(mm Hg)

200
150
100

ECG

10 seconds

99
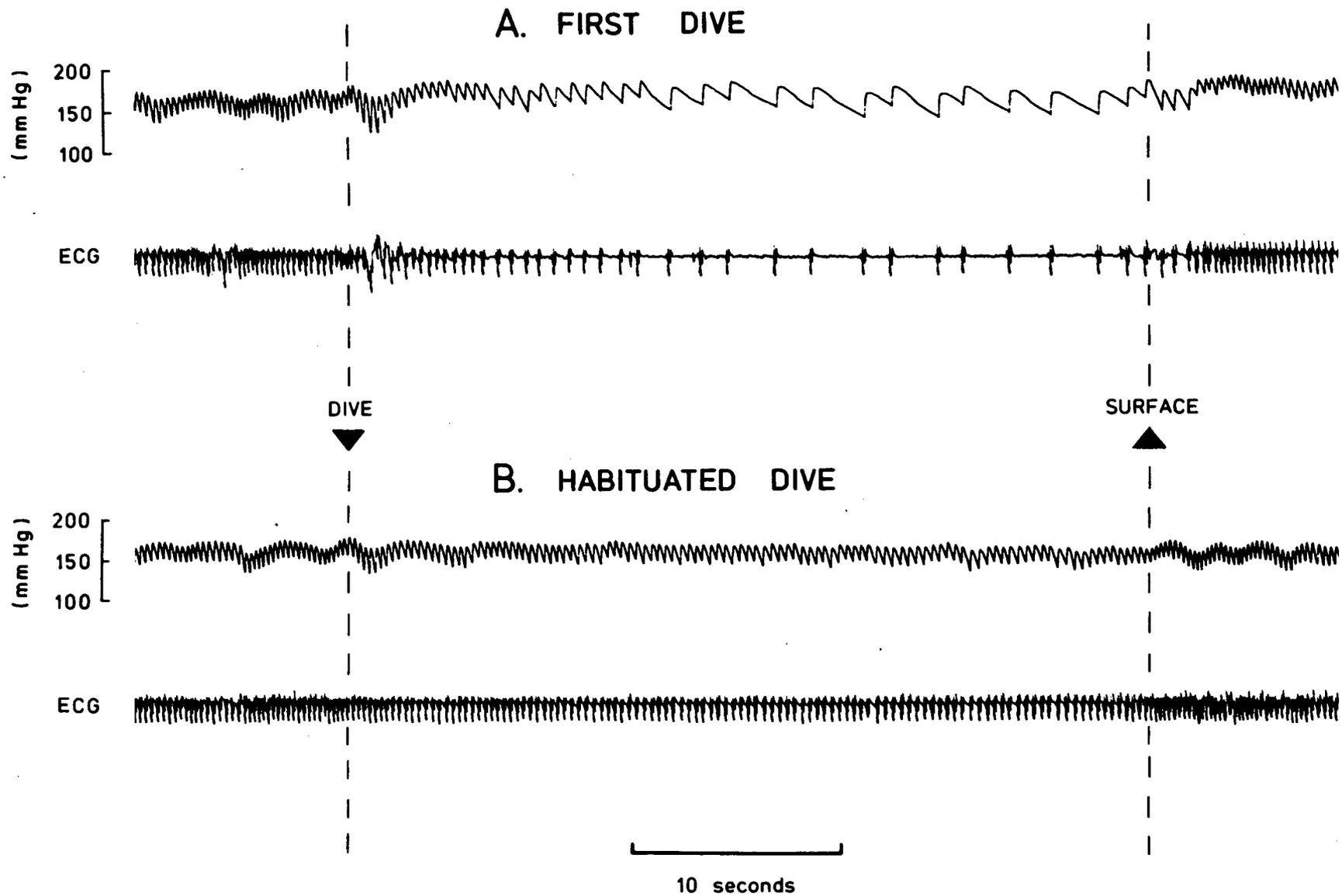
Table III. Effect of Habituation on the Cardiovascular System.
Values are means ±S.E.. Naive values were obtained from the first dive of the first training session. Habituated values were obtained from the first dive to exhibit a fall in HR less than or equal to 20% of pre-dive HR. PaO_2, $PaCO_2$ and MAP in mm Hg. (n=N)

Table III. Effect of Habituation Training on the Cardiovascular
System

	n	NAIVE		HABITUATED	
		Pre-Dive	Dive	Pre-Dive	Dive
PaO$_2$	4	95.13±1.42	55.70±2.99	93.83±7.30	56.48±2.74
PaCO$_2$	4	29.15±2.39	38.50±2.19	27.80±4.96	35.33±5.78
pHa	4	7.44±0.02	7.40±0.01	7.45±0.04	7.41±0.03
MAP	5		157.5±7.4		157.2±12.4

included for statistical analysis but they showed the same trends as the other ducks and the results from one of these animals is graphically represented in Figure 18.

C) i.) Effect of Breathing Pure Oxygen on Diving Bradycardia

In the preliminary study on six animals breathing pure oxygen before submersion, the fall in heart rate was always substantially less than after the same animals had breathed air. This was true whether the oxygen dive was the first or second dive of the day. In the majority of cases, the heart rate during oxygen dives exceeded the pre-dive values. There was no significant difference in the end-dive HR's between oxygen and sham-dives, but all were significantly different (P<0.05) from the end-dive HR's of air-dives (Figure 19).

ii.) Effect of Hypoxia on the Habituated Response.

The mean fall in HR during submersion before habituation was 70% for the five animals studied (Figure 20). After training, the mean fall of the habituated response was 22% (Figure 20). When these habituated animals were then given air with low oxygen (15% O_2) prior to forced submergence, the mean fall in HR was 69%. If the oxygen content was lowered further (10% O_2), pre-dive breathing increased and HR fell by 70% during submersion. After breathing pure oxygen (100% O_2), the mean fall in the habituated animals only amounted to 24%, similar to

Figure 18.   Effect of habituation on end-dive blood gas
     levels.   Graph (A) represents the changes of end-dive
     $PaCO_2$ and graph (B) represent the changes of end-dive
     PaO_2.   The solid line in each graph represents heart
     rate.   The changes in blood gas levels are plotted as
     the differences (in mm Hg) from the value obtained for
   the first dive on the first day.
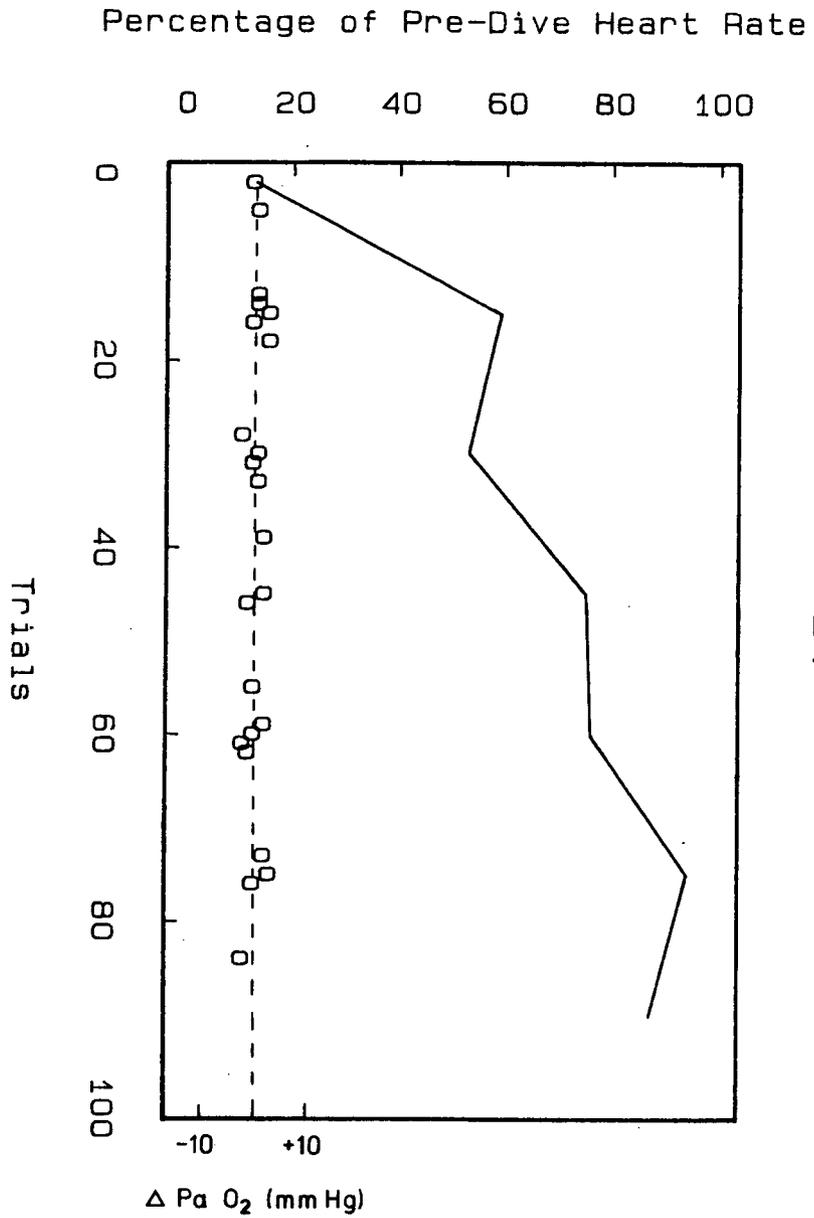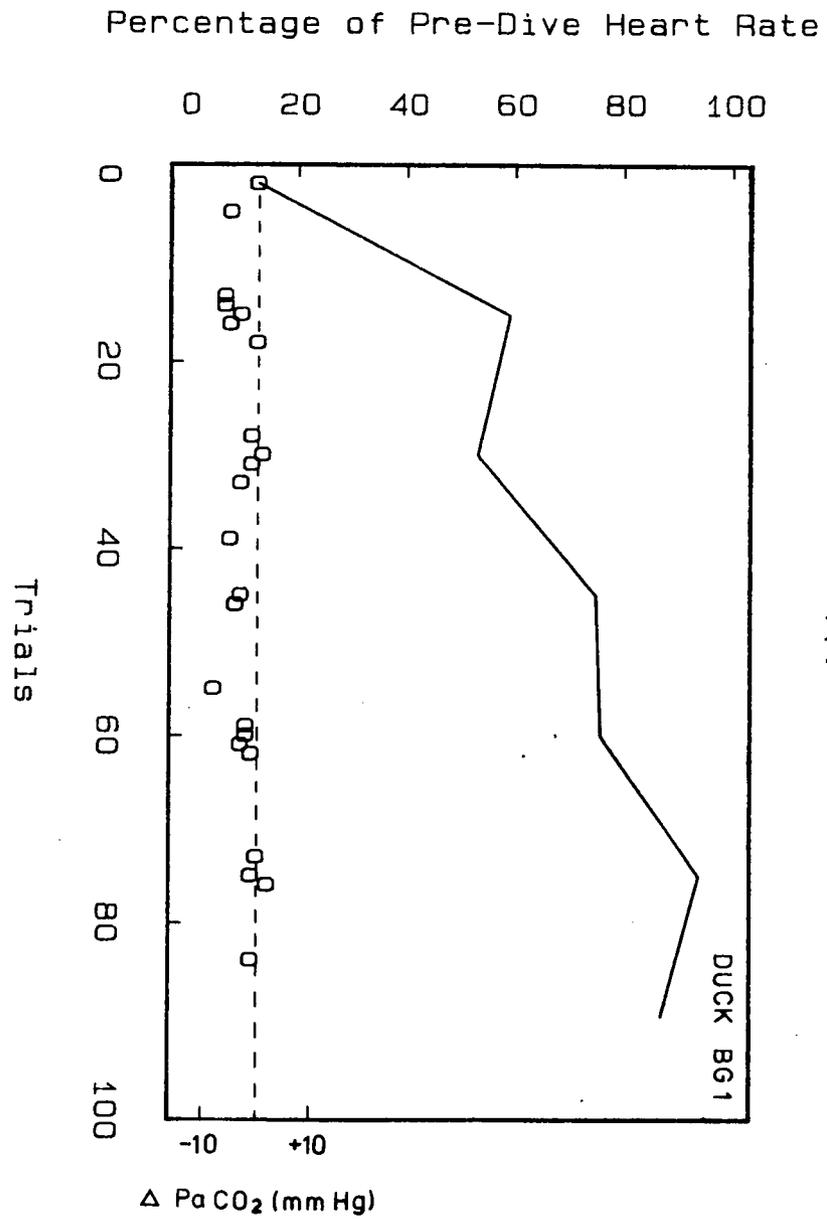
Figure 19.   Effect of breathing pure oxygen before 40 second
      dives.   '1st','2nd' and '3rd' refer to the sequence of
      tests as described in the text.(Error bars ±S.E.; n=6)
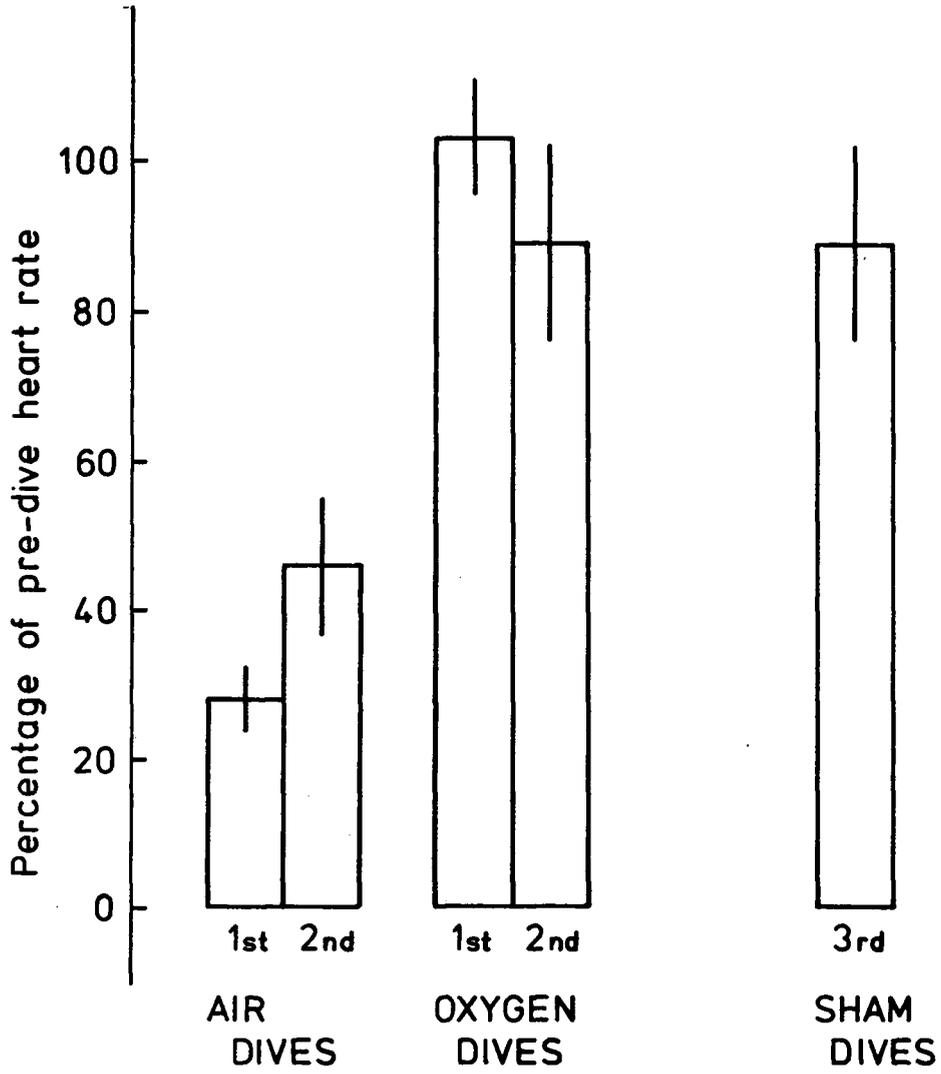
Figure 20.   Effect of various inspired oxygen contents on the
         habituated response to submersion.   The hatched bar
         represents values for untrained animals; open bars
         represent values for habituated animals.   Values
         beneath each bar represent inspired oxygen content.
         (n=6)
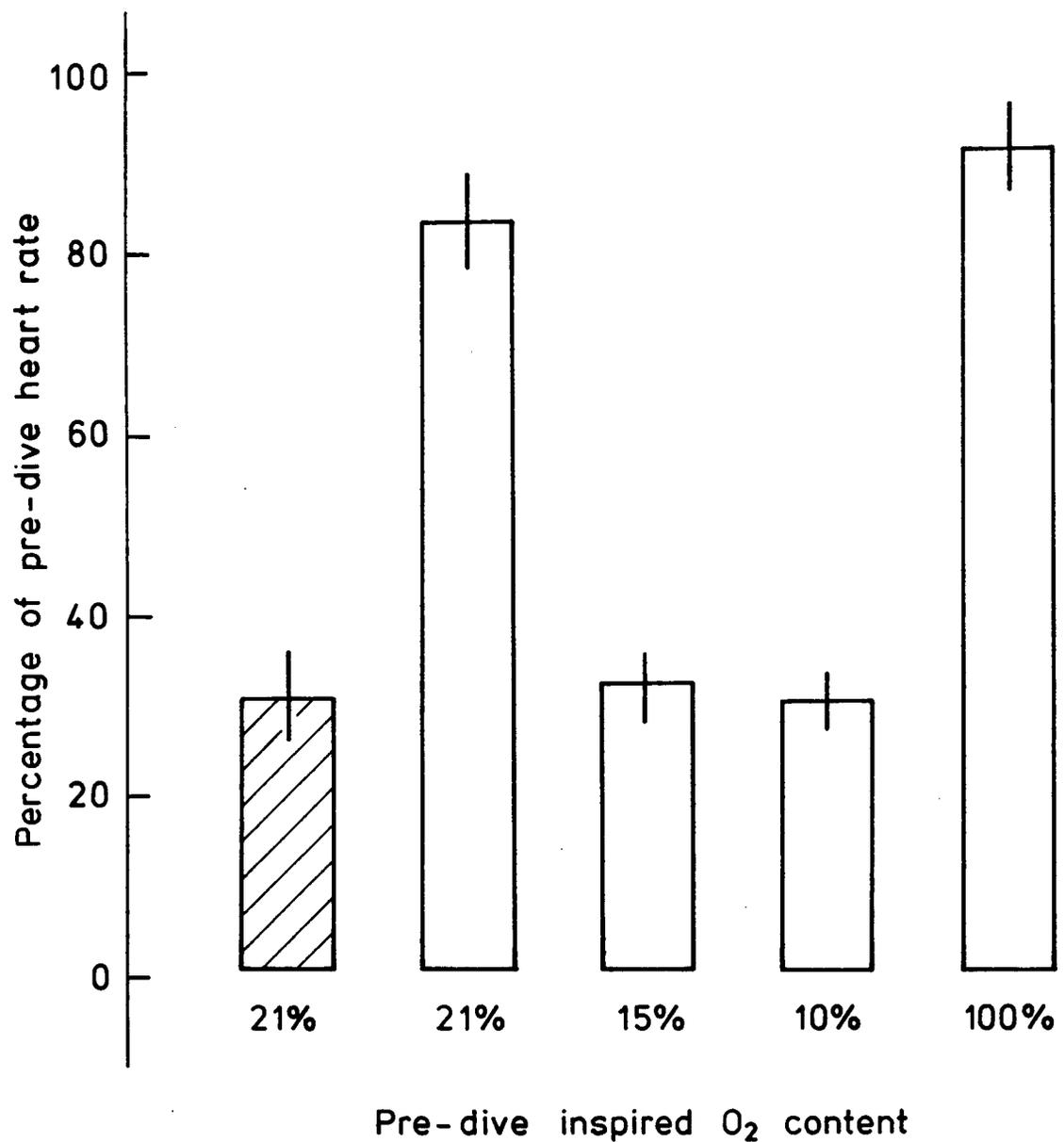
the values for habituated animals on normal air (Figure 20). The effect of the hypoxic test was not permanent as the very next trial with normal oxygen content (breathing room air) evoked the usual habituated response (Figure 12).

D) Effect of Habituation on the Oxygen Breathing Test.

In naive animals, the ventilatory response to pure oxygen and 10% oxygen was a decrease and increase, respectively, in comparison to air breathing. After training each animal achieved a level of habituation where only a 10% drop in HR was elicited by submersion. When these animals were given the low and high oxygen tests, the changes in minute ventilation were of the same magnitude and direction as before. The data from the tests were compared using a two-way analysis of variance with $P<0.05$. The ventilatory responses to the tests were significantly different from one another, but the values obtained prior to habituation training were not significantly different from those after habituation (Table 4).

E) Habituation of the Cardiac Response in Diving Ducks.

The redhead duck responds to submersion with an immediate and rapid drop in HR. Although the pre-dive HR in these animals is considerably lower than in Pekin ducks (ranging from 100 to 130 beats/min) they achieved a 70% fall in HR within the first

Table IV.   Effect of Habituation on the Oxygen Breathing
        Test.
        'After Habituation' values were obtained after 5
        training sessions for each animal

Table IV. Effect of Habituation on the Oxygen Breathing Test.

	AIR	100% O_2	10% O_2
Before Habituation	0.32±0.02	0.22±0.05	0.57±0.03
After Habituation	0.33±0.04	0.23±0.04	0.59±0.06

N=3:n=6. Values are $\dot{V}_E$ (l (BTPS)/min/kg) ± S.E.

10 seconds of immersion. As with the Pekin ducks, the effect of repetitive submersion was the gradual reduction in the degree of bradycardia. All animals showed some spontaneous recovery overnight but it was insufficient to prevent them from achieving almost complete habituation of the cardiac response after only about 100 trials, over 3 days of training (Figure 21).

Figure 21.   Effect of training on diving bradycardia in 3
        Redhead ducks (Aythya americana).

Percentage of Pre-Dive Heart Rate

Trials

Trials

Trials
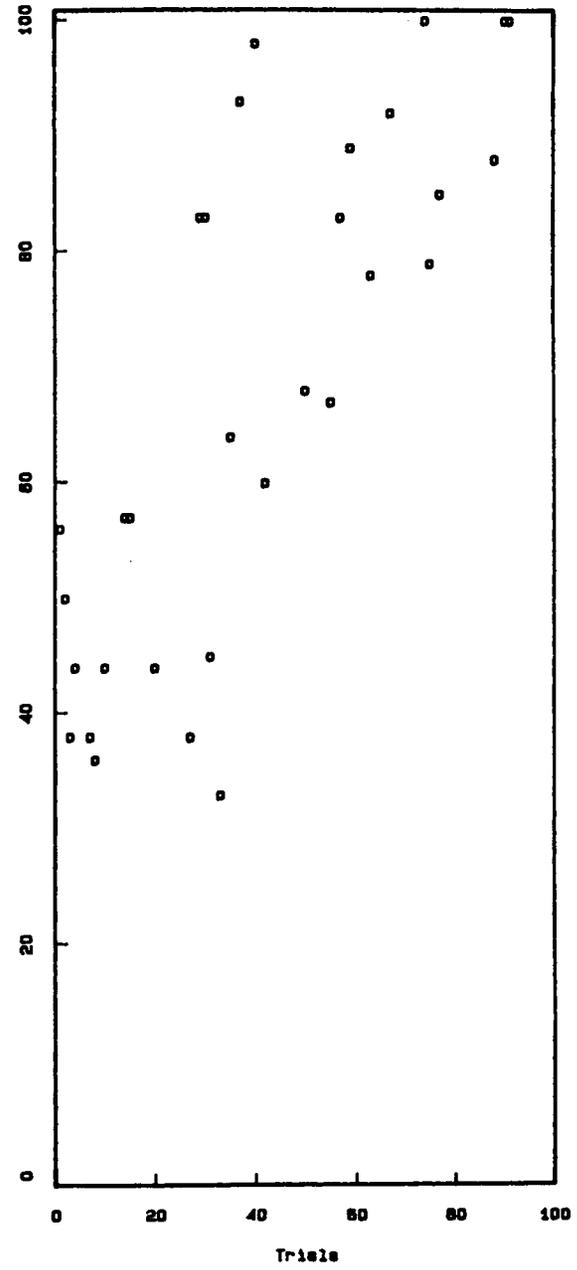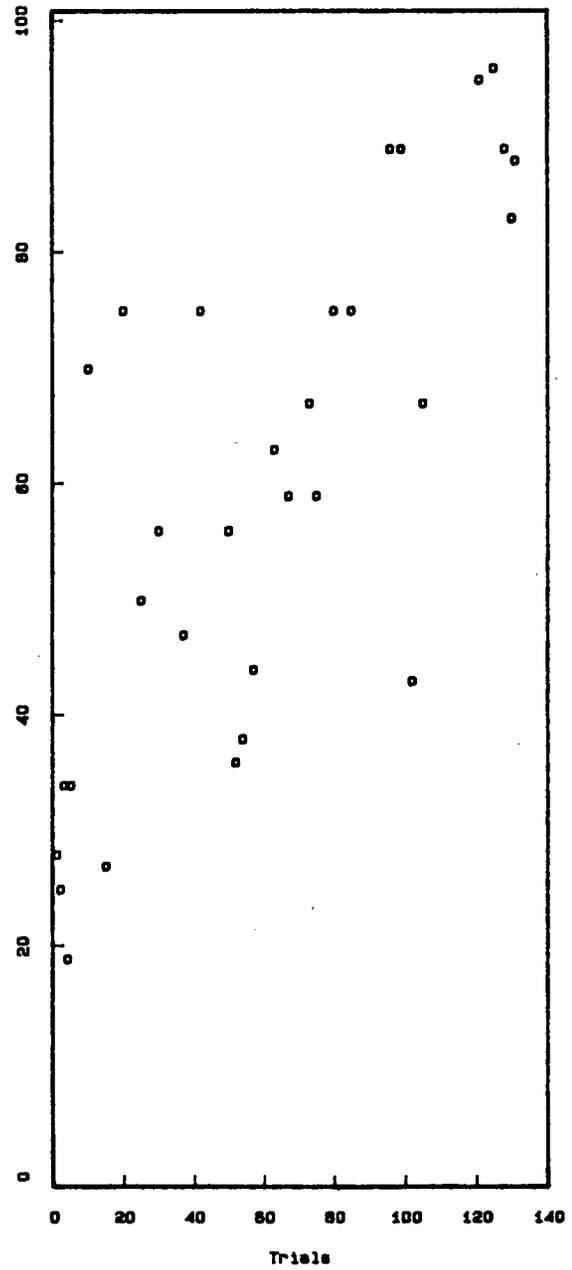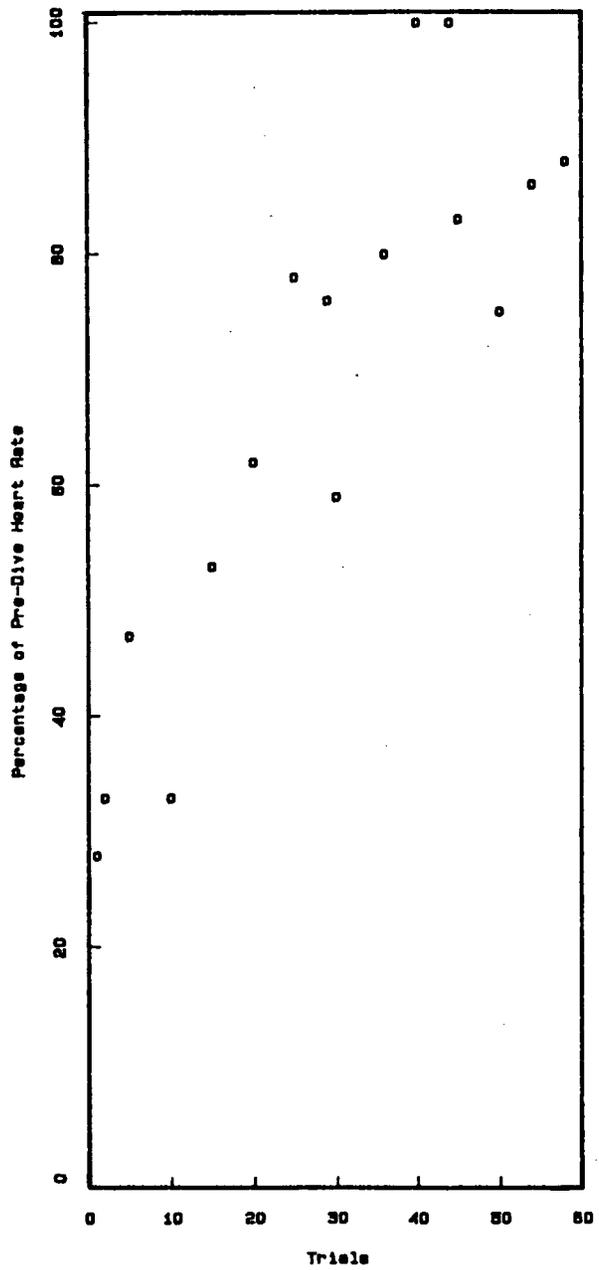
DISCUSSION

Repeated exposure to the stimuli evoked by submersion results in habituation of the normal or naive cardiac chronotropic response (bradycardia). This is so pronounced that in spite of some spontaneous recovery between training sessions, the bradycardia in many animals could be completely abolished. The instances where the heart rate response appears to reverse during submersion, that is, to develop tachycardia, may be a reflection of the fact that having abolished chemoreceptor activation of the vagal deceleratory system, the reduced bradycardia is the result of inhibition of the vagal system (a form of cardiac disinhibition) or direct sympathetic acceleratory effects. Before habituation the acceleratory effects on the heart due to agitation or struggling were largely overridden by vagal deceleratory control, but following habituation, the vagal drive is either diminished or abolished.

In a previous study, habituation of diving bradycardia was not observed despite a training regime of thirty 60-second dives each day for up to 5 months (Rey 1971). It is surprising that at the end of such an extensive training period, there was no significant difference in the diving bradycardia between trained animals and their untrained controls. From the present study, it was shown that stimulus intensity is a crucial factor for habituation. If dive times exceeded 40 seconds (Figure 15 and 16), or if the inspired oxygen content was only slightly reduced (Figure 12 and 20), habituation of diving bradycardia was abolished or considerably reduced. It seems likely that the

dive time in the study done by Rey (1971) was too long and that arterial PO_2 was consequently too low for habituation to occur.

The argument could be raised that training sessions served to familiarise the animals to an otherwise "fearful" situation. When viewed in this light, the gradual reduction in the diving bradycardia could be because the animals are less frightened. As accomodating as this interpretation sounds, it ignores the following observations:

a) Naive ducks show little or no bradycardia during submersion after breathing pure oxygen;

b) The same animals that habituate with 40 second trials will not habituate if the time is extended to 60 seconds, even if the number of training sessions is increased;

c) A fully habituated animal develops as deep a fall in heart rate as a naive animal after breathing air with only a 5% reduction in oxygen content before the dive.

Over the period of habituation, MAP remains unaltered in spite of the reduction in bradycardia. This suggests that either equal and parallel habituation of the vascular constrictor response must occur, or alternatively the barostatic reflex can continue to maintain MAP. In any event the vasomotor component of the submersion response also attenuates with training. This was demonstrated for one animal which showed habituation of both the bradycardia and the increase in HLVR (Figure 22; Gabbott and Smith unpublished observations).

The efficacy of efferent control was confirmed in the tests where the habituated animals breathed slightly hypoxic gas

Figure 22.   Effect of training on hind limb vascular flow.
        Open circles represent values for end-dive heart rate.
        Closed circles represent values for blood flow measured
        with a Doppler flow probe on the ischiatic artery.

Percentage of Pre-Dive Heart Rate and Pre-Dive
Hind Limb Vascular Resistance

DUCK S1

117
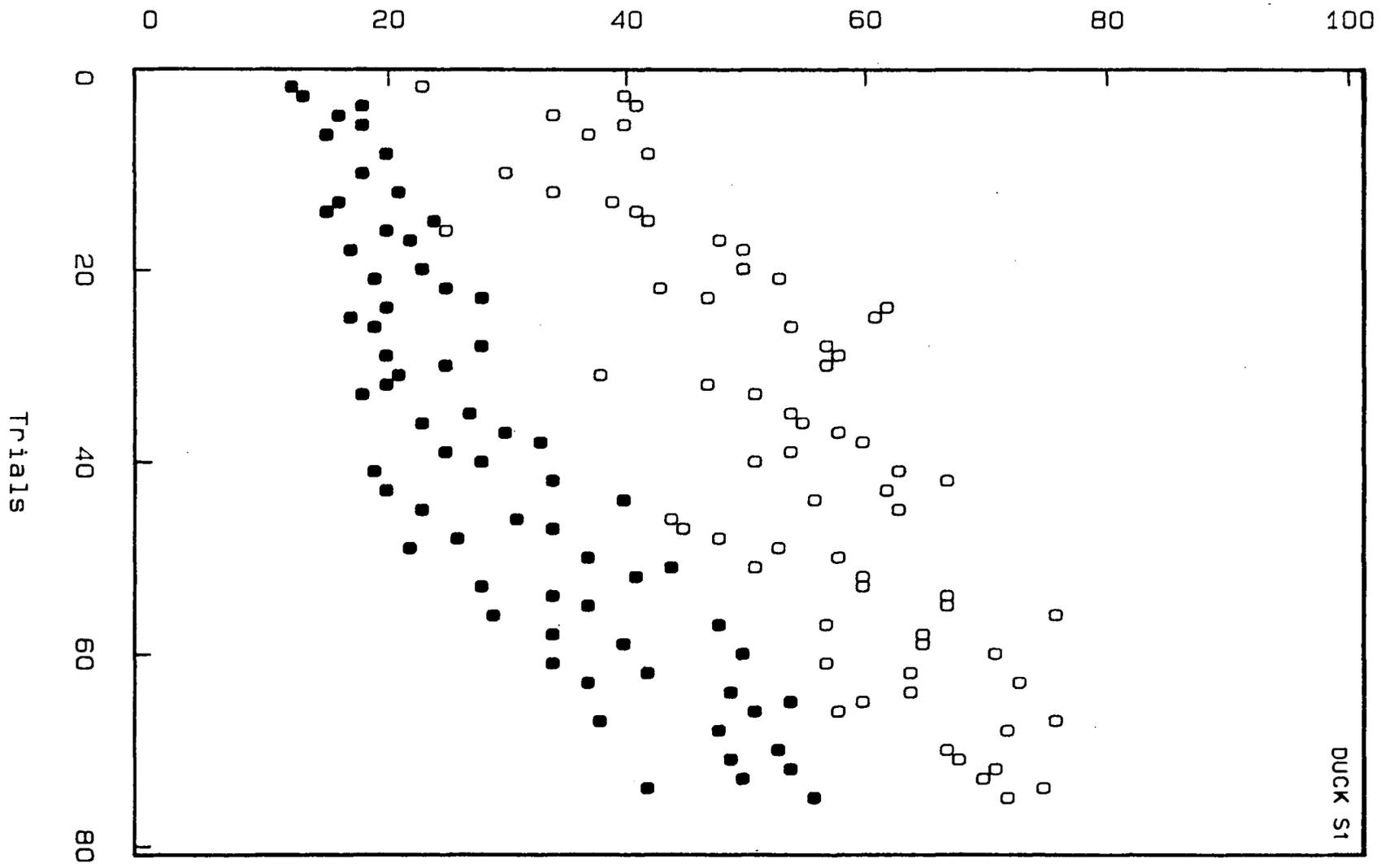
mixtures before submersion. Though the effect on breathing prior to submergence was negligible, levels of bradycardia during dives were approximately the same as in naive control dives. Only when the inspired oxygen content matched or exceeded 21% did habituation of the bradycardia occur. The effect of hypoxia trials was not permanent since the very next trial with normal air (21% oxygen content) resulted in the return of the habituated response. This suggests that vagal output remains just as potent in the habituated condition as in the naive control.

Tests of receptor sensitivity are more difficult to accomplish. However, an attempt was made to detect a change in chemosensitivity by comparing the effect on breathing of the "Oxygen Test" before and after habituation. The changes in minute ventilation in response to either pure oxygen or 10% oxygen appear to be the same after five days of training sessions which resulted in successful cardiac habituation. In order to accept the notion that receptor sensitivity is virtually unaltered by habituation, it is necessary to examine the propriety of such a test for chemosensitivity to the effects of submersion. Obviously the test is an indirect measure of sensitivity and involves changes in an entirely different effector system. Nevertheless, it is well established that the carotid body chemoreceptors are the prime mediators of ventilatory change in response to the Oxygen Test (Dejours 1975). As with bradycardia during submersion, the removal of carotid body afferent activity also abolishes the ventilatory

response to hypoxia (Bouverot et al. 1965; Jones and Purves 1970; Bouverot and Leitner 1972; Lillo and Jones 1982).

During a dive cardiovascular adjustments function to conserve oxygen; the elimination of these responses during submersion (i.e. habituation) ought to provoke greater oxygen depletion. However, the fall in PaO_2, is the same in the habituated condition as in the control. This suggests that, at least within 40 seconds of submersion, the blood flow adjustments are not sufficiently developed to prevent oxygen loss. This is corroborated in experiments where the cardiovascular changes are eliminated pharmacologically by atropine and a-adrenoreceptor blockers (Butler and Jones 1971; Bryan and Jones 1980). Thus the rate of oxygen depletion in the first 30 to 40 seconds of submersion is similar whether the cardiovascular responses are present or not. It is only after this time period when the diving responses are fully developed that the loss of oxygen is retarded.

Since, in these experiments, the stimulus intensity for eliciting the cardiovascular responses to submersion appears to be maintained, and because decrement in neither receptor chemosensitivty nor efferent potency could be demonstrated in habituated animals, it suggests that the locus of habituation is within the CNS. This is in agreement with evidence from other diverse studies of habituation that support and have even demonstrated a CNS mechanism of habituation (Thompson and Spencer 1966; Kandel 1976).

Habituation has been considered one of the main mechanisms

by which an animal copes with the onslaught of diverse trivial stimuli to which it is exposed in its natural habitat (Glaser 1966). Each stimulus may evoke complex patterns of somatic and visceral responses, the magnitude of which depend on the physiological parameters of the sensory input as well as the behavioural context of the stimulus. Often these signals may elicit responses of increased magnitude disproportionate to the situation. Habituation, however, may serve to maintain CNS sensitivity to biologically significant stimuli through suppresion of more meaningless and inappropriate responses (including reflexes). When animals dive, asphyxic defense mechanisms are readily evoked to restrict the loss of oxygen. However, this study has revealed that the cardiac responses to submersion can be suppressed, whether they are provoked in diving ducks by "narial type" receptors or in dabbling ducks by chemoreceptors.

Data collected by telemetry from animals diving voluntarily has shown that the cardiovascular responses are not always provoked. This variability appears particularly common for brief excursions under water (Jones et al. 1973). It seems reasonable to suggest that oxygen conserving adjustments are unnecessary in animals diving for periods which are well within their aerobic capacity. Clearly the means by which behavioural responses are determined are many and varied, however, this study has suggested an option in the control of cardiac function during forced submersion that has hitherto never been demonstrated.

SECTION 3.

Demarcation of the Neural Substrate of the Diving Response.

Introduction

The complex temporal and spatial organisation of the cardiovascular response pattern to submersion is well integrated and demonstrates the involvement of higher levels of the CNS. Evidence indicates that during chemoreceptor-driven changes induced by submersion, baroreceptor reflexes may be attenuated (Kobinger and Oda 1969; Jones et al. 1982). Though the neurophysiological basis of this attenuation is unclear, some evidence suggests that in mammals a similar interaction is mediated within the hypothalamus (Hilton 1966; Gebber and Snyder 1970; Hilton and Spyer 1971; Takeuchi and Manning 1973; Coote 1978). Suprabulbar regions (areas rostral to the pons) have also been implicated in the integration of arterial chemoreceptor-driven cardiovascular responses in the cat (Bacelli et al. 1964,1965; Thomas and Calaresu 1973).

The specific involvement of the hypothalamus in the modulation of chemoreceptor-driven bradycardia was first reported by Korner and his co-workers (Korner et al. 1969; Uther et al. 1970). They demonstrated that, in rabbits, bradycardia in response to arterial hypoxia was abolished following transection of the brain below the level of the hypothalamus (Korner et al. 1969; Uther et al. 1970; Korner 1971). Certainly, anatomical studies have revealed direct

hypothalamic projections to and from areas of the caudal brainstem that mediate cardiovascular function (Conrad and Pfaff 1976; Evans 1976; Swanson 1977; Ricardo and Koh 1978; Ciriello and Caverson 1984). Furthermore, hypothalamic neurones appear to be sensitive to cardiovascular-related afferents (Brickman et al. 1977; Ciriello and Calaresu 1980, Calaresu and Ciriello 1980). These observations lend support to the concept of hypothalamic control of cardiovascular function (Hilton 1965,1975; Smith et al. 1974).

From another point of view, higher CNS control of cardiovascular responses to submersion has been suggested by the similarity to responses for a particular form of the defence reaction (Smith et al. 1974; Smith and Woodruff 1980; Kanwisher et al. 1981; Smith and Tobey 1983). Often designated the Type II fear response or "freezing" response, this behaviour has only recently become the focus of attention (Folkow and Neil 1971; Evans 1976; Gabrielsen et al. 1977; Smith et al. 1981a,b). Instead of the changes evoked in the Type I fear response, classically named the "flight or fight" response, the changes in Type II are almost diametrically opposite. Whereas the Type I response features tachycardia, hypertension and hyperventilation, Type II involves bradycardia, hypotension and often greatly reduced ventilation (Zanchetti and Bartorelli 1977; Azevedo et al. 1980; Smith et al. 1981a and b). Support for the contention that laboratory-induced submersion responses are ostensibly the result of a Type II fear response comes from a study in which rats were startled when either restrained or

unrestrained (allowed to escape). In the restrained condition, the fear response included bradycardia whereas in the unrestrained condition, the startle resulted in tachycardia.

In the cat, regions have been mapped in the rostral brainstem (including the hypothalamus, the central grey matter and the mesencephalic tegmentum) from which active cholinergic vasodilatation in the muscles can be evoked by electrical stimulation (Abrahams et al. 1960, 1964; Uvnas 1960). These regions are not merely "vasodilator" areas, as many of the characteristic features of the defence reaction are also elicited, including vasoconstriction in skin and intestine, pupil dilatation, pilo-erection, respiratory changes, arching of the back and swishing movements of the tail. Although the cat does not normally display the Type II response, stimulation of certain areas of the anterior cingulate gyrus cause widespread sympathetic inhibition together with vagal bradycardia, resulting in hypotension. In addition, there is reduction of skeletal muscle tone and depression of breathing (Lofving 1961). In another study, stimulation of regions in the caudal hypothalamus of the rabbit corresponding to 'defence areas' of the cat, provoke apnoea, bradycardia, vasoconstriction resulting in hypertension and various other responses including exopthalmus, pupil dilatation and erection of ears (Evans 1976). From these studies, it seems likely that expression of the defence reactions is mediated via the rostral brainstem, in particular the hypothalamic area.

In a study attempting to resolve the question of the

voluntary or reflex nature of the apnoeic response of ducks to submersion, Huxley demonstrated that the apnoeic and bradycardiac response persisted following the destruction of both cerebral hemispheres (1913). Some time later, Andersen (1963) demonstrated that both responses to submersion persisted even after section of the brain below the level of the diencephalon. But owing to the unfortunate inadequacy of Andersen's description of brain transection, it is not clear if the entire diencephalic region was removed, and the argument could be raised that some remaining caudal diencephalic centres were still viable and maintaining the diving response.

This investigation was designed to re-examine the involvement of higher nervous centres in the control of the diving responses. A technique was developed whereby local Xylocaine infusion was used to impose a reversible transection of the brain at the rostral level of the mesencephalon just below the hypothalamic region. Xylocaine blocks both synaptic transmission and conduction along fibres of passage and has been used successfully to cause reversible inactivation of discrete areas of brain tissue (Malpeli and Schiller 1979). Experiments were also conducted to examine the effect of habituation training on diving bradycardia in decerebrate ducks.

METHODS

Experiments were performed on seven White Pekin ducks (<u>Anas</u> <u>platyrhynchos</u>) weighing between 2.2 and 4.0kg. Two female and 5 male ducks were used. Experiments were carried out at room temperature (21-22°C) and the ducks were acclimated to this temperature for at least 1 week before experimentation.

Preliminary Surgery

Decerebrations were done under general anaesthesia induced by administration of Halothane (Fluothane, Ayerst Laboratories) into the air stream of unidirectionally ventilated (UDV) animals. For this purpose a resealable large bore cannula (1.0 cm i.d.) was first sewn into the interclavicular air sac which would serve as the exit port from which expired gases and halothane could be safely eliminated. The right brachiocephalic artery and vein were cannulated with PE 90 tubing. Cannulation techniques were described in the Methods for Section II, and all surgery up to this stage was done under local anaesthesia (2% Xylocaine, Astra Pharmaceuticals). The animal was allowed a minimum of 2 hours to recuperate from surgery before any experiments.

Decerebration

The animal was positioned within a modified Narishige stereotaxic table and arranged for UDV. This entailed intubation of the trachea and connection of the interclavicular cannula to an exhaust system. 1.8 to 2.2 volumes % Halothane was administered into the inspired airstream (50% oxygen:balance Air) by means of a Halothan Vaporiser (Dragerwerk, Lubeck, W.Germany). To augment the hypotensive action of the anaesthetic, a 0.142 μl/ml sodium nitroferricyanide solution (in 5% dextrose) was infused intravenously by means of an infusion pump (Model 901 Harvard Apparatus Co., Millis, Mass.). With careful control of the rate of infusion, blood pressure could be maintained low enough to reduce bleeding from brain tissue.

An extensive craniotomy was performed, taking care not to damage the underlying dorsal sinus. Once the dura was opened, the central segment of the sinus was ligated and removed. The cerebral hemispheres were extirpated to a level above the thalamus (Figure 24a) by suction through a blunt tipped hypodermic needle, the force of suction being controlled by varying the amount of occlusion of a small side-hole in the handle carrying the needle. In this way brain tissue could be removed rapidly with minimal brainstem distortion and blood loss.

After bleeding had stopped, blood pressure was gradually raised by reducing the rate of ferricyanide infusion. If bleeding occurred the infusion rate was increased to drop blood pressure again until bleeding ceased. The operation usually

required an hour and a half to complete and no more than 20 ml ferricyanide solution.

The cut edges of the scalp were liberally coated with vaseline and the cranial opening covered with plastic film to prevent dehydration. Several layers of gauze padding were placed over the skull and secured with tape. The animal was left to recuperate for a minimum of 24 hours. Body temperature (Tb) was continuously monitored via a rectal thermistor and maintained at $41\pm1.0°C$ with an electric heating pad and infra red lamps mounted over the animal. In three animals (DC5, DC8 and H), the scalp incision was sutured closed after decerebration and these animals were used in Habituation Training experiments (see later). Subsequently, use of two of these animals (DC5 and DC8) was continued for the brain transection experiments.

Most experiments were conducted with the use of a temperature- controlled body plethysmograph (described in Section II) and consequently in the latter stages of recovery the animals were kept in the plethysmograph so that the body temperature (Tb), blood pressure, HR and respiration could be monitored.

Reversible Section Of The Brainstem Using Xylocaine

Areas of the hypothalamus in the chicken (Richards 1970, 1971) and the duck (Simon et al. 1978,1981) are responsible for thermoregulatory control. For this reason, confirmation of

complete transection across the brainstem below the hypothalamus would be indicated by loss of thermoregulatory function.

Decerebrate animals were positioned in the stereotaxic apparatus. Since the plethysmograph could not be used at the same time, blood pressure, HR and Tb only were monitored. The dorsal brainstem was exposed and a Xylocaine-filled glass micropipette (tip diameter: $30\mu m$) positioned by means of a micromanipulator directly over the caudal end of one optic lobe. (See Appendix I for details of the micropipette injecting system.) The longitudinal axis of the pipette was tilted at $54°$ from vertical in alignment with the intended plane of transection passing from the posterior commissure to a point just below the hypophyseal foramen. The micropipette was advanced 8 mm into the brain tissue. As it was slowly withdrawn, 0.5 μl Xylocaine was deposited for every 1 mm raised. Once lifted clear the micropipette was moved 1.5 mm laterally and advanced into the tissue again, with Xylocaine being deposited as before in 0.5 ul quantities for every 1mm raised. In this manner, Xylocaine was deposited in a planar array across the brainstem. The injection procedure usually took up to 8 minutes to complete. Tb was continuously monitored and usually began to drop toward the end of the injection period.

The animal's head was freed from the apparatus and immersed into a beaker of water for diving, but only if a drop in Tb had occurred during the Xylocaine blockade. Care was taken to immerse the head to just above eye level and to ensure that no water spilled into the cranial cavity. Control experiments were

carried out using saline injections (0.9% NaCl) and although Tb did not drop, dives were done at about the same time after saline injection that Tb would have fallen were Xylocaine used.

Mechanical Brainstem Transection

To accomplish transection between the mesencephalon and the diencephalon, the animals were re-positioned in the stereotaxic apparatus. A narrow scalpel blade was exchanged for the micropipette in the micromanipulator and the blade drawn through the brain in exactly the same plane as the Xylocaine blockade. Section of tissue down to the ventral side of the brain was completed with a hand-held scalpel.

Experimental Protocol

Prior to decerebration, the animals were placed within a temperature-regulated body-plethysmograph and were subjected to 2 minute submersions during which HR, BP and Tb were recorded. Pre-dive and post-dive ventilation was also recorded except during dives following Xylocaine blockade. Samples for blood gas analysis were taken before submersion and in the 10 seconds just before the end of the dive.

The animals were allowed a minimum of 1 day to recuperate from decerebration during which they were regularly force-fed water. Following brain transection, the animals were allowed at

least 6 hours to recover during which time Tb was closely monitored and maintained at $41\pm1.0°$ C by means of electric heating pads and infra red lamps.

The dives were replicated in each condition for every animal. In addition to the test dives, two of the mesencephalic preparations were subjected to a few 4 minute submersions.

At the end of the experiments, the animals were killed with an overdose of anaesthetic (Sodium Pentobarbital i.v.) and the heads removed and placed in 5% formal-saline. Two weeks later, the brains were removed and processed for paraffin section as described in Appendix II. The plane of transection was confirmed by inspection of 12 μm midline sagittal sections stained with luxol fast blue 'G' and neutral red.

RESULTS

Xylocaine blockade

The temporary removal of hypothalamic control following Xylocaine blockade was marked by a loss of thermoregulatory function. The fall in Tb reached a maximum approximately 18 - 20 minutes from the end of the injection period. The mean fall in Tb for all animals was 1.2±0.2°C and this value is similar to the fall in Tb produced approximately 20 minutes after brain transection (Figure 23). Body temperature usually returned gradually to normal by about 45 minutes to an hour after injection. Control experiments with saline injection never showed a drop in Tb. In one animal Tb was monitored for 2.5 hours after death and it was found that it took 1hour 20 minutes for Tb to fall 1°C.

Plane of transection

Figure 24a is a composite diagram of a sagittal view of the brain of a Pekin duck showing the level of decerebration and the plane of section between the mesencephalic and diencephalic regions. Figure 24b is a photograph of a mid-sagittal section from a brain transected at the rostral level of the mesencephalon.

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Figure 23.   Effect of Xylocaine blockade and transection on
        body temperature.   Closed circles represent values
        obtained after infusion of Xylocaine across the rostral
        mesencephalon.   Open circles represent values obtained
        after mechanical transection.   Vertical dashed line
   indicates the time of Xylocaine injection and mechanical
        transection.
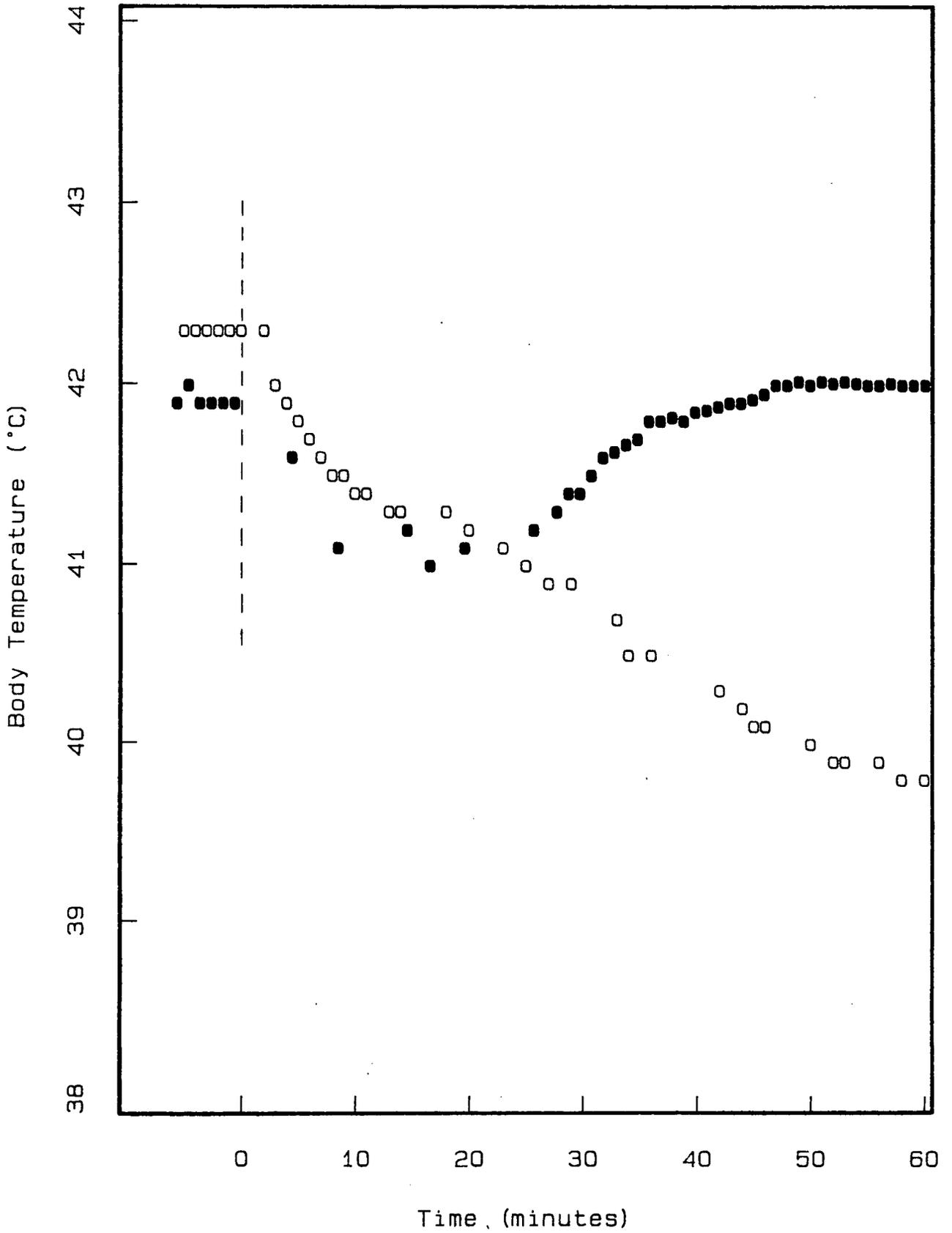
Figure 24.   A) Schematic view of a mid-sagittal section of
        intact brain from the Pekin duck (Anas platyrhynchos)
        indicating level of decerebration and mesencephalic
        transection.   B) Photograph of mid-sagittal section
        from a mesencephalic preparation.   (Stained with luxol
        blue and neutral red)

DECEREBRATION

TRANSECTION
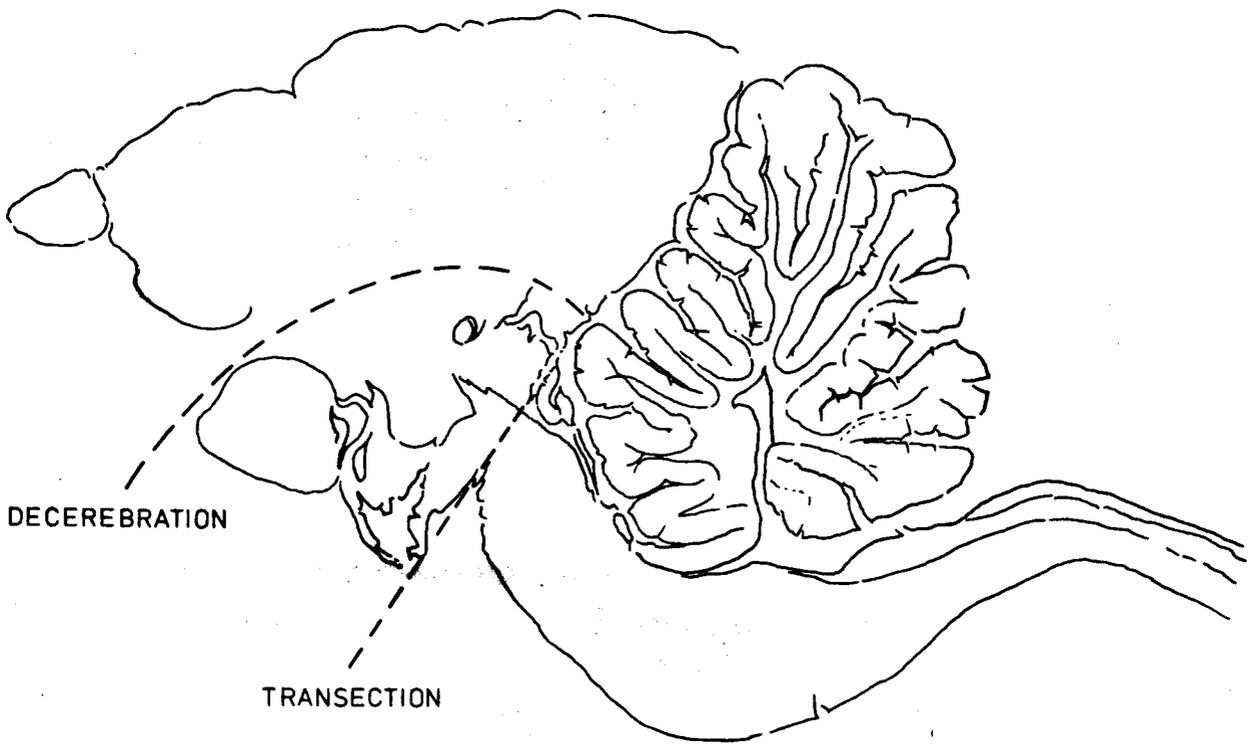
Diving performance

Resting HR and MAP usually decreased after decerebration and the ducks, though still responsive, were much more passive. 24 hours after surgery, they would often walk spontaneously and would occasionally flex their wings. Following transection below the diencephalon, the ducks never exhibited spontaneous activity although they would sometimes stand if provoked. Differences between resting values from intact and transected animals for HR, MAP and blood gases were not significant at the 0.05 probability level.

The profile of the fall in HR during submersion was the same for all intact and experimental conditions. There was no significant difference between the rates of fall for the first 30 seconds nor the maximum levels of bradycardia achieved at the end of the dives (Figure 25). Despite the considerable bradycardia, MAP for all animals was maintained and matched pre-dive levels (Figure 26). The mean fall in PaO_2 by the end of 2 minutes submersion for the intact, decerebrate and mesencephalic (including both Xylocaine and mechanically transected) animals was 43.5, 40.6 and 43.1mm Hg respectively; these were not significantly different (P>0.05). $PaCO_2$ rose some 13 - 16mm Hg during submersion and the rise was identical under all experimental conditions (Figure 27).

There was a profound difference in the breathing pattern during recovery from submersion between intact and decerebrate animals when compared with mesencephalic preparations (Figure 28). Typically, minute ventilation increased immediately after

Figure 25.   Effect of various levels of brain transection on
        the cardiac response to 120 seconds submersion.   A.
        Intact animals (N=7, n=14), B.   Decerebrate animals
        (N=7, n=14), C.   'Xylocaine' animals (N=6, n=6), D.
        Mesencephalic animals (N=7, n=14).   (Error bars ±S.E.)
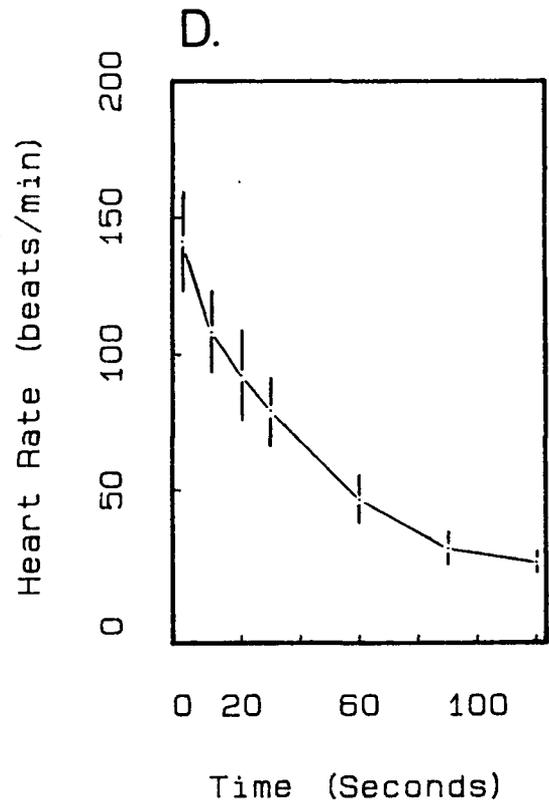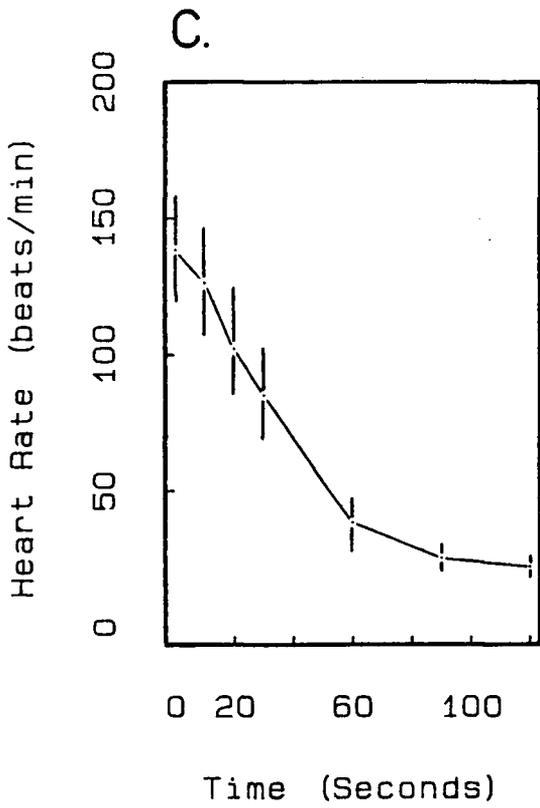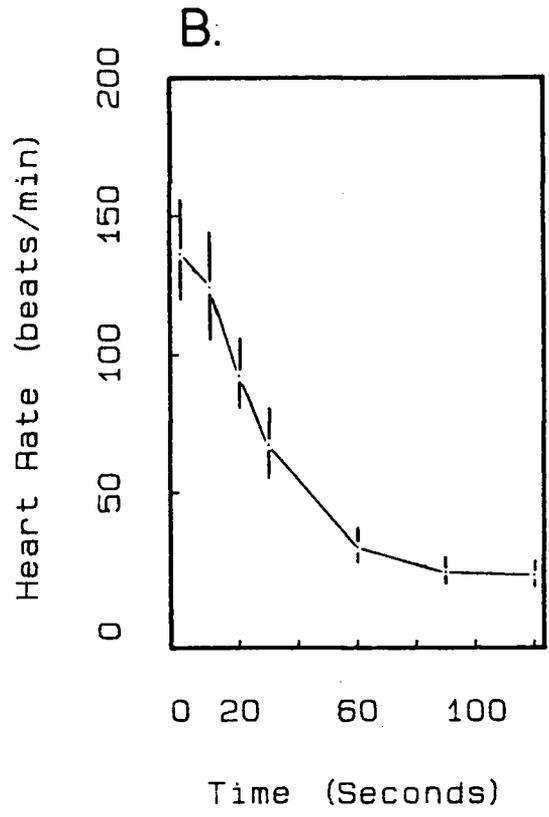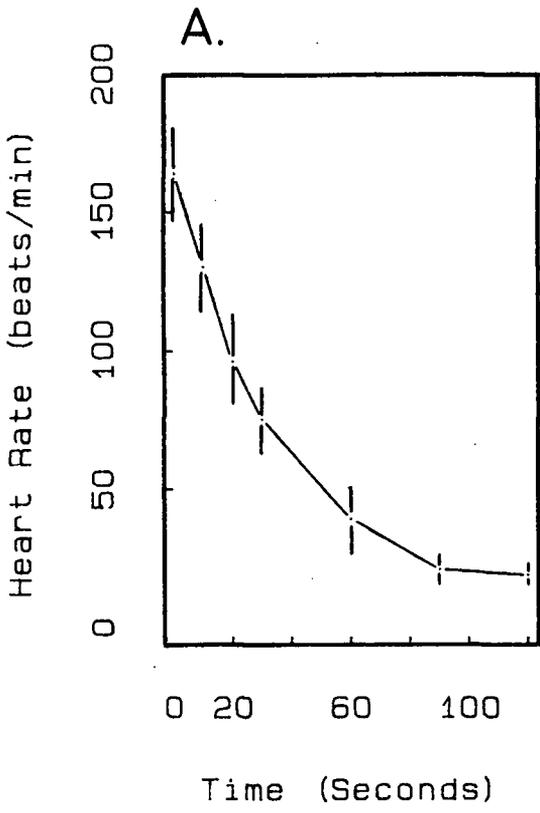
Figure 26.   Effect of various levels of brain transection on
     arterial blood pressure during 120 seconds submersion.
     A.   Intact (N=3, n=6) B.   Decerebrate (N=3, n=6) C.
     'Xylocaine' (N=3, n=6) D.   Mesencephalic (N=3, n=6)
   (Error bars ±S.E.)

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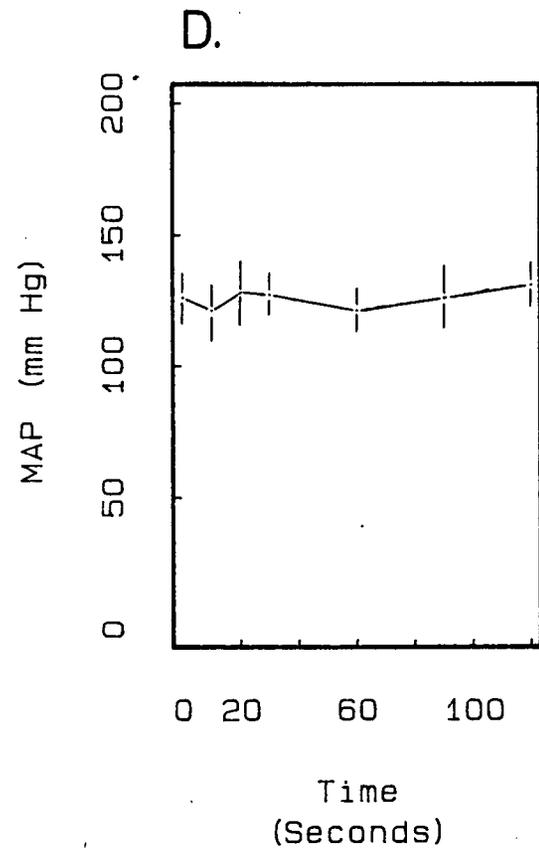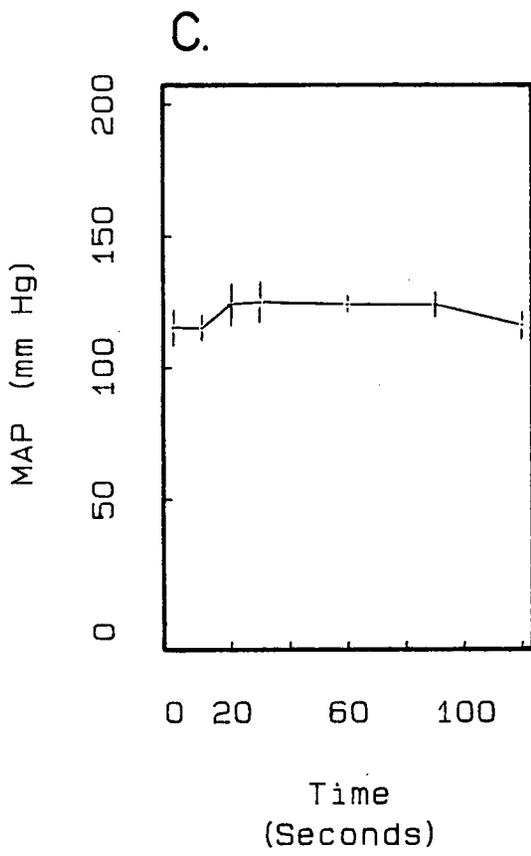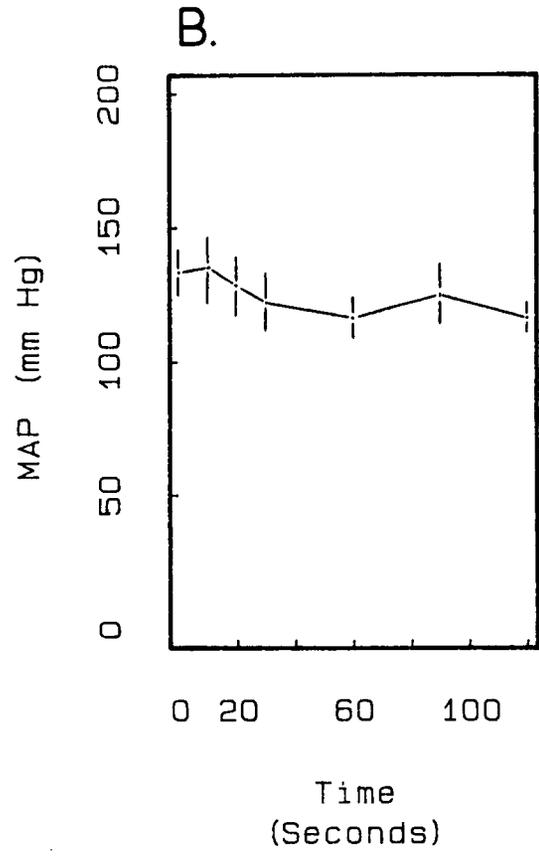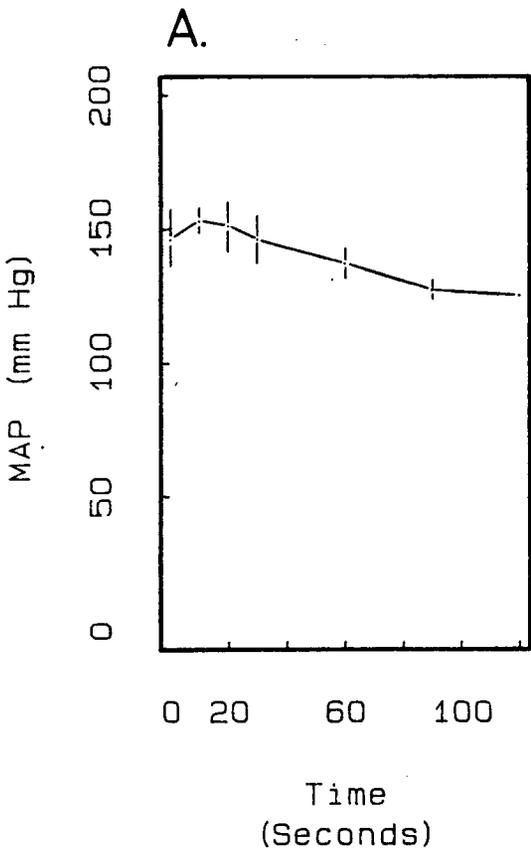
Figure 27. Effect of brain transection on end-dive blood gas
     levels and pH.   Hatched bars represent values obtained
     at the end of 120 seconds submersion.   (Error bars
     ±S.E.)
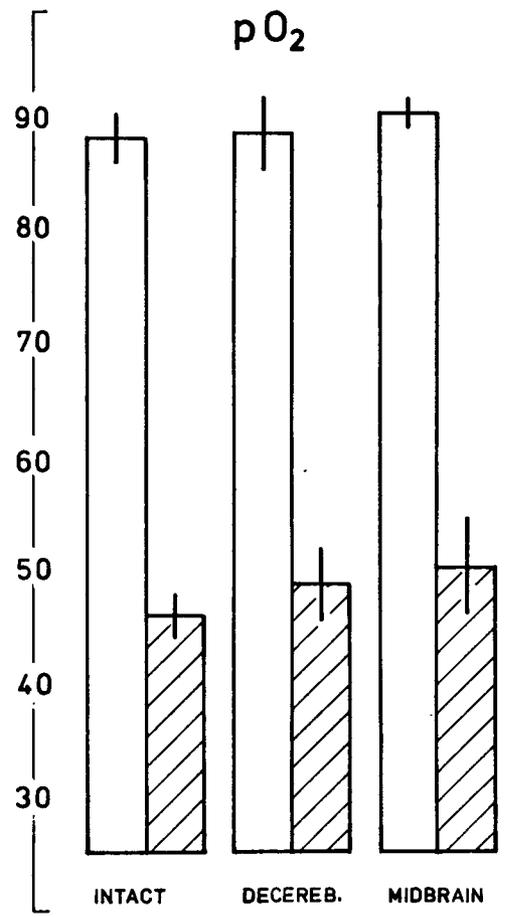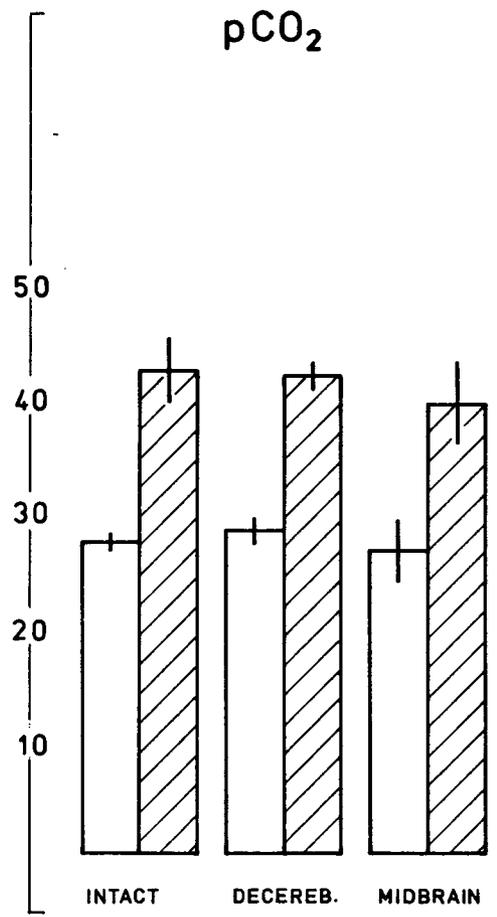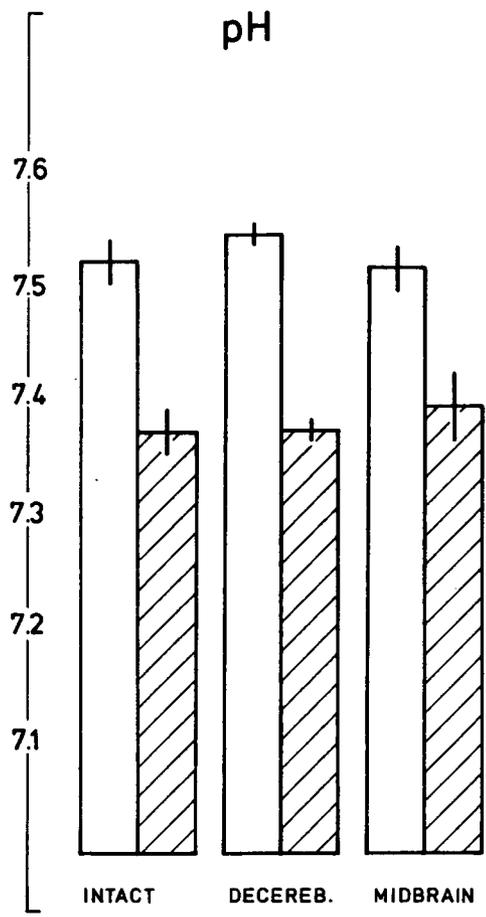
Figure 28.   Effect of brain transection on breathing in the
        recovery period following 120 seconds submersion.   A.
        Intact animals B.   Decerebrate animals C.
        Mesencephalic animals Vertical dashed line indicates the
        end of submersion; the point left of this line on each
        graph represents pre-dive values for ventilation.
        (N=3, n=6.   Error bars ±S.E.)

A.
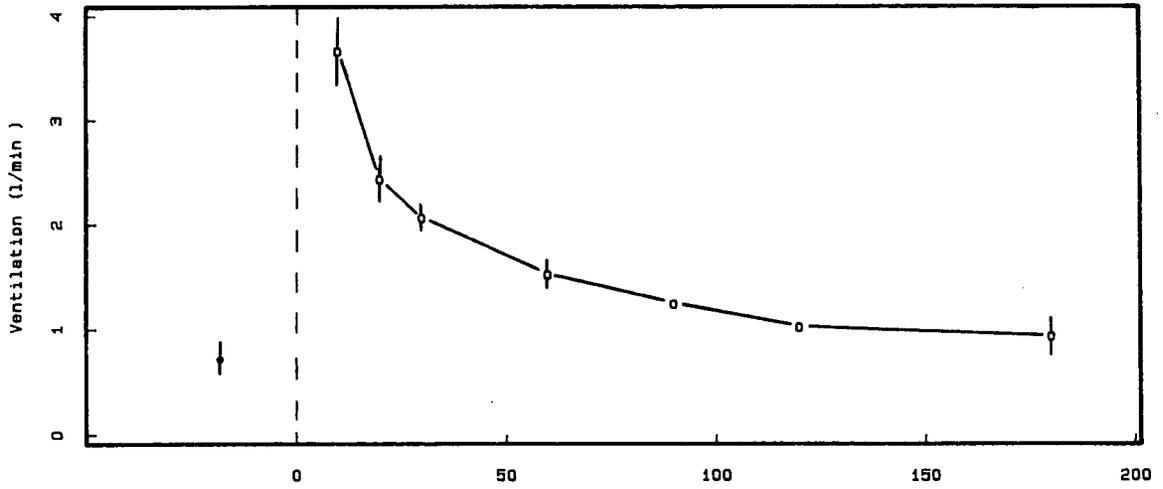
B.
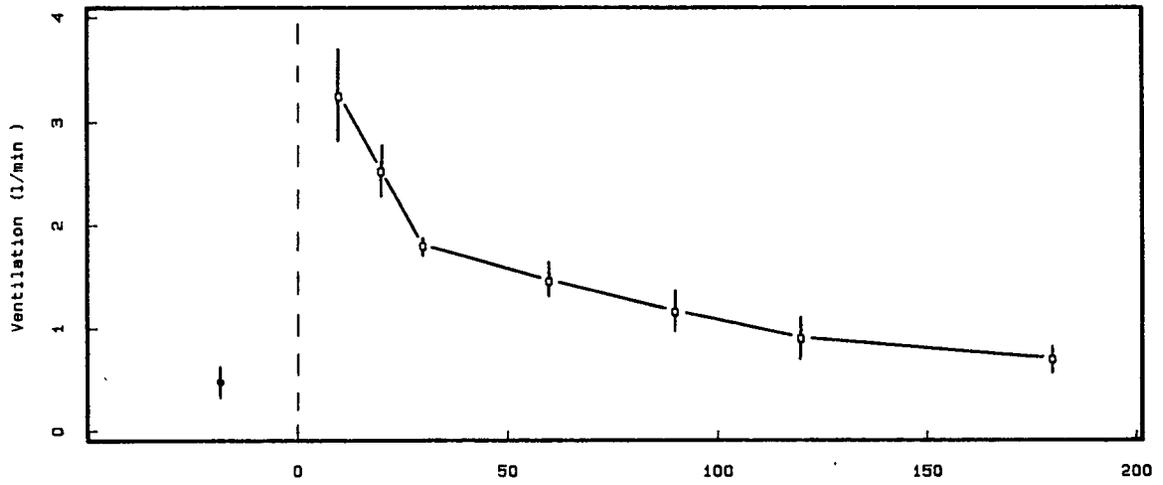
C.
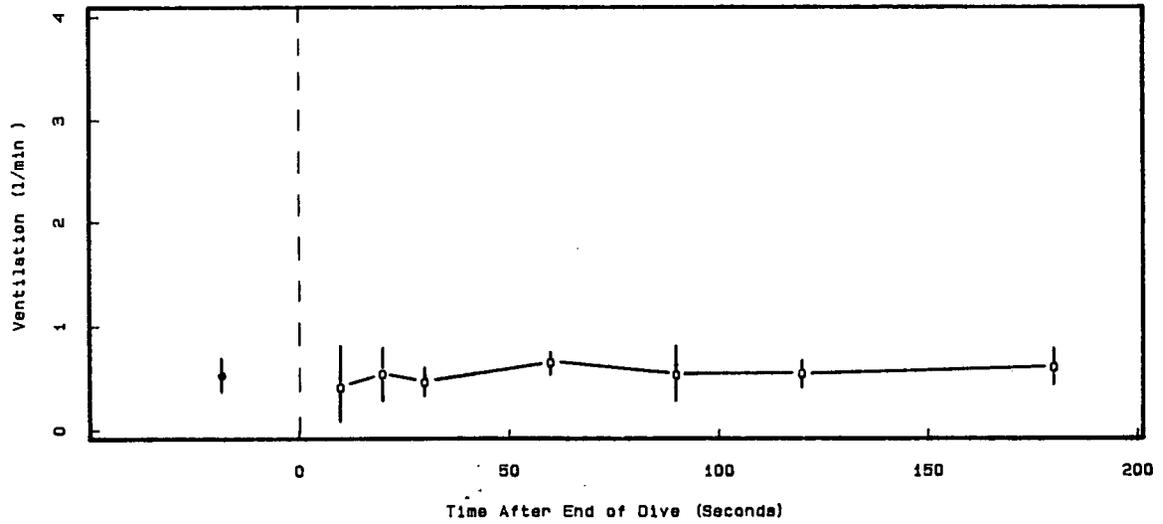
Time After End of Dive (Seconds)

surfacing by about 4 to 5 times, falling gradually to normal after about 3 minutes. This was not the case for mesencephalic preparations which never exhibited hyperventilation. The pattern of breathing was irregular and sometimes mildly gasping. The mesencephalic animals survived 4 minute submersion periods all the while maintaining bradycardia, but again following these extended dives, minute ventilation was little different from resting values.

Effect of Habituation Training of Decerebrate Ducks

METHODS

Three ducks (DC5,DC8 and H) were decerebrated as described in the preceeding experiments and allowed to recover for a minimum of 6 weeks. 65 mg antibiotic (Penbritin, Ayerst Laboratories) dissolved in 1.5 mls saline were administered intramuscularly for the first 6 days of recovery. The animals were regularly force-fed food and water and were kept in a temperature controlled room. Within a few days after surgery all these animals walked spontaneously when released from their cages and would respond, though somewhat sluggishly to loud noises. One animal was allowed to recover for much longer and after a few months was feeding and drinking voluntarily. This animal (H) was eventually self sufficient enough to be returned to the Animal Care Unit with the stock animals where it remained for 4 years.

Each of the ducks underwent habituation training as described in Section II Methods.(DC5 and DC8 for 3 days, 15 trials per day; H for 2 days, 15 trials per day) Heart rate was obtained from the ECG in the usual manner.

RESULTS

The two ducks (DC5 and DC8) allowed a shorter post-operative recovery showed very little, if any, habituation of diving bradycardia (Figure 29). Duck H, however, showed substantial reduction of bradycardia on the first day and further habituation on the second day. Whereas the first dive evoked a 70% drop in HR, by the sixth trial of the second day, HR dropped by only 4% of the pre-dive HR (Figure 29).

Figure 29.   Effect of habituation training on three
        decerebrate animals.   Duck H: 4 years after
        decerebration, ducks DC5 and DC8: 6 weeks after
        decerebration.

duck: H   duck: DC8   duck: DC5

Percentage of Pre-Dive Heart Rate

Trials
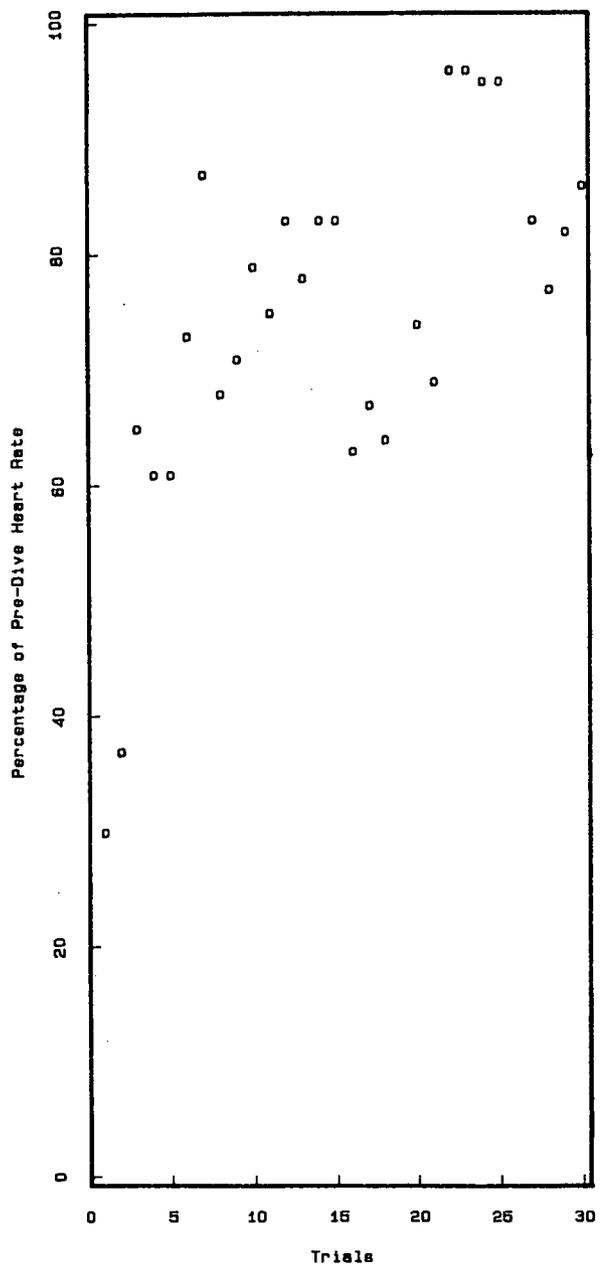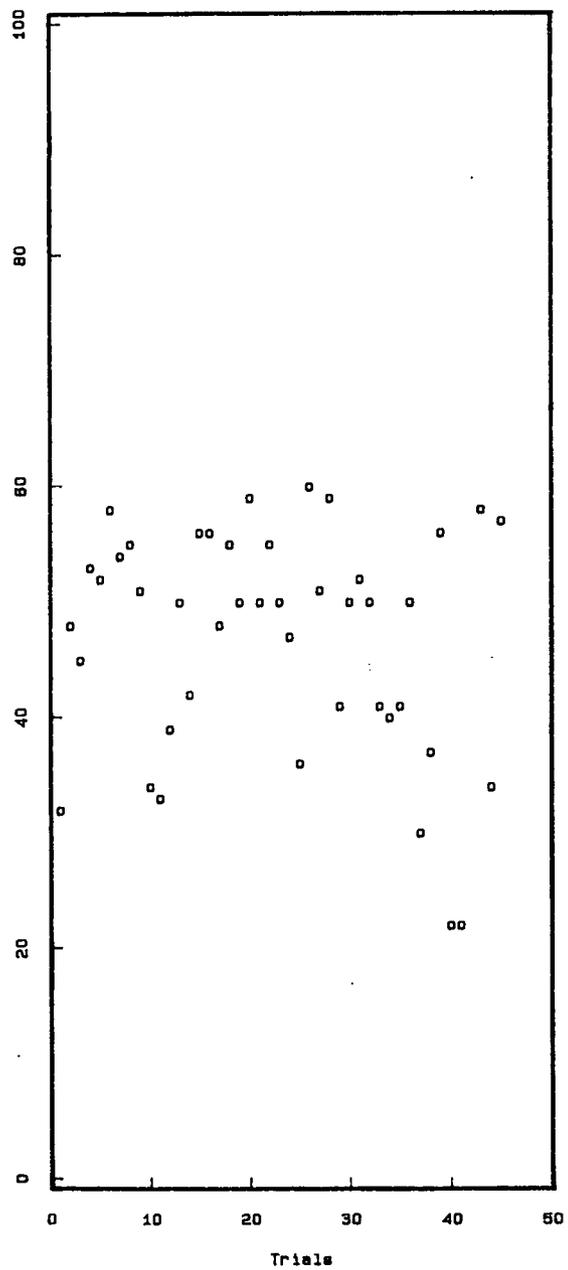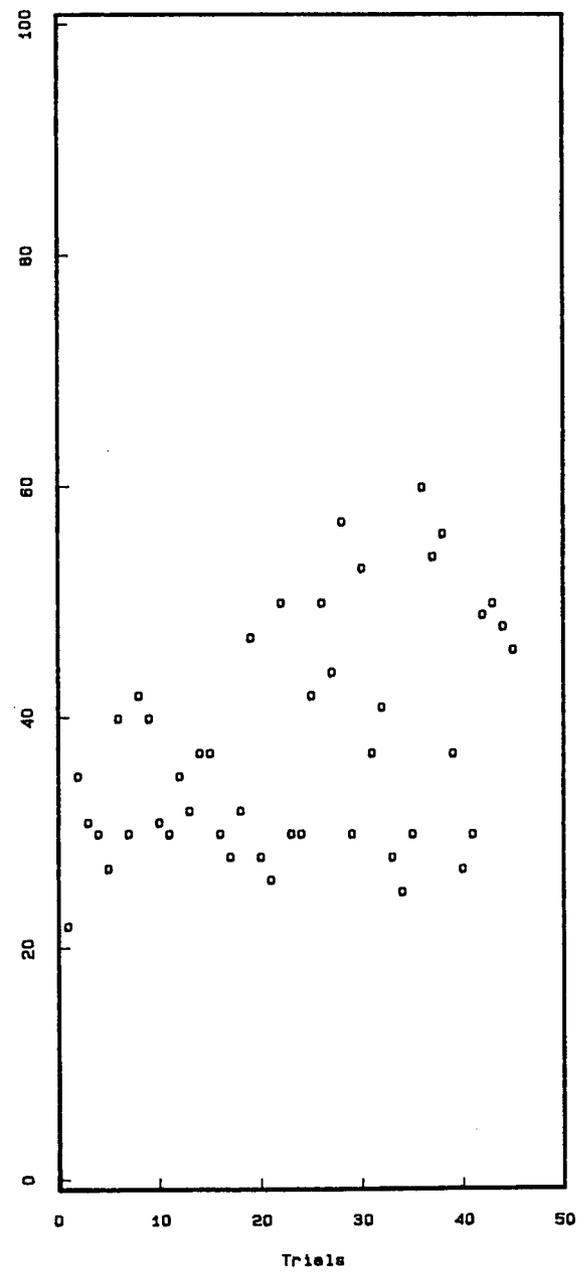
DISCUSSION

The results of this study confirm those of Huxley (1913) and Andersen(1963). Regardless of the absence of diencephalic regions, it was demonstrated that in addition to bradycardia during submersion, MAP is maintained at pre-dive levels despite a 70% drop in HR. This suggests that a compensatory increase in peripheral vascular resistance must occur which serves, as part of the diving response, to limit the loss of stored oxygen. Similar end-dive blood gas values and the fact that mesencephalic preparations exhibited cardiovascular adjustments to two-minute submersions that were indistinguishable from intact animals, confirm that the diving response is not lost. Moreover, the ability of these preparations to survive four-minute periods of submersion demonstrates the effectiveness of the responses (Kobinger and Oda 1969; Bryan and Jones 1980). Thus, the control of cardiovascular adjustments and apnoea appears to be localised within the caudal brainstem, below the rostral border of the mesencephalon. Consequently, these results argue against a diencephalon-mediated chemoreceptor-driven bradycardia (Korner 1971) and they demonstrate that fear (mediated through the diencephalon) is not an essential component of the diving response (Smith et al. 1974; Smith and Woodruff 1980; Kanwisher et al. 1981).

The hypothesis that suprabulbar regions are essential for the diving response must be reassessed. It has been assumed that suprabulbar involvement is necessary for multidirectional and differential changes in vascular resistance (Uther et al.

1970; Korner 1971; Janig 1975; Manning 1977); however, the present data suggest that the concept of a highly differential control of various vascular beds in the diving response should be rejected. The predominant feature of vasomotor change during submersion is the widespread increase in peripheral vascular resistance. Consequently, the suggestion could be made that, during submersion, sympathetic discharge is increased to vessels throughout the vascular system. That is, the level of sympathetic activation would be close to maximum. Complete vascular shutdown does not occur because of the uneven density of vascular innervation and the presence of adrenergic and cholinergic vasodilatatory fibres. Muscular, mesenteric and renal vessels, for instance, are densely innervated with sympathetic fibres and this is reflected in the greatest decrease to flow in these tissues (Jones et al. 1979; Zapol et al. 1979; McKean 1982). Cerebral, coronary and lung vessels on the other hand are sparsely innervated and blood flow to these tissues has been variously reported as either increasing, remaining the same or decreasing slightly. It also seems likely that autoregulatory adjustments to hypoxia and hypercapnia in the heart and brain will counter neurally mediated vasoconstriction and increase blood flow. Dilatation during extreme hypoxia has been reported to occur predominantly in coronary and cerebral vessels in contrast to the minimal response of renal and limb vessels (Daugherty et al. 1967; Heistad et al. 1975). Thus, one of the consequences of hypoxic hypercapnia during submersion is almost complete vascular

sympathetic activation causing widespread vasoconstriction, excluding a few specific tissues requiring maintained or even increased blood flow.

In diving some degree of differential control, mediated by higher nervous centres, may exist in intact animals as part of the cardiovascular adjustments but its occurrence is likely to be limited. For instance, it is uncertain how cutaneous vascular beds respond to submersion. Djojosugito and co-workers (1969) have demonstrated that blood flow in the web of a duck is preserved during submersion primarily through arterio-venous shunts. Maintained cutaneous flow was also demonstrated in the diving seal (Zapol et al. 1979). Studies of skin blood flow in the rabbit ear (Chalmers and Korner 1966) and dog paw (Heistad et al. 1975) have revealed vasodilatation in response to chemoreceptor stimulation by arterial hypoxia. Strong evidence has been obtained to suggest that this is due to differential sympathetic activity (Iriki et al. 1972; Iriki and Kozawa 1975, 1976). A redistribution of blood flow away from skeletal muscle toward the skin can be visualised as part of oxygen conserving adjustments in view of the abundance of A-V anastomoses in skin and the low rate of oxygen consumption of this tissue. In rabbits, control of this differential activity resides in suprabulbar regions as it is lost following removal of the hypothalamus (Iriki and Kozawa 1976). It is uncertain, however, what proportion of blood flow is affected by these changes in cutaneous vascular resistance and therefore how much of it contributes to oxygen conservation. Much less is known

regarding parasympathetic activity, but vagal cardioinhibitory output appears maximal as it is difficult to decrease heart rate further without provoking vagal escape.

The picture that begins to evolve is that during submersion, both the sympathetic and parasympathetic divisions are maximally activated, with very little in the way of differential control. Adequate perfusion continues to the brain, owing to its sparse vascular innervation and powerful autoregulatory control (Berne et al. 1981; Kontos 1981). If the diving response demands some bidirectional control, such as a possible vasodilatation of the adrenal glands (McKean 1982) and cutaneous beds, then the effect on overall MAP is immeasurable.

Stimulation induced by submersion does not demand much of the CNS: just maximal widespread activation of the autonomic system, quite within the capacity of lower brainstem control. Small regional differences that may be peculiar to the diving response would thus be relatively insignificant by comparison to the large scale shutdown. Transection experiments will not reveal the detailed features of higher CNS control, unless specific and highly localised changes in flow are measured. In the case of responses requiring greater vascular differentiation, such as the defence reaction, vasodilatation is more easily measured in the large muscle beds and would be noticeably abolished following transection.

An interesting observation was the loss of post-dive hyperventilation. Whereas the pattern of breathing after a dive

appeared similar for each level of section, it was absent in only the mesencephalic preparations. Unfortunately, it is not known how long it took for arterial blood gas levels and pH to return to normal as these were not measured during the dive recovery period. Although the plane of section corresponded to the rostral border of the mesencephalic region, certain damage may have spread caudally to known respiratory control centres. Areas within the midbrain mediating polypnoea have been identified with respect to thermoregulation in the pigeon (Richards 1971) and stimulation of these sites results in increased respiratory frequency in the duck (Kotilainen and Putkonen 1974). The loss of the normal post-dive respiratory pattern in mesencephalic animals confirms that important respiratory control resides in regions at or above this level of the brainstem.

With regard to the effect of habituation training on diving bradycardia in decerebrate animals, the results from the animals tested are far from conclusive. The finding that the two ducks allowed to recover for 6 weeks post-decerebration showed no habituation suggests that modification of the chemoreceptor driven bradycardia originates in rostral CNS regions. However, when allowed a far greater recovery period, one duck demonstrated considerable habituation by only the second day of training. From these results, it is difficult to say whether the first two animals were still suffering post-surgical trauma or that perhaps some degree of "plasticity" may have occurred in duck H. The suggestion could even be made that, on the basis of

the speed of habituation in duck H, habituation of the
bradycardia in intact ducks is inhibited by rostral regions of
the CNS. Clearly, these findings indicate the need for further
investigation of CNS control following long term recovery from
brain transection.

GENERAL DISCUSSION

From the preceeding experiments in the Pekin duck, cardiovascular adjustments to prevent asphyxiation during submersion are instigated primarily by the stimulation of central and peripheral chemoreceptors. The peripheral receptor group, located within the carotid bodies, is responsive to both the rising $PaCO_2$ and the declining PaO_2, and causes both bradycardia and a substantial part of the increase in peripheral vascular resistance. The fall of PaO_2 appears to be the more potent stimulus to the peripheral chemoreceptors and accounts for approximately two-thirds of the total change in heart rate. The rise of $PaCO_2$, on the other hand, contributes by decreasing heart rate a further 20%. Other inputs, such as baroreceptive reflexes, account for the remaining bradycardia (approximately 12%). Peripheral chemoreceptor activation also serves to increase vascular resistance, with an apparently similar ratio of contribution from both PaO_2 and $PaCO_2$. The cumulative change in resistance caused by these receptors amounts to some 60% of the total. The rest of the vascular resistance change appears to be due to central chemoreceptors. This group is extremely sensitive to an increase in $PaCO_2$, and causes some 30% of the total increase in vascular resistance. Surprisingly, the central region also appears to be affected by a falling PaO_2, and 10% or so of the change in vascular resistance can be attributed to low PaO_2 acting centrally. Baroreceptor input appears far less significant in the development of cardiovascular responses to submersion and serves mainly to

mitigate the changes and maintain arterial blood pressure via the barostatic reflex. Peripheral and central chemoreceptors are thus of paramount importance in the generation of responses to forced submersion.

Although central and peripheral chemoreceptors predominate for the cardiovascular responses seen in forced dives, it is difficult to quantify and assess the role of chemoreceptors in animals in natural settings because they may be responding to so many more inputs. The moment of submergence is crowded with diverse sensory information including that from pulmonary receptors, vestibular receptors and facial or narial receptors. On the basis of the immediate change in heart rate with submersion, it seems that diving ducks (such as redheads), unlike dabbling ducks (such as Pekins), respond to receptors other than chemoreceptors when diving. This is supported by recent experiments on diving ducks, where the initial bradycardia evoked in forced submersion is substantially reduced following application of a local anaesthetic to the mucous membranes of the nasal passages (Furilla and Jones 1985). However, there is little doubt that chemosensory reflexes remain important as hypoxia and hypercapnia develop during prolonged submersion. The role of peripheral chemoreceptors was demonstrated in experiments studying the cardiac response of spontaneously diving ducks that had the nerves to their carotid bodies sectioned. The initial drop in heart rate appeared unaltered while bradycardia was slightly reduced at the end of longer dives (Butler and Woakes 1982 a,b).

It is apparent that diverse reflex pathways contribute to the development of the diving responses; however, it is also certain that higher CNS centres impose powerful control or modification of the cardiovascular system (Folkow et al. 1965; Folkow and Neil 1971; Korner 1971). For instance, anticipatory reduction in heart rate before submergence and increase before surfacing is indicative of some form of associative learning (Jones et al. 1973). Moreover, the failure of some birds and mammals to exhibit as great a fall in heart rate during voluntary dives compared with forced dives could mean that, under natural conditions, peripheral sensory inputs are "ignored" either by conditioning or by habituation.

The demonstration, in this study, that the cardiac chronotropic response to forced submersion will readily habituate following repetitive diving lends support to the contention that diving responses can be modified by learning. It seems reasonable to suggest that the oxygen conserving adjustments evoked by submersion may be unnecessary in animals diving for periods which are well within their aerobic capacity. Habituation of these responses may therefore be an important means by which these animals adapt to their environment.

Another, though perhaps less tangible, feature of higher central nervous control is the arousal state or disposition of the animal. In this regard, suggestions have been made that the responses incurred by forced submersion may have less to do with diving than with the defence or fear reaction (Smith and Woodruff 1980; Kanwisher et al. 1981; Smith and Tobey 1983).

However, the demonstration that decerebrate animals sectioned at the rostral border of the mesencephalon continue to exhibit profound diving bradycardia and can even withstand four-minute periods of submersion seems contrary to the view that the response is a defence mechanism. This is also supported by the finding that bradycardia does not develop in naive ducks submerged after they have been breathing pure oxygen. It seems clear that the observed responses are engendered by submersion per se and that, on occasion, these responses may be profoundly altered by higher centres and not vice versa.

In view of the finding that only a minimal amount of neural substrate is required to generate effective cardiovascular responses to submersion, it is tempting to speculate on the degree of neural control involved in the responses. Descriptions in the literature frequently refer to the "selective redistribution" of blood away from hypoxia-tolerant tissues to the brain and heart which are especially susceptible to lack of oxygen. The response is characterised as being the result of differential output of sympathetic activity so that discriminative vasoconstriction produces regionalised blood flow to or from select capillary beds (Johansen 1964; Folkow et al. 1965). Bidirectional control of this type is further suggested to be derived from suprabulbar centres, such as the hypothalamus (Korner 1971; Hilton 1975; Manning 1977). Thus, the occurrence of the intact diving response in the absence of these rostral brainstem centres suggests an alternative hypothesis for the generation of blood redistribution. For example, vasodilatation

evoked locally by hypoxia is not uniform in all vascular beds, and is instead selective (Heistad and Abboud 1980). For instance, dilatation occurs predominantly in coronary and cerebral vessels, whereas the response in limb vessels is weak, and renal vessels do not even dilate at a PaO_2 of 20 mm Hg (Daugherty et al. 1967; Heistad et al. 1975; Grubb et al. 1977,1978). In addition to this, the density of sympathetic innervation of the vasculature varies between tissues. Therefore, the chemoreceptor information impinging on the CNS during submersion may provoke the simultaneous activation of both components of the autonomic nervous system. Parasympathetic discharge would cause extreme bradycardia and sympathetic discharge would produce intense vasoconstriction of the more densely innervated vessels. Selective redistribution of the blood flow is thus achieved through a balance between powerful autoregulatory vasodilatation and neural vasoconstriction.

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APPENDIX I

A method is described whereby localised regions deep within brain tissue were reversibly inactivated with injections of lidocaine hydrochloride (Xylocaine 2%, Astra Pharmaceuticals). The injection system is illustrated in figure 24.   It consisted of a glass micropipette which was connected by polyethylene tubing (P.E.   90, Clay Adams) to a three-way stopcock.   One port of the stopcock was connected to a micrometer-driven syringe (AGLA, Burroughs Wellcome Co., England) which was filled with mineral oil.   The other port was connected to a syringe filled with Xylocaine.   After the micropipette was backfilled from the Xylocaine syringe, the stopcock was turned to connect the micropipette with the mineral oil in the second syringe. In this way, as the micrometer was turned, the oil/xylocaine interface could be easily seen moving along the P.E. tubing. Advancing the micrometer by 0.1mm increments ejected $0.5\mu l$ aliquots of xylocaine from the pipette tip.   The micropipette was mounted in a standard micromanipulator clamped on one rail of the stereotaxic apparatus (Narishige, Japan).

Figure 30.   Appendix I.   Schematic diagram illustrating
    arrangement for Xylocaine injection.
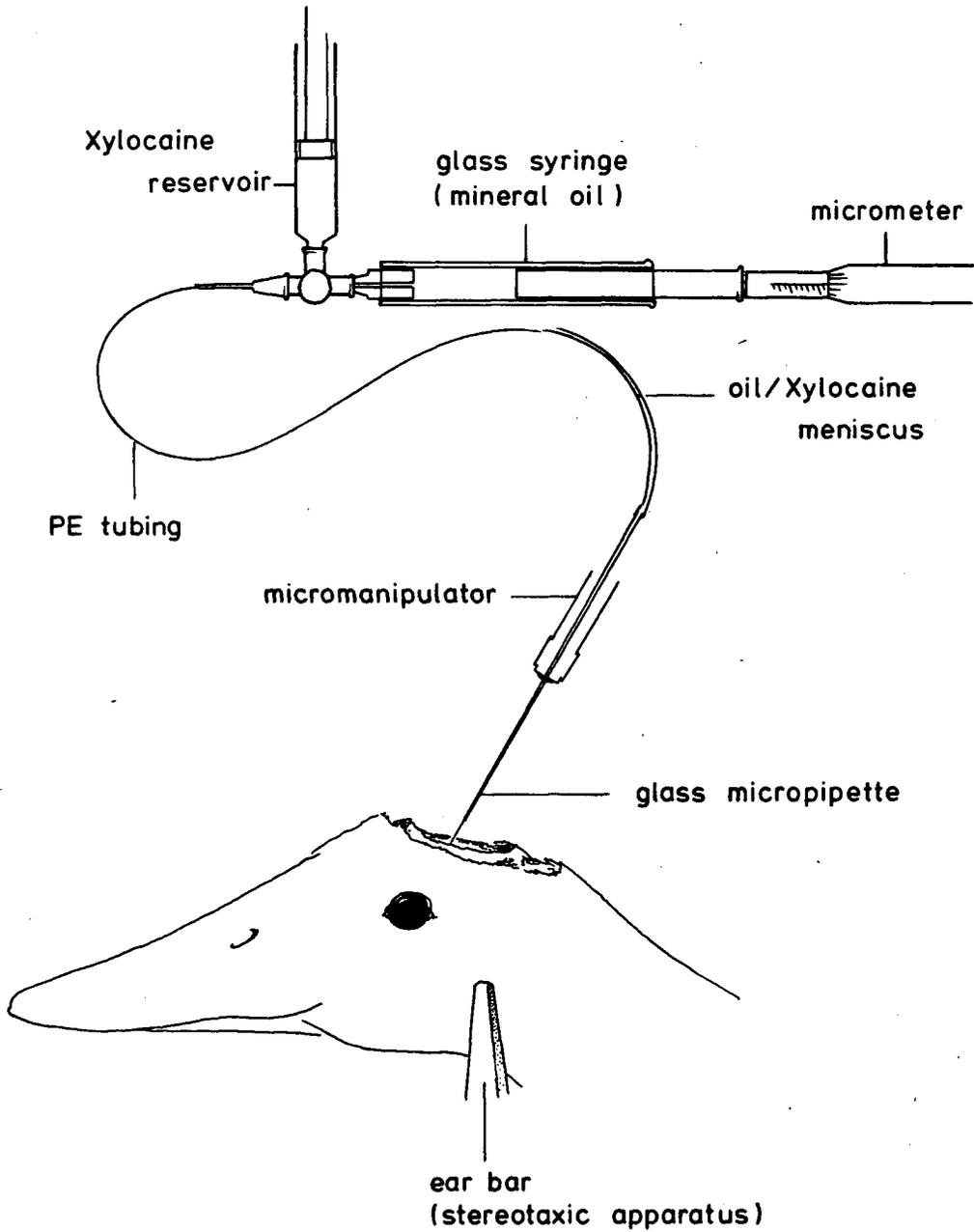
APPENDIX II

Immediately after death the animal was decapitated. Skin, feathers, and soft tissue around the skull were removed. As much brain tissue as possible was exposed by carefully clipping away skull bone with ronguers. The lower jaw was completely removed and the ventral surface of the brain exposed. The rest of the beak, the neck muscles, trachea and eyeballs were also removed and the head was then immersed in 4-5% formaldehyde in 7.5% saline.

After 3 weeks, the hardened brain was carefully removed from the skull and replaced in fresh fixative until ready for processing.

Clearing: The brain was washed in running water for 24 hours.

Dehydrating:

Soaked in the following sequence of solutions:

a) 50% ethanol overnight;

b) 70% ethanol for 24 hours;

c) 80% ethanol for 24 hours;

d) 95% ethanol for 24 hours;

e) repeat;

f) repeat;

g) 85% amyl acetate for 24 hours;

h) repeat;

i) toluene for 24 hours;

j) repeat;

k) 50% toluene: 50% melted paraffin overnight at 60°C;

l) melted paraffin (paraplast "+") for 24 hours at 60°C under 15 lbs vacuum;

m) repeat with fresh paraffin;

n) repeat;

o) embed in fresh de-gassed paraffin.

Sectioning: Saggital sections were cut, 12 um thick, on a rotary microtome (Spencer 820). Sections were floated in a water bath at 54°C and mounted on double coated chrome/alum subbed slides. Slides air dried overnight at 35°C.

Staining: Sections were stained with Luxol Fast Blue 'G' and Neutral Red counterstain.