VENTILATION AND DIVING APNOEA IN *RANA PIPIENS*

by

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ABSTRACT

Two types of ventilation cycle were recorded in unanaesthetised but restrained frogs (*Rana pipiens*); one concerned with ventilation of the buccal cavity alone (buccal cycle) and the other with lung ventilation (lung cycle). During the former the nares were open and the glottis closed so that only small pressures were generated by the movement of the buccal floor. The onset of a lung ventilation was signalled by activity in the laryngeal dilator muscle and when the glottis opened lung pressure and volume fell while buccal cavity pressure and volume increased. After narial closure the buccal floor was rapidly raised and gas was forced into the lungs from the buccal cavity. At peak pressure in the lungs and buccal cavity the glottis closed and nares opened, the recovery stroke of the pump being passive. Air flow recordings made at the external nares showed two phases of flow during each buccal cycle, while four phases accompanied each lung ventilation cycle.

By plotting pressure/volume loops from the buccal pump an analysis was made of the mechanical work performed in one lung ventilation cycle, and the proportion of this work available for lung inflation after various losses against viscous and flow resistive forces in the pump itself; while measurement of the areas of typical sequences of such loops together with respiratory frequency enabled the mechanical work output of the pump to be determined for frogs ranging in size from 24 to 86 grams. Using Hill's classical equation for muscle efficiency, it was possible to estimate mechanical efficiency for single respiratory cycles by calculating the heat of maintenance and heat of shortening of the buccal floor muscles, while simultaneously measuring mechanical work output. Calculated efficiencies of lung ventilation cycles rose as mechanical work performed increased from 7.4% at 0.65 gram.cm/cycle to
19.3% at 2.73 gram.cm/cycle.

Diving apnoea in *Rana pipiens* was induced by the presence of water at the level of the external nares, at which point the nares closed, no water entering the buccal cavity during the dive. Occasional ventilation cycles occurred during the dive in which gas entered the buccal cavity from the lungs, an equal volume then being pumped back into the lungs, but there was no ventilatory exchange with the external medium. Bilateral section of the trigeminal nerves resulted in an abnormal response to submersion, in that water entered the buccal cavity, and in some cases the lungs, while surfacing often did not result in resumption of ventilation. Skin mechanoreceptors in the region of the external narial openings serving the ophthalmic branch of the trigeminal were found to be capable of responding to the minimum stimulus encountered on submersion, movement of a water meniscus across the narial region, while a tonic response to hydrostatic pressure occurred in some preparations. In control experiments cutaneous mechanoreceptors innervated by the spinal nerves were shown to have no response to a water meniscus passing across their receptive fields, suggesting that they possess higher thresholds than the narial receptors. Periods of apnoea could be induced in air in *Rana pipiens* by bilateral or unilateral stimulation of the cut peripheral ends of the ophthalmic branch of the trigeminal nerve at threshold voltages as low as 30 mv, at a frequency of 200 Hz. Increase in stimulating voltage resulted in longer periods of apnoea before ventilation "broke through", and in these periods the external nares were closed and buccal pressure was held independent of atmospheric pressure. Reduction of the stimulation frequency by a factor of ten after the initiation of apnoea, simulating adaptation of the sensory nerves, proved as effective in maintaining apnoea as continued stimulation at the original frequency.
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1

GENERAL INTRODUCTION

Although apnoea is a central feature of the periods of submergence undergone by diving vertebrates, much less is known about the stimuli which inhibit or modify the rhythmic activity of the respiratory centre during diving than about the respiratory and cardiovascular adjustments which occur as a result of such stimulation. Huxley (1913b), working on ducks, proposed a "postural reflex" on the basis that ducks become apnoeic on assuming certain positions in air but the significance of this observation was lessened when Koppanyi and Kleitman (1927) found that ducks never assume these positions when freely swimming underwater. Andersen (1963) presented evidence that in the duck apnoea was produced by water immersion of the area of the beak surrounding the nostrils. The response could be abolished by bilateral section of the trigeminal nerve to the beak, in which case ducks would resume breathing through a tracheal cannula during periods of head submersion. Butler and Jones (1968) considered however, that the most important factor in initiating apnoea during diving in the duck was the contact of water with the respiratory passages at the level of the glottis, for they found that ducks continued to breathe (via a tracheal cannula) when water covered the external nares, whereas raising the water to the level of the glottis caused apnoea. Electrical stimulation of the branches of the glossopharyngeal nerve serving this area was also found to cause apnoea (Jones and Purves, 1970). Moreover some doubt has been cast upon the involvement of the trigeminal nerves as an afferent pathway in reflex apnoea in the duck by the recent finding that beak mechanoreceptors were not capable of a response even when the hydrostatic pressure at the beak surface was raised and lowered between 0 and 50 cm H₂O simulating diving and emersion (Gregory, 1973).
Tchobroutsky et al (1969) have shown that apnoea produced by head immersion in the newborn lamb, adult rabbit, and adult sheep was abolished when the glottis was anaesthetised, or when the superior laryngeal nerves were severed. They have suggested that a reflex initiated by the contact of the amniotic fluid with the airways of the foetus may be responsible for the inhibition of respiratory movements in utero, and that release of the reflex at parturition ensures the prompt onset of ventilation.

Reflexes from the area of the nasal mucous membrane have been shown to cause apnoea in response to mechanical, electrical and chemical stimulation in non-diving vertebrates. Recently Angell James and Daly (1972a) have demonstrated in dogs that the respiratory responses were diminished by division of the ethmoidal or maxillary branches of the trigeminal and abolished when both nerves were bilaterally divided. Water or saline in contact with the nasal mucosa was effective in producing apnoea, although the liquid had to be in motion, suggesting that information from mechanoreceptors was primarily involved.

No detailed information is available concerning the ventilatory adjustments of amphibians to submergence, although many amphibians have diving habits and can remain submerged for extremely long periods, Rana esculenta surviving submersion for 2-3 weeks at 14-15°C (Serfaty and Guental, 1943). Indeed the general field of amphibian ventilatory control has aroused little interest. Smyth (1939), investigated the respiratory response to altered concentrations of O₂ and CO₂ in inspired air, while De Marneffe-Foulon (1962) studied the effects of shifting the blood pH on the frequency of lung and buccal ventilation cycles. Neil et al (1950), and Taglietti and Casella (1966) have demonstrated neurophysiologically that lung stretch receptors are active during lung inflation and are probably involved in the
reflex termination of lung filling. Some of the mechanoreceptor population also appear to be sensitive to lung deflation (Taglietti and Casella, 1968). More specifically, information on the initiation of diving apnoea in anuran amphibians is limited to observations of Lombroso (1913), and Willem (1920) on *Rana esculenta*. Lombroso (1913) noted that contact of the external nares with water was sufficient to inhibit ventilation, this inhibition being so powerful in submerged batrachians deprived of access to the surface that death ensued from anoxia before any attempts were made at ventilation, while Jones (1967) confirmed an observation of Willem (1920) that occasional ventilation movements in which gas was moved from the lungs to the buccal cavity and back again occurred during the course of the dive, although the external nares remained closed underwater.

The increased oxygen consumption that follows a period of submergence in anurans has been considered to be partly due to the need to replenish the blood oxygen supply and partly due also to the increased work done by the respiratory muscles in post dive hyperventilation. Jones (1967) pointed out that animals showing bursts of hyperventilation but which were otherwise quiescent consumed a larger amount of oxygen in these periods, indicating that the cost of hyperventilation is high. An estimate of the oxygen cost of ventilation must therefore be obtained before the true oxygen debt after a dive can be determined. The energy cost of ventilation has been studied in two main ways; by measuring the energy put into the respiratory pump, and by measuring the external work performed by the pump. Oxygen consumption is a measure of energy input to the pump, every litre of oxygen consumed producing an average of 4.825 calories. In man, at least, it is possible to allow an experimental subject to ventilate at a series of minute volumes up from resting $V_E$ while measuring oxygen consumption. It can then be assumed that
the respiratory muscles account for the additional oxygen consumption above the basal rate, and that this additional consumption gives an indication of oxygen cost. Liljestrand (1918) first studied the metabolic cost of ventilation in man, obtaining a series of minute volumes by both voluntary hyperventilation and stimulation with CO₂ and found that voluntary hyperventilation had a greater oxygen cost, apparently due to tetanus in muscles usually unrelated to breathing.

A typical technique for estimating the oxygen cost and efficiency of human ventilation was that of Campbell et al (1957) who increased \( V_E \) by placing dead space between subjects and a closed circuit spirometer. By measuring oxygen consumption at rest and at several levels of minute volume a curve was produced from which the oxygen cost of resting ventilation could be obtained by extrapolation. All recent studies in man have shown that the oxygen cost of resting ventilation is small and is almost certainly less than 2% (Campbell et al, 1970).

The efficiency of the respiratory muscles was obtained in Campbell et al's (1957) study by measuring the increased oxygen consumption associated with increased mechanical work loads obtained by increasing inspiratory resistance. Oxygen consumption was converted to its mechanical equivalent using the appropriate calorific equivalents. Efficiency was found to vary from 5-10% in young normal subjects, with efficiency remaining constant in any one subject over the range of imposed workloads. Other estimates of the mechanical efficiency of breathing in man have been made by plotting pressure-volume loops while recording oxygen consumption. By this method Fritts et al (1959) found efficiencies of from 1-7% in normal subjects, whereas Milic-Emili and Petit (1960) found efficiencies of from 19-25%, which is in the range of muscular efficiency. It is self-evident that these efficiency
determinations in man are extremely variable, partly due to unavoidable difficulties in the methods employed. For example, small amounts of negative work may be done on the system which would not appear in the pressure/volume loops, i.e. some muscles may be forcibly lengthened during their contraction periods. Secondly, mechanical work may not be the only parameter which should be considered as an output of the system; for example, if a weight is supported, no mechanical work is done on the system but the oxygen consumption of the supporting muscles increases as a function of the weight supported. An analogous situation in breathing would occur if the resistance to flow is increased as in the case of an airway obstruction. In this case there may be an increase in oxygen consumption with no corresponding increase in mechanical work out of the system, as the respiratory muscles would need to contract more forcibly to produce the same pressure and volume changes.

It is partly for this reason that the energy cost of gill irrigation in fish is thought to be high, for the flow resistance of airways and gill curtains depends on the viscosity of the medium, and water is 55 times more viscous than air. Also, inertial forces depend on the mass of the medium and water is 840 times more dense than air, although, presumably, if there is no flow reversal in the system, inertial forces will be small.

The methods that have been used to determine the energy cost of breathing in man are very difficult to apply to fish. For example, it is difficult to measure O_2 consumption at different levels of gill irrigation in fish, without producing side effects, such as increased excitability, so that it is unwise to assume that the increased oxygen consumption at elevated levels of irrigation is due only to the increased respiratory work. Cameron and Cech (1970) considered that the study of Van Dam (1938) is open to criticism on these grounds. Van Dam used CO_2 to increase irrigation rate, and
assumed that the resultant increase in oxygen consumption was solely due to the increased irrigation, although he noted that 2% CO₂ produced struggling of the fish, and presumably increased metabolic oxygen demand. The oxygen cost of breathing in the Tench has been studied by Schumann and Piiper (1966). Highly anaesthetised fish were used and respiratory water flow and oxygen uptake were measured. The cost of breathing in terms of O₂ uptake was measured using spontaneous changes in irrigation rate. With a respiratory flow rate just sufficient to supply the O₂ required by the fish, i.e. at resting irrigation rate, the O₂ cost of breathing was 30% of total uptake and with a ventilation rate 3 times higher, 50% of total O₂ uptake. Schumann and Piiper (1966) contend that non-ventilatory oxygen demand was constant, the fish being in a "plateau" of anaesthesia, but there is little evidence to support this conclusion (Cameron and Cech, 1970). Measurements of the differential pressure between the buccal and opercular cavities of trout (Hughes and Shelton, 1958) have been used to estimate the mechanical work of gill irrigation by Alexander (1967). Combining these estimates with an assumed mechanical efficiency of 20%, Alexander (1967) arrived at a figure of 1% for the oxygen cost of irrigation. However, he made many assumptions which have been shown to be unwarrantable, such as constant flow through the gills, constant gill resistance, and a constant pressure gradient across the gills. Many of the objections to the work of Van Dam (1938) and Schumann and Piiper (1966) have been avoided by Jones and Schwarzfeld (1974), who changed the work of irrigation in unanaesthetised trout by altering the pressure head across the gills, and then measuring the resulting changes in oxygen consumption, enabling the energy cost and efficiency of irrigation to be measured.

No studies comparable to those on fish and man have yet been made on
amphibians. In this study, however, measurement of the mechanical work of the buccal pump proved to be feasible in the anuran, *Rana pipiens*, by the use of pressure/volume loops. A method for the estimation of mechanical efficiency in single respiratory cycles by the determination of respiratory muscle tension and degree of shortening during a respiratory cycle was also developed in order that an estimate of the oxygen cost of ventilation could be made. During preliminary experiments the ventilation mechanism of *Rana pipiens* was found to be sufficiently different from published accounts of other anurans (e.g. Sholten, 1942; De Marneffe-Foulon, 1962; De Jongh and Gans, 1969) to warrant further investigation. Internal gas exchange in most vertebrates is brought about by the use of pressure pumps or vacuum pumps, which in many cases are bimodal, operating in the pressure mode in one phase of the ventilatory cycle, and the suction mode in another. In fish for example, where more than one pump is functional, the phase relationships between the pumps must be precisely controlled to achieve adequate irrigation of the exchange surfaces (Shelton, 1970).

Hughes and Shelton (1958) first used modern techniques to record pressures from the buccal and opercular cavities of teleosts and showed that a differential pressure was maintained from buccal to opercular cavities, to provide for water flow through the gill resistance. However, in periods of transition between the buccal and opercular pumps very low pressure differentials occur, and as the suction pump takes over from the pressure pump the differential may become negative so that flow over the gills may tend to reverse, although probably never does so due to inertial forces. Hughes and Shelton (1958) point out that the idea of a separate pre-gill and post-gill pump is a simplification of the situation in that anatomically it is difficult to separate the functional parts of each, and that in many species one pump
predominates. Bottom living fish seem to depend on a suction-pump mechanism, and in some, for example the gurnard, the exit of water is restricted to a small, dorsally directed aperture. The exhalent phase of ventilation tends to be increased also, so that water is ejected rapidly and at high velocity. In both the teleost and elasmobranch flat fish the opercular openings and openings to the gill slits can be closed actively, perhaps so that entry of debris into the gill chambers is prevented. In pelagic fish the pumps appear to be balanced, although the buccal pump predominates in the horse mackerel and the opercular pump in the whiting. It appears that in other pelagic fish active respiratory movements are not made, but that they rely on the current entering the mouth, a kind of ram-jet method of irrigation (Brown and Muir, 1970). The mechanism of gill irrigation in elasmobranchs has been shown to be essentially the same as that found in teleosts (Hughes, 1960), although recently heterogeneity of function within the elasmobranch respiratory mechanism has been emphasized (Hughes, 1973), based on the finding in the dogfish that water entering the spiracle, and mouth, exits through the anterior and posterior gill slits respectively, and that the relative sizes of the positive and negative pressure phases in irrigation vary between gill slits.

In modern lungfish the mechanism of air ventilation consists of a straightforward adaptation of an aquatic irrigation cycle, with inspiration being due to the buccal force-pump, while expiration is passive and consists of the release of compressed lung gas which is aided by the elasticity of the lung tissue (Bishop and Foxon, 1968; McMahon, 1969). As pointed out by Hughes (1973), the adaptations of modern lungfish to air breathing may be misleading if it is naively considered that they also apply to the ancestral tetrapods, since there is evidence that the Dipnoi are a specialized group
(White, 1966), a point of particular interest being that the modern Dipnoi do not possess the dorsal ribs from which the plural ribs of tetrapods are derived (Goodrich, 1930).

Maximum specialization of the buccal force-pump for air ventilation occurs in frogs (De Jongh and Gans, 1969), the Amphibia being the only modern group in which the nares rather than the mouth are used as a path for air entering the buccal cavity. McMahon (1969) proposed a possible scheme for the incorporation of the nares into the buccal force-pump mechanism. He suggested that buccal filling must have been difficult in early crossopterygians as the gape was long, necessitating raising the head clear of the surface to obtain air. One solution to the problem, taken by the Dipnoi line, was to reduce the gape; while another solution, taken by the amphibian ancestors, was to use the nares as a path for buccal filling. Use of the nares also appears to have conferred on the amphibia the advantage of finer control over inspired air, although at the expense of increased resistance to flow.

Although this is an attractive possibility, it appears doubtful that the buccal force-pump was important in the lung ventilation of ancestral amphibians, for the fossil record shows that they possessed well developed ribs, associated in modern vertebrates with an aspiration mode of ventilation (Romer, 1972). This evidence suggests then that the buccal force-pump of modern amphibia is a secondary adaptation associated with rib reduction, which itself probably came about in response to a comparatively recent return to a more aquatic habit, the ancestors of the modern amphibia having the main attribute needed for aspiration, a well developed rib-cage. Amphibians superficially appear to possess a less complex breathing mechanism than that of most fish, anurans at least ventilating by means of a buccal force-pump. However, the addition of variably active valves (external nares and glottis)
greatly increases its functional complexity if only from the point of view of the controlling mechanisms involved. It is perhaps for this reason that there has been little agreement between authors on the precise relationships of the mechanical events of anuran ventilation.

This study therefore, naturally fell into three sections. In the first, the mechanism of lung and buccal ventilation was examined. Buccal and lung pressures were measured in frogs that were restrained but unanaesthetised and combined with measurements of the associated volume changes and respiratory muscle activity to give an overall picture of the breathing mechanism; in the second section the mechanical work and efficiency of ventilation were determined to give the energy cost; in the third section experiments were performed to determine the stimulus or stimuli responsible for the initiation and maintenance of diving apnoea, and the sites sensitive to such stimulation. Experiments were also performed to determine the capability of receptors in these areas of responding to the stimuli presented on submersion and attempts were made to initiate periods of apnoea in air by stimulation of the appropriate sensory nerves.
PART I. THE MECHANICAL EVENTS ASSOCIATED WITH LUNG AND BUCCAL VENTILATION IN THE FROG, RANA PIPIENS

INTRODUCTION

Although it has long been recognised that anuran amphibians ventilate by means of a buccal force-pump, there is surprisingly little agreement as to the relationship of the respiratory events in these animals. This is due, in part, to the difficulty of recording the pressure and volume changes within the lungs and buccal cavity along with the associated activity of the nares, glottis, and respiratory muscles. Furthermore, as Foxon (1964) points out, many early experiments were made on pithed or anaesthetised animals often placed on their backs, so that the results probably do not relate very closely to events in the conscious, undisturbed animal, for anuran respiration is notoriously labile (Gaupp, 1896).

Virtually all studies show the existence of two types of respiratory movement in the buccal pump, an ongoing series of movements that ventilate only the buccal cavity, interspersed by movements which ventilate the lungs (Martin, 1878; Wedenski, 1881; Willem, 1919, 1920; Sholten, 1942; De Marneffe-Foulon, 1962; De Jongh and Gans, 1969). Das and Srivastava (1956) claim a constant ratio between the frequency of buccal and lung ventilation movements in Rana tigrina, but others have found no evidence for this. On the other hand, Gnanasathu (1936), and Cherian (1956) hold a radically different view of the ventilatory process, claiming that the glottis never remains completely closed, and the lungs are ventilated by every buccal cycle. For instance Cherian (1956), working with Rana hexadactyla, found no experimental proof for solely buccal ventilation, considering that the glottis never completely
closed, although the nares closed twice in each respiratory cycle. (Unfortunately their experiments were performed with the frogs fastened on their backs, so that the results were of doubtful value.)

Although most authors now accept the validity of separate lung and buccal ventilating cycles, there is much disagreement on the precise sequence of events during lung ventilation, in particular the timing of glottal opening and narial closure, and whether the exit of gas from the lungs on exhalation is due to elastic fibres in the lung walls, or an active contribution from the flank muscles. Further debate centres around the sequences of lung inflation and deflation, during which the lungs are inflated by a series of lung ventilation movements of increasing peak pressure, isolated from the buccal cavity by the closed glottis and then deflated again. Willem (1919, 1920), Sholten (1942), and De Marneffe-Foulon (1962) considered these sequences to be caused by stress, excitement or pain, but De Jongh and Gans (1969) observed them in cannulated but otherwise unrestrained *Rana catesbeiana*.

The present study was undertaken to clarify the breathing mechanism in the North American grass frog, *Rana pipiens*, by recording the pressure and volume changes in the lungs and buccal cavity during normal breathing in unanaesthetised animals. Volume changes were recorded in such a way as to place no mechanical load on the breathing mechanism. Records were also taken of the activity of the main respiratory muscles during breathing, and of the pattern of flow through the nares during both lung and buccal ventilation cycles.
METHODS

The experiments were performed on 30 grass frogs (Rana pipiens), ranging in weight from 19 to 90 grams, the average weight being 65 grams. All the experiments were performed at 24°C ±1°C, the frogs being allowed to acclimate in tanks for at least a week at this temperature before use. All operative techniques were done under surgical anaesthesia obtained by immersion in MS 222 (Sandoz) solution (400 mg/l). Anaesthesia occurred within 30 minutes and the animals remained anaesthetised for a further 30 minutes. All recordings were taken from animals at least 2 hours after recovery from the anaesthesia.

For lung cannulation a small slit was made in the skin and body wall of the posterior flank, and the tip of a lung (usually the left) was exposed. A short cannula of flexible 2 mm diameter urinary catheter tubing was then inserted into the apex of the lung and tied in place. To prevent occlusion the cannula was cut into a taper, and the tip was blunted to prevent possible penetration of the lung wall (Jones, 1970). The lungs were usually found to be in a semi-inflated state on cannulation. If they were collapsed they were re-inflated by introducing air into them via the trachea, to facilitate insertion of the cannula. The slits in the skin and the body wall were closed separately after cannulation. Blood loss caused by this operation was negligible.

The buccal cavity was cannulated using P.E. 90 tubing with one end flattened to form a flange. It was introduced through the left tympanic membrane from inside to outside and held in position by a washer of large bore P.E. tubing which was fed over the cannula and crimped the tympanic membrane by pressing against the flange. This prevented it from pulling out of the
tympanum. Lung pressure was usually recorded by a Hewlett-Packard model 270 pressure transducer, and buccal pressure by a model 268 BC pressure transducer, although in a few cases both lung and buccal pressures were recorded by model 268 BC transducers. The transducers were air-filled and had a frequency response of 50-70 Hz with damping of less than 0.1 of critical when tested by a Hansen "pop-test". Following insertion of the cannulae the frogs were positioned on a cork board and were restrained by pinning, care being taken to position the pins such that limb circulation was not impeded.

Changes in lung volume were measured by means of a Biocom model 991 impedance converter, used as an impedance pneumograph (Geddes and Baker, 1968). Two fine copper wire electrodes, insulated except for 1 mm at the tip, were introduced using a hypodermic needle into the body wall, one in each flank. Lung volume was calibrated by injecting small volumes (usually 0.1 ml) of air in steps into the lungs via a three-way tap in the lung cannula. Both lungs were inflated by this method, as the bronchi connect to each other posterior to the larynx. If a linear response was not obtained on calibration, the positions of the electrodes were adjusted until the calibration was linear. The impedance converter was also used in 2 frogs to monitor narial opening by recording impedance mechanographs. This was accomplished by inserting fine copper wire electrodes, insulated except for the last 1 mm which was bent into a hook, into the medial and lateral borders of the external nares and measuring the impedance change across the electrodes. Decrease in the measured impedance was considered to represent narial closure, and an increase in impedance, narial opening (this in fact was confirmed by visual observation). This method of recording narial movements possessed the advantage of placing no mechanical load on the nares as would a strain gauge type of transducer.
Buccal volumes were recorded by means of an E.E.L. 1 inch diameter selinium photocell (Jones, 1970). The position of the photocell was adjusted so that a shadow of the buccal floor was thrown onto the face of the photocell by a variable intensity microscope lamp. Changes in buccal volume were measured as changes in output from the photocell. Volume changes were calibrated after the experiments by withdrawing and reinjecting small volumes of air (0.1 ml) into the buccal cavity, the frog having previously been anaesthetised in position by injecting 0.25 ml of MS 222 solution in saline (400 g/l) directly into the dorsal lymph sac. During this calibration procedure the nares were blocked with vaseline.

Gas flow through the nares during lung ventilation cycles was investigated in 2 frogs by means of a bead thermistor connected to a resistance bridge circuit. The thermistor, which was 1 mm in diameter, was positioned as close as possible to one external narial opening, without making contact with the skin. Exhaled gas produced a deflection in the resulting oscilloscope trace, while inflow deflected the trace in the opposite direction. No attempt was made to quantify flow rates, and output at zero flow was established during portions of buccal cycles when the nares were closed.

Electromyograms (EMG) were recorded bipolarly. Fine insulated copper wire electrodes, the last 1 mm of which were bared and bent into a hook shape, were inserted into the appropriate muscle using a fine hypodermic needle. The position of the electrodes was checked post mortem. When EMGs were recorded from the glottal region, the leads were either led out of the angle of the jaw, or through an aperture in the right tympanic membrane which was sealed to prevent gas leakage.

All recordings were made on a Technirite TR 722 two channel pen recorder,
a Technirite TR 888 eight channel recorder, a Hewlett-Packard 1201 storage oscilloscope, or a Telequipment D 54 oscilloscope.
THE MORPHOLOGY OF THE PRINCIPAL RESPIRATORY MUSCLES

The muscles of the buccal floor have been described in detail for Rana esculenta (Ecker, 1889), while descriptions of the glottal muscles of Rana pipiens are summarized by Schmidt (1972). For the purposes of comparison the terminology used by De Jongh and Gans (1969) will be followed here.

The superficial muscles of the buccal floor, the anterior and posterior intermandibular and interhyoids, run transversely between the mento-meckalian cartilages and angulars of the lower jaw (Fig. 1a). Between these transverse muscle sheets and the tongue lie two pairs of geniohyoid muscles which run sagittally from the mento-meckalian and anterior angulars to insert ventrally into the posterior processes of the hyoid (Fig. 1b). The large sternohyoid muscles insert between the insertions of the geniohyoid muscles and arise from the coracoid and xiphisternum, thus running inside the pectoral girdle, while the omohyoids arise from the scapulae and are inserted laterally into the ventral surface of the hyoid (Fig. 1b). The anterior petrohyoid muscles (Fig. 2) arise from the pro-otic bones and are inserted at the lateral margins of the hyoid cartilage.

The muscles of the glottis and larynx (Fig. 2) have been divided into constrictors and dilators on purely morphological grounds. The lips of the glottis are formed by the arytenoid cartilages, into the lateral apices of which are inserted the laryngeal dilator muscles, which originate laterally on the hyoid. There are three groups of muscles which have been described as constrictors; the anterior, posterior and external laryngeal constrictors. The external constrictors surround the arytenoid cartilages ventrally while the anterior and posterior constrictors are dorsal in position (Fig. 2).
Figure 1. (a) The superficial muscles of the buccal floor.

1, pectoral girdle; 2, interhyoid muscle; 3, posterior intermandibular; 4, angular of lower jaw; 5, connective tissue; 6, anterior intermandibular muscle (under connective tissue).

(b) The deeper muscles of the buccal floor.

1, sternohyoid muscle; 2, omohyoid; 3, medial geniohyoid; 4, lateral geniohyoid; 5, angular of lower jaw; 6, anterior intermandibular muscle.
Figure 2. The muscles of the glottis and larynx.

1, process of cricoid cartilage; 2, posterior laryngeal constrictor; 3, anterior laryngeal constrictor; 4, laryngeal dilator; 5, arytenoid cartilage; 6, external laryngeal constrictor; 7, anterior petrohyoid muscle; 8, hyoid cartilage.
Dorsal

Ventral

1
2
3
4
5
6
7
8
(a) Pressure and volume changes in the lungs and buccal cavity.

It was possible to distinguish two distinct types of breathing movements in *Rana pipiens*: ventilation movements which did not involve the active participation of the glottis and nares (buccal cycles), and movements in which large positive pressures were generated by their coordinated activity (lung ventilation cycles). The pressures developed during buccal ventilation cycles, with the glottis closed and the nares open, ranged in amplitude from ±0.1 to ±0.5 cm H$_2$O in individual frogs. The volume changes of the buccal cavity associated with buccal ventilation averaged 0.56 ml in a 50 gram frog, and were approximately 2/3 of the magnitude of the volume changes which occurred during lung ventilation (Fig. 3a). They were simple in form, consisting of a fall to a minimum volume, followed by a volume increase at almost the same rate as the fall. The volume changes occurred at the same frequency as the associated pressure changes, but were not in phase with them; the pressure maxima preceding the volume minima by 80-120 m secs in individual frogs.

The pressure and volume changes which occurred in the buccal pump during lung ventilation were more complex and of greater amplitude than those occurring during buccal ventilation. As shown in Figure 4, a simultaneous fall in lung pressure and lung volume occurred as the glottis opened and lung gas flowed into the buccal cavity. Coincident with this buccal pressure rose, and there was also a small increase in buccal volume. Buccal volume at this point always increased by an amount smaller than the volume decrease of the lungs, suggesting that the nares were at least partially open at this point in the cycle, and that part of the gas contained in the buccal cavity passed
Figure 3.  
(a) Pressure and volume changes recorded from the buccal cavity. (Increase in buccal volume is up on the trace.)
(b) Slow speed recording to show sequences of lung inflation in *Rana pipiens*. Top trace, buccal pressure; lower trace, buccal volume. (Increase in volume is up on the trace.)
(c) Pressures recorded from the lungs and buccal cavity. Top trace, lung pressure; lower trace, buccal pressure.
Figure 4. Pressure and volume changes in the lungs and buccal cavity, together with activity from the muscles of the respiratory valves.

(a) E.M.G. from narial closer, M. *lateralis narium*.
(b) E.M.G. from laryngeal dilator muscles, M. *dilatator laryngis*.
(c) Lung volume, increase in volume is up on the trace.
(d) Lung pressure.
(e) Buccal volume, decrease in volume is up on the trace.
(f) Buccal pressure.
through them. Lung and buccal pressure equilibrated at the end of this phase and often produced a marked step or inflexion in the buccal pressure curve (Fig. 3). In some frogs the pressure in the system stayed constant for up to 160 m secs or even fell slightly, presumably due to gas leakage through the nares. The nares then closed and a large decrease in buccal volume occurred which caused a rapid elevation in buccal pressure. The rise in lung pressure at this point closely paralleled the rise in buccal pressure, and as illustrated by Figure 5a and b, no evidence was found for a large differential pressure across the glottis in this phase. The increase in lung pressure was associated with a similar increase in lung volume. At peak joint lung/buccal pressure the lungs maintained the new pressure level due to glottal closure, while buccal pressure fell back to atmospheric, typically in 50-60 m secs, and undershot to give a pressure in the buccal cavity of -0.25 to -0.4 cm H$_2$O before slowly returning to atmospheric pressure, at which point the buccal ventilations restarted. The attainment of maximum buccal pressure slightly preceded the point of minimum buccal volume in lung ventilation, and its fall to atmospheric pressure occurred either just before or at the point of minimum buccal volume. In either case the fall to atmospheric pressure was nearly isovolumetric because of its extreme rapidity (50-60 m secs). It appeared to be due to two factors, firstly the opening of the nares synchronously with glottal closure at the point of peak buccal pressure, and secondly the simultaneous cessation of activity of the respiratory muscles of the buccal floor with the result that buccal floor tension rapidly fell to zero. Buccal pressure therefore also fell rapidly and equilibrated through the open nares with atmospheric pressure. The slow buccal volume increase (250-300 m secs) which occurred once minimum buccal volume was attained then served to drive buccal pressure below atmospheric pressure, until finally the
Figure 5. The relationship between lung and buccal pressure during (a) lung inflation; (b) lung deflation. Top trace, lung pressure; lower trace, buccal pressure.
buccal pressure again equilibrated with atmospheric pressure, at which point the next respiratory cycle commenced.

Lung ventilation cycles in the frogs investigated did not occur randomly, but in well defined sequences of 9-30 such movements interspersed with buccal ventilation cycles, the sequences being separated from each other by pauses during which the lungs remained inflated and only buccal ventilation cycles were performed. Figure 3b and c illustrates a series of such sequences, while Figure 5a and b shows the details of one such sequence of lung inflation and deflation. During the last few lung ventilation cycles of a sequence the pressures developed by the buccal pump increased in a way so that each cycle produced a lung pressure 10 to 20 percent greater than that achieved by the previous cycle, and at the end of a sequence the lungs were fully inflated at a pressure of 4-5 cm H$_2$O and remained inflated through the period of buccal ventilation (Figure 3c). Lung deflation was usually brought about by the first few lung ventilation cycles of the subsequent sequence, each of which attained a lower peak pressure than the preceding one, until a relatively constant lung pressure of 1.5 to 2 cm H$_2$O obtained which was maintained prior to the next lung inflation sequence. Lung deflation proved to be more variable than inflation, on some occasions the lungs were deflated on the first lung ventilation cycle after a period of inflation, while on other occasions this occurred over two or three cycles (compare Figures 3c and 5a and b).

The frequency with which lung inflation occurred varied according to the condition of the frog and in frogs which were obviously disturbed lung inflation cycles were suppressed. In the majority of frogs studied however the lungs were inflated approximately once per minute and remained fully inflated for 10 to 20 seconds. Although the lungs were isolated from the
buccal cavity by the closed glottis during periods of lung inflation small amplitude pressure fluctuations still occurred in the lungs (Figs. 3c, 5a and b). These small amplitude fluctuations varied in size with the magnitude of the buccal oscillations (Fig. 3a) and occurred at the same frequency as the buccal ventilation cycles. They could have been caused either mechanically (due to the muscle insertions of the buccal floor pulling on the flanks) or by pressure changes of the buccal cavity being transmitted indirectly through the tissues to the thoraco-abdominal cavity, in which the lungs lie. The small pressure peaks recorded from the lungs lagged behind the pressure peaks of the preceding buccal cycles by 350-400 m secs so this transmittance of pressure was a relatively slow process which tends to favour the notion of indirect transmittance. Further long term pressure changes occurred in the lungs during the inflated period in many frogs. Figure 3c illustrates a typical example in which lung pressure gradually rose and then fell, while in some cases there was a gradual fall in lung pressure throughout the inflated phase. A simple calculation indicates that a long term (20 sec) fall in pressure is unlikely to indicate uneven respiratory exchange ratios in the lung, since the volume change was generally greater than that which would be predicted from the metabolic rate of the frogs used. It seems most probable that these changes were due to small postural changes in the frogs concerned, although the frogs were restrained throughout the experiments, or to changes in the tonic activity of the pulmonary smooth muscle, controlled perhaps by the level of interpulmonary CO$_2$ (Kobayasi et al, 1961).

(b) The nares and glottis

The external nares and glottis provide the valves of the buccal pump. The positions of the external nares were monitored by recording EMC's from
the M. lateralis narium, which occupies the space between the anterior portion of the maxillary bone and the ascending process of the premaxilla (Ecker, 1889). Muscle potentials recorded from this region were coincident with narial closure as could be deduced from the buccal pressure traces. Glottal opening was monitored by recording EMG's of high amplitude from the laryngeal dilatory muscles, the M. dilatator laryngis, which are inserted laterally on the arytenoid cartilages which form the cartilaginous walls of the vertical slit-like glottis.

Figure 4 illustrates the relationship between electrical activity in these muscles and the pressure and volume traces from both the buccal cavity and the lungs. No activity was recorded in either set of muscles during buccal ventilation, the nares remaining open, while the glottis was closed. In lung ventilation however, the onset of activity in the laryngeal dilator muscles either slightly preceded or was synchronous with the beginning of the initial fall in lung pressure and volume, and activity continued until peak lung/buccal pressure was reached. Throughout this period the glottis remained open and the lungs retained the new pressure level due to glottal closure. No activity could be found in those muscles anatomically described as glottal closers, i.e. the anterior, posterior and external laryngeal constrictors, although several attempts were made to record from them. It appears that ordinarily in Rana pipiens the glottis is held open by muscular activity but that glottal closure occurs passively. The glottis was always found to be closed in curarised (0.1 mg/10 g wt d-tubocurarine chloride) frogs indicating that the inherent elasticity of the laryngeal cartilages and their connections was sufficient to maintain glottal closure. Furthermore, if air was pumped into the lungs of these frogs via a cannula, the glottis was able to support lung pressures of 10-15 cm H₂O before opening. This pressure is
2-3 times greater than that developed across the glottis during normal lung inflation cycles. In 3 pithed frogs stimulation of the M. dilatator laryngis (1.5 v, 4 Hz, 10 m sec impulses) resulted in glottal opening. Immediate passive closure occurred when stimulation ceased.

The onset of activity in the M. lateralis narium signalled narial closure which occurred at the start of the phase of active elevation of buccal pressure, typically 50-60 m secs after glottal opening, and activity continued until peak buccal/lung pressure was reached, at which point it stopped, indicating narial opening. No activity could be recorded from the M. dilatator narium which by its anatomical position has been considered to be a narial opener (Ecker, 1889), suggesting that elastic forces are sufficient for narial opening. The nares were always found to be open in curarised frogs, confirming that no muscle activity is required for opening. Figure 6 illustrates the relationship between the impedance mechanograph of the external nares, and the associated buccal pressure trace. The records show that the start of narial displacement coincided with the beginning of the rapid rise in buccal pressure which is due to the active contraction of the muscles of the buccal floor. The nares presumably closed very soon after the beginning of narial displacement and remained closed up to the point of the minimum impedance measured, which was coincident with the point of peak buccal pressure. During the falling phase of buccal pressure impedance across the nares increased, indicating narial opening; maximum impedance indicating maximum narial opening occurred at the point when the buccal pressure trace had fallen back to zero. No narial displacements were recorded during buccal ventilation cycles, the nares remaining open.

Figure 7 illustrates flow at the nares during buccal ventilation cycles, followed by the first phase of a lung ventilation cycle. Flow was biphasic
Figure 6. The relationship between narial closure and glottal opening.

Top trace, impedance mechanograph of nares. Middle trace, E.M.G. from glottal openers, *M. dilatator laryngis*. Lower trace, buccal pressure.
during the buccal cycles, consisting of an initial outflow of buccal gas, as the volume of the buccal cavity decreased, followed by an inflow as the buccal floor fell passively. Between the two phases of flow there was a short period (100 m secs) of negligible flow, while the duration of the entire cycle was approximately 1 second. Figure 7b shows narial air flow during 2 lung ventilation cycles. During the first phase of each cycle, A-B, there was a major outflow of buccal gas through the nares. This is due to glottal opening, which allowed lung gas to flow into the buccal cavity raising its pressure above atmospheric, so that an outflow of gas occurred via the nares. At the start of the next phase, B-C, narial closure occurred, and there was no narial flow, although buccal pressure was being rapidly elevated by the muscles of the buccal pump. Narial opening at peak buccal pressure C, coincident with glottal closing, resulted in a second phase of outflow of buccal gas through the nares, as buccal pressure fell back to atmospheric at D. Once buccal pressure fell below atmospheric however, flow reversal occurred at the nares and air entered, refilling the expanding buccal cavity (D-A) preparatory to the next ventilation cycle.

(c) The activity of the principle respiratory muscles

Muscle activity during the buccal cycles occurred only during the phase of the cycle in which pressure rose and none was present during the falling pressure phase. EMG's were recorded from the posterior intermandibular muscle almost immediately buccal pressure rose above atmospheric, but usually stopped before peak pressure was reached. On the other hand, activity in the medial and lateral geniohyoid muscles continued to the point of peak pressure (Figure 8a and b).

Figure 9a illustrates the activity recorded from the sternohyoid muscle and the anterior petrohyoid, together with a trace of pressure. No activity
Figure 7. Narial gas flow during (a) four buccal ventilation cycles; (b) two lung ventilation cycles. A-D' correspond to phases in schematic diagram, Figure 10.
Figure 8. (a) Top trace, buccal pressure. Lower trace, activity in posterior intermandibular muscle. (b) Top trace, buccal pressure. Lower trace, activity in medial geniohyoid muscle.
was present in either of these muscles during the buccal cycles, but both showed strong activity during lung ventilation cycles. The sternohyoid, which is inserted anteriorly to the hyoid and attached posteriorly to the pectoral girdle was the first muscle to show activity during a lung inflation cycle. Activity in this muscle started 15-20 m secs before the glottis opened, as judged by the accompanying pressure trace and continued until lung and buccal pressures had equilibrated. A second burst of activity was sometimes recorded during the rising phase of buccal pressure, especially when a high peak pressure was generated. From the position of the sternohyoid it has been proposed that its contraction serves to distend the posterior portion of the buccal cavity, increasing buccal volume (Das and Srivastava, 1957; De Jongh and Gans, 1969). This suggests that the volume increase of the buccal cavity which occurred in the first phase of the lung ventilation cycle did not result solely from gas leaving the lungs, but also had an active component, although the significance of the second burst of activity observed in some cycles during the rising phase of lung pressure is obscure.

During the rapid elevation of buccal pressure, in lung ventilation cycles, EMG activity was recorded from the anterior petrohyoid muscle, the medial and lateral geniohyoid muscles, the posterior intermandibular muscle, the omohyoid, and the interhyoid (Fig. 9b), all of which commenced firing at the beginning of the rapidly rising phase of buccal pressure, after lung and buccal pressures had equilibrated and activity continued until or slightly after peak buccal pressure was reached. The amplitude of the potentials recorded from the posterior intermandibular muscle during lung ventilation cycles was always very much larger than that of those obtained during buccal ventilation cycles, indicating that greater numbers of motor units were involved in lung ventilation cycles (Fig. 8). This was also generally true for the lateral
Figure 9. (a) Top trace, activity in sternohyoid muscle. Middle trace, activity in anterior petrohyoid muscle. Lower trace, buccal pressure.

(b) Top trace, activity in omohyoid muscle. Middle trace, activity in interhyoid muscle. Lower trace, buccal pressure.
and medial geniohyoid muscles, although EMG amplitude increased during large buccal ventilation movements (Fig. 8b). No muscular activity was found to occur in the falling pressure phase which followed peak pressure; the relatively slow increase in buccal volume which occurred during these phases therefore presumably resulted from gravitational and elastic forces.
DISCUSSION

The present study has permitted a reappraisal of the mechanism of anuran ventilation, as well as providing new information on the volume changes of the buccal cavity and lungs associated with the ventilatory cycles, which is necessary for a more complete understanding of the respiratory events. A description of the sequence of events occurring during a typical lung ventilation cycle will clarify the relationships between them, and will enable an overview of the mechanism to be made (Fig. 10). The beginning of the first phase of a lung ventilation cycle (A-B) occurs, whether the preceding cycle was a buccal or a lung ventilation cycle, when buccal pressure is at atmospheric. The beginning of this phase is indicated by (1) a simultaneous fall in lung pressure and volume due to opening of the glottis; (2) an increase in buccal pressure; (3) an increase in buccal volume (which is never as great as the associated decrease in lung volume); (4) electrical activity in the M. dilatator laryngis, which opens the glottis; and (5) activity in the M. sternohyoideus, contraction of which distends the posterior portion of the buccal cavity (Das and Srivastava, 1957; De Jongh and Gans, 1969). Equilibration of the lung and buccal pressure traces is accomplished before the start of the second phase of the lung ventilation cycle (B-C). The start of this phase is marked by (1) narial closure, indicated by EMG activity in the M. lateralis narium, and also by impedance mechanographs of the external nares; (2) EMG activity in the main respiratory muscles of the buccal floor; (3) a rapid decrease in the volume of the buccal cavity; (4) a rapid rise in the buccal pressure driven by the decrease in buccal volume; and (5) a simultaneous rise in lung pressure and volume, as the buccal cavity and lungs are in communication through the open glottis. The third phase of the cycle
Figure 10. Semischematic diagram of lung and buccal pressure and volume changes in a buccal and a lung ventilation cycle. Up on trace is an increase in lung volume, decrease in buccal volume, muscular activity in all frogs investigated, muscular activity in some frogs during this phase.
A buccal laryngeal dilator
narial constrictor
medial geniohyoid
lateral geniohyoid
post. intermandibular
sternohyoid
ant. petrohyoid
omohyoid
interhyoid

lung vol.
buccal vol.
lung p.
buccal p.

0:5ml.
cms. H₂O

0-5 sec.
(C-D) is commenced by, (1) the end of activity in the M. dilatator laryngis, indicating closure of the glottis, and isolation of the lungs from the buccal cavity; (2) narial opening; (3) a rapid fall of buccal pressure to atmospheric; (4) an associated, but much slower increase in buccal volume which drives buccal pressure considerably below atmospheric (D'-D''), before equilibration occurs through the open nares. At this point the next ventilation cycle, whether buccal or lung, commences.

The volume decrease of the buccal force pump (Fig. 10, B-C), and the action of its valves in lung ventilation are due to active muscular contraction, while the subsequent increase in buccal volume is driven by gravitiational and inertial forces. The most simple control mechanism which can be suggested for the lung ventilation cycle is the sequential excitation of the valves and respiratory muscles by means of a group of self-exciting respiratory motor neurones. In this respect it is interesting that discharges have been recorded from the frog medulla associated with the increasing pressure phase of lung ventilation cycles (Ito and Watanabe, 1962; Jones, 1970).

This respiratory sequence resembles that of Rana catesbeiana (De Jongh and Gans, 1969), but differs in several important respects. In Rana pipiens the buccal cycle preceding a lung ventilation cycle never shows an exaggerated negative pressure phase and cannot be considered the first phase of the lung ventilation cycle; nor is there electrical activity of the M. sternohyoideus during this phase as reported for Rana catesbeiana. Activity in the M. sternohyoideus in Rana pipiens, commenced at A, Figure 10, and continued throughout the phase A-B, when buccal volume was increasing, but no activity occurred before this phase.

As soon as buccal and lung pressure equilibrate, they are both rapidly raised by the decrease in volume of the buccal cavity. No large pressure
differential was recorded between the lungs and buccal cavity during this phase (Willem, 1919, 1920; De Marneffe-Foulon, 1962), although a significant pressure differential has been demonstrated in *Rana catesbeiana* (De Jongh and Gans, 1969). This is puzzling, considering the large size and evidently low flow resistance of the open glottis, although De Jongh and Gans speculate that it may be "due to the post contraction relaxation of the smooth muscles of the lung walls".

The opening of the glottis, which initiates the pressure events of the lung ventilation cycle, is due to the contraction of the *M. dilatator laryngis*, while closure is normally passive. EMG activity continued in the *M. dilatator laryngis* throughout the period of time when the glottis was open, as deduced from the pressure record, but no activity could be recorded from the positions of those muscles described anatomically as glottal closers during normal breathing. Schmidt (1972) supports the view that closure of the glottis can occur passively in *Rana pipiens*, although he showed that the constrictor muscles are active during calling, and suggests that their function may be to reduce the duration of the call trills. Earlier electromyographic studies throw little light on the subject because the authors fail to discriminate between buccal and lung ventilating cycles (Kato, 1951; Oka, 1957). Shinkai and Narita (1957), however, found large spike discharges from the *M. dilatator laryngis* during lung ventilation cycles, but failed to find activity in laryngeal constrictors. In *Rana catesbeiana* the situation appears to be different in that electrical activity in the *M. dilatator laryngis* does not continue throughout the period when the glottis is open, but only occurs on the onset of glottal opening. Closure of the glottis is coincident with activity from the region of the anterior laryngeal constrictor muscles (De Jongh and Gans, 1969). Since in *R. catesbeiana* the glottis is opened actively,
remains open without tonic muscular activity in the dilators, and is then closed by active constrictor activity, then some form of click-stop mechanism must be involved. In this study it has been demonstrated that passive closure of the glottis follows stimulation of the M. dilatator laryngis muscles, and that the inherent elasticity of the laryngeal cartilages is sufficient to maintain closure of the glottis even if there is a large pressure gradient between the lungs and the buccal cavity. If the laryngeal muscles are removed and the glottis is then forced open, immediate passive closure of the glottis will still occur (Schmidt, 1972).

Narial closure in Rana pipiens was monitored by measuring the impedance change across the external narial opening and by recording the activity of the M. lateralis narium (Ecker, 1889), which continued throughout the period of narial closure, as deduced from the pressure records. Electrical activity in these muscles, coincident with narial closure has been previously demonstrated (Jones, 1970), and striated muscle fibres have been observed in the area of the nasal cartilages (Shinkai and Narita, 1957). No concrete evidence was found for the frequently proposed mechanism of narial closure by the indirect action of the muscles of the lower jaw on the premaxilla (Gaupp, 1896; Willem, 1919, 1920; De Jongh and Gans, 1969), although manual elevation of the premaxilla certainly does cause external narial closure in anaesthetised animals. The claim of Baglioni (1900) that the anterior processes of the hyoid close the internal nares appears to be without foundation.

Flow measurements, recorded at the external nares, demonstrate the existence in Rana pipiens of two phases of flow during buccal ventilation cycles and of four phases of flow during the lung ventilation cycle. The initial major outflow of buccal gas, which occurs in phase A–B of the lung
ventilation cycle (Fig. 10) must be associated with a similar outflow as lung
gas exits. During B-C, there is no flow at the nares, as the nares are
closed, but flow through the glottis must reverse as the lungs are inflated,
until the glottis closes at C. The four phases of flow at the nares are
therefore associated with two phases at the glottis, for each ventilation
cycle. The pattern of narial flow reported here is essentially similar to
that deduced from pressure records for Rana catesbeiana (De Jongh and Gans,
1969), although no evidence was found for a "major inflow" of air preceding
a lung ventilation cycle.

Lung inflation sequences occurred in all the frogs studied, the lungs
remaining inflated for 10 to 20 seconds. Although the sequences were noted
by early workers (Martin, 1878; Wedenski, 1881), they generally have been
considered to be symptomatic of stress or pain brought about by securing the
frog (Willem, 1919, 1920; Scholten, 1942; De Marneffe-Foulon, 1962). Their
existence has however been demonstrated in cannulated but unrestrained
Rana catesbeiana, and also in unrestrained Rana pipiens and Rana temporaria
in which buccal movements were recorded by means of a photocell (De Jongh
and Gans, 1969; West, 1969, unpublished). In a series of lung ventilation
cycles during which lung pressure and volume is maintained at a steady level,
the net outflow from the lungs during phase A-B of each cycle is balanced by
the inflow during phase B-C (Fig. 10), and the volume of the lungs remains
the same. In lung inflation sequences the outflow is suppressed by shortening
the time available for outflow, narial closure and decrease in buccal volume
occurring soon after opening of the glottis. In many of the frogs investi-
gated the last one or two lung ventilation cycles of an inflation sequence
showed a decrease in buccal volume before opening of the glottis so that the
outflow of gas from the lung was reduced to a minimum or eliminated. These
movements were very rapid, and often accompanied by postural movements of the frog. They approximate to "pure filling movements", in which gas flows only into the lung (Wedenski, 1881; Scholten, 1942). Lung deflation following an inflation period is accomplished by lengthening the outflow phase A-B (Fig. 10) sufficiently to allow a major fall in lung volume and pressure. The rate of outflow is obviously also dependent on the previously established pressure level in the lungs, so that this period may not be significantly longer than in ventilation cycles which merely maintain lung pressure and volume. The lungs are then reinflated to a lower pressure level during phase B-C. Occasionally deflation occurs by means of a series of rapid cycles in which the increase in pressure during phases B-C is very small. These cycles approximate to the "pure emptying movements" described by Wedenski (1881) and Scholten (1942). As pointed out by De Jongh and Gans (1969), the existence of sequences of lung inflation and deflation in anurans means that the concept of tidal volume is of doubtful value when applied to these animals unless the type of cycle is specified, as the volume of gas pumped into the lungs in any cycle will depend on whether the lung is being inflated or deflated.

Hutchinson et al (1967) found a wide range of tidal volumes in Rana pipiens at 25°C.

The initial emptying of lung gas into the buccal cavity during a lung ventilation cycle means that some mixed gas is almost certainly returned to the lungs (Gans et al, 1969), the gas volume passing into the buccal cavity from the lungs during A-B (Fig. 10) being typically 30 to 50% of total buccal volume. It would be more efficient to empty the lungs, completely refill the buccal cavity with air, and then pump this into the lungs if acquisition of oxygen was at a premium, eliminating the possibility
of mixed gas being returned to the lungs. Jones (1972) found that PaCO₂ fell from 9.48 mmHg in normally breathing frogs (*Rana esculenta*) to 7.77 mmHg in frogs artificially ventilated to the same volume (although arterial pH and PaO₂ did not change significantly) suggesting that lung ventilation with mixed gas may serve to maintain and regulate blood PCO₂ in adult anuran amphibians. H₂CO₃ is only weakly ionized in solution and behaves as a weak acid in the blood, but its conjugate base HCO₃⁻ is strong enough to serve as a principal buffer for H⁺ and may be used therefore to control body fluid pH in the face of, for example, the combined respiratory and metabolic acidosis which occurs during submergence in frogs (Jones, 1972). Ventilatory control of blood PCO₂ may be especially significant in frogs in view of the fact that the skin may represent a surface for CO₂ exchange which is not capable of precise regulation in the face, for example, of changes in temperature. Poikilotherms appear to direct their regulation of acid-base balance towards a stabilization of OH⁻/H⁺ ratio in the face of changes in ambient temperature (Howell *et al.*, 1970; Rahn and Baumgardner, 1972), and the data of Reeves (1972) suggests that bullfrogs could regulate ventilation in order to maintain a stable CO₂ content of the blood plasma over a wide range of body temperatures.
PART II. THE MECHANICAL WORK AND EFFICIENCY OF VENTILATION IN RANA PIPIENS

INTRODUCTION

No attempt has previously been made to study the detailed mechanics and dynamics of ventilation in an amphibian, or to measure the mechanical work involved in breathing. Furthermore only one series of experiments has been performed which provides information on the oxygen cost of ventilation in anurans (Jones, 1972), although it has been stated that lung ventilation is the most important single factor in their respiratory exchange (Hutchinson et al., 1968).

The energy cost of ventilation in horse and in man has been measured by elevating the ventilation rate with CO$_2$ stimulation or voluntarily, and measuring the associated increase in oxygen consumption (Zuntz and Hagemann, 1898, cited from Liljestrand, 1918; Otis, 1954). Spontaneous increases in ventilation volume due to hypoxia have been utilized in fish (Van Dam, 1938; Schumann and Piiper, 1966). Extrapolation of the increase in oxygen consumption back to the resting ventilation rate has then been used to give an estimate of the energy cost of breathing at rest. The results of these studies suggest that the cost of resting ventilation in man is less than 2 percent, while in fish it is from 5 to 20 percent (Cameron and Cech, 1970; Campbell et al., 1970; Shelton, 1970). This technique is difficult to apply to amphibians, however, due to the lability of ventilation and the unpredictable ventilatory response to CO$_2$ (Smyth, 1939). Also it is possible that significant changes in cutaneous gas uptake could occur due to a rise in the number of open skin capillaries in response to CO$_2$ if hypercapnia
was used to stimulate breathing (Poczopko, 1956).

An alternative method of determining energy cost is to calculate the mechanical work performed by the respiratory pump which may then be combined with an assumed or experimentally determined value for the mechanical efficiency of the pump to give the cost of ventilation. The mechanical work of ventilation in man has been estimated by measuring tidal volume or flow, and interoesophageal pressure, and constructing pressure-volume loops (Milic-Emili and Petit, 1960; Tenny and Reese, 1968). Alexander (1967) has calculated the mechanical work done in ventilation in fish on the basis of differential buccal and opercular pressures and mean flow rates, while Jones and Schwarzfeld (1974) have used pressure and flow measurements in trout to measure the mechanical work of ventilation, which, combined with measurements of oxygen consumption under various applied pressure heads, gave mechanical efficiency and thus the oxygen cost of ventilation.

Consequently in the present experiments, the mechanical work of ventilation in restrained but unanaesthetised *Rana pipiens* was measured and an attempt was made to determine experimentally the mechanical efficiency of the buccal pump. These values have been used to give an estimate of the energy cost of ventilation, and of the metabolic rate of the respiratory muscles.
METHODS

The experiments were carried out on thirty-four *Rana pipiens* weighing between 20 and 90 g. The frogs were held in tanks at room temperature for at least a week before the start of the experiments which were performed at 24°C ± 1°C.

In order to measure the mechanical work output of the buccal pump, buccal pressure was recorded by means of a cannula inserted through the tympanic membrane, and volume by means of a photo cell, as described in the previous section. Care was taken to ensure that the volume responses were linear over the physiological range, and if non-linear responses were obtained the photocell position was adjusted until linearity was achieved. The pressure and volume changes were displayed on a Hewlett-Packard 1201A storage oscilloscope operated in X-Y mode to produce pressure-volume loops (Jones, 1970). By operating the oscilloscope at maximum persistence it was possible to display up to eight of such loops on the storage surface at one time, which were then photographed by a Hewlett-Packard oscilloscope camera. The pressure and volume traces were recorded simultaneously on a Technirite 2 channel pen recorder run at 1 mm per sec in order to measure ventilation frequency. Sequences of loops which were stored on the oscilloscope were marked by means of the recorder event marker.

To measure mechanical work appearing in the lungs during lung inflation sequences, lung pressure was recorded in 4 frogs by means of a cannula of urinary catheter tubing as described in Section 1, while changes in lung volume were measured by means of the Biocom 991 impedance converter used as an impedance pneumograph. Linearity of the system proved to be ±5 percent.
Several lung inflation sequences were recorded for each frog, together with the frequency of lung inflation.

Data from 5 frogs whose weights ranged from 72 to 87.5 g were used to estimate the mechanical efficiency of individual ventilation cycles. The mechanical efficiency of the buccal pump for one ventilation cycle was considered to be given by the following modification of Hill's (1939) equation for muscular efficiency.

\[
\text{Mechanical Efficiency} \% = \frac{W}{W + a \times x + \int F dt} \times 100
\]

Where \( W \) = mechanical work measured from the buccal pump in one ventilation cycle (gram.cm)

\( a \times x \) = the heat of shortening of the muscles of the buccal floor for the cycle (gram.cm)

\( \int F dt \) = maintenance heat (the heat production associated with the force exerted by the muscles of the buccal floor throughout the ventilation cycle) (gram.cm)

In order to measure mechanical work performed in single ventilation cycles buccal pressure was recorded on the Technirite 2 channel pen recorder run at 25 or 50 mm per sec, while the volume changes of the buccal cavity were simultaneously filmed in side-view on 16 mm Tri X reversal film using a Bolex cine camera running at a nominal 24 or 32 frames per sec. To enable extreme close-ups to be taken a short extension tube was placed between the lens and camera, while a mirror included in the frame displayed a front-view of the buccal cavity. The frog was illuminated by two photofloods which were lit only when filming was taking place. The film frames were synchronised with the pressure records by including a rotating 1 cm diameter disc divided into a black and white sector in the camera field, which closed a switch in
the event marker circuit of the pen recorder once per revolution. A further switch connected to the second channel of the pen recorder was depressed when filming commenced, and a time signal was also displayed on this channel. Following a sequence of filming volume changes in the buccal cavity were calibrated by withdrawing air from the buccal cavity (with the nares and glottis blocked) in 0.2 ml steps and filming each step. Tracings of the lateral view of the buccal cavity were then made on constant density graph paper, the tracings weighed to establish the weight difference represented by the 0.2 ml calibration steps, and a calibration curve was drawn. Over the physiological range there was a linear increase of weight with volume ± 5 percent. Buccal volume was measured from the cine frames at every frame during the chosen cycle by tracing the lateral view of the buccal cavity onto constant density graph paper, weighing the tracings on a Sartorious milligram balance and reading volume from the calibration curve. Buccal pressure at each point was read from the synchronised pressure traces and plotted against volume, to give a volume-pressure loop, the area of which represented the mechanical work performed in the buccal cavity during the individual cycle.

The heat of maintenance of muscle in which twitch summation, or tetanus is occurring is the sum of the heats of activation of the muscle fibres due to successive stimuli, and is proportional to the force exerted by a muscle and the time for which it is maintained (∫Fdt). In order to estimate the heat of maintenance of the muscles of the buccal floor, it was first necessary to determine tension in the floor throughout the respiratory cycle. This was accomplished by the application of Laplace's law to the buccal floor. The two major radii of curvature of the buccal floor (longitudinal and transverse) were traced from the film frames each time buccal pressure and volume were
recorded for the mechanical work plot and substituted together with buccal pressure at that time into:

\[(ii) \quad P = \frac{T \left( \frac{1}{R_1} + \frac{1}{R_2} \right)}{R_1 R_2} \text{ grams per cm}^2 \quad \text{grams per cm} \quad \text{cm} \quad \text{cm} \]

which is the general form of the Law of Laplace (Figure 11). This gave the tension in the buccal floor at the point of intersection of the two radii (Burton, 1957). The two measured radii of curvature were taken to be constant throughout the floor so that for a given buccal pressure, tension produced by the longitudinal and transverse muscles of the buccal floor would be the same at any point on the floor. The principal respiratory muscles (medial and lateral geniohyoids, intermandibulars and interhyoids) were assumed to be attached uniformly to the borders of the buccal floor, and to lie along the radii of curvature. To estimate the total force exerted by these muscles in order to produce a given buccal floor tension, the buccal floor was considered to approximate a square in plan view, the length of one side being the distance from jaw-bone to jaw-bone at the angle of the jaw, the muscles being uniformly attached along the sides of the square. For the production of a given floor tension the total force on the muscle attachments was thus:

\[(iii) \quad F = T \times abcd \quad (\text{Figure 11}) \]

\[\text{grams} \quad \frac{\text{grams}}{\text{cm}} \quad \text{cm} \quad \text{cm} \]

and the force produced by the muscles, \(T \times 1/2 \ abcd \ \text{cm}\). Force, \(F\), was calculated each time pressure and volume were recorded and the integral of force.time was plotted graphically as the area under the curve for each respiratory movement investigated. Only the area under that part of the curve during which the respiratory muscles were active (Section 1) was used in the calculations and the heat (gram.cm) associated with this value of force.time
Figure 11. Tracings from film frames of 80 g *Rana pipiens* illustrating the 2 major radii of curvature of the buccal floor, and the application of Laplace's law to the floor.
\[ P = T \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]
was read from a calibration curve constructed from data of Fales (1972) on
the heat production of *Rana pipiens* skeletal muscle under various conditions
of force-time.

The heat of shortening of amphibian muscle is $a \cdot x$, where $a$ is a constant
with a mean value of 400 grams/cm$^2$ cross-sectional area for amphibian muscle
(Abbot, 1951) and $x$ is the shortening (cm) of the muscle. The shortening of
the longitudinal and transverse buccal floor muscles was measured externally
from the film frames as the difference between the circumferential distance
between a and b, a and d (Figure 1) at the maximum and minimum radii of
curvature during the ventilation cycle. The cross-sectional area of the
appropriate muscles (medial and lateral geniohyoids, intermandibulars and
interhyoids) was measured after sectioning and staining in haemotoxylin and
eosin. No corrections were made for muscle shrinkage in fixation.

The heat of recovery, liberated after muscular contraction, has been
found to be almost exactly equal to the initial heat (consisting of the sum
of the heat of shortening and the heat of maintenance) liberated during the
contraction, and must be included in the efficiency equation (1) in order to
give a true measure of muscular efficiency. It was therefore necessary to
multiply $a \cdot x$ (heat of shortening) in the denominator of the equation (i) by a
factor of 2, although the values of maintenance heat were not doubled as the
values determined by Fales (1972) included the associated heat of recovery.

The mass of the respiratory muscles was measured in three frogs by
weighing the frog and then dissecting out the muscles used in ventilation after
double pithing. These muscles were the medial and lateral geniohyoids, the
intermandibulars, the interhyoids, the sternohyoids, anterior petrohyoids and
omohyoids (Part I). Once dissected out, the muscles were dropped
into a previously weighed container of amphibian saline, which was reweighed in order to obtain the wet weight of the muscles. This was then expressed as a percentage of body weight.
RESULTS

(a) The mechanical work appearing in the buccal cavity and lungs in one inflation cycle.

Figure 12a and b is a diagrammatic representation of the pressure and volume events in the buccal cavity and lungs respectively, during a lung ventilation cycle. The cycle commences at A (12a), P (12b) when the glottis opens, and lung pressure and volume fall while buccal pressure and volume simultaneously rise (A to B, P to Q). The nares close at the end of this phase so that a common pressure level is attained at the end of it. Active elevation of the buccal floor then occurs in the phase B to C, Q to R, which is terminated when the glottis shuts at C, R. The nares open simultaneously, so that although the lungs maintain the new pressure level, buccal pressure drops rapidly to zero and overshoots before the loop is completed.

The total amount of work potentially available for lung inflation is represented by area F B C D A F. The amount of work performed in the cycle by the buccal pump is represented by area A B C D E A. Area B D B, however, is performed against viscous forces in the pump itself and is lost as heat, while area D E A D represents work performed against flow-resistive forces in drawing air through the nares in order to refill the pump. It, too, is lost as heat. Area B F A represents a contribution from the previous lung inflation cycle. On glottal opening the lungs release the energy stored by elastic stretch during the previous cycle; area P Q U T represents the total work done by the lungs, while B F A is the fraction effectively contributed to lung inflation in the next cycle. The remainder is done in expelling lung gas through the partially open nares, and is lost as heat. Therefore the net amount of work available for lung inflation in the cycle
Figure 12. (a) Diagrammatic representation of pressure and volume changes in the buccal cavity in a lung ventilation cycle. (b) Pressure and volume changes in the lungs during the same cycle.
is represented by area F B C D A F-B D B and the net work done in the lungs is represented by area Q P R S T U Q.

The decrease in buccal volume is mirrored by a similar volume increase in the lungs when the two are in contact through the open glottis (B to C, Q to R). Furthermore in *Rana pipiens* there is no large differential pressure across the glottis (Section 1). Therefore F B C D A F-B D B = Q P R S T U Q, little work being lost as heat in overcoming flow resistance through the glottis, which must therefore be a pathway of low resistance compared to the nares.

The magnitude of the viscous losses in the pump was investigated by passively inflating and deflating the buccal cavity of curarised frogs (0.1 mg. per 10 grams weight) with sealed nares (Figure 13a and b). The loop travels clockwise and the area of the loop over the appropriate pressure range represents the amount of work performed against viscous forces in the pump, which proved to be 5.4-6.5 percent of total buccal work in the frogs investigated.

The possibility that active flank muscle contraction aided lung deflation (Section 1) was further investigated by comparing normal lung inflation cycles with cycles obtained in the same frog after curarisation of skeletal muscle by inflating and deflating the lungs via a cannula inserted into the tip of one lung. The area of the loops obtained were the same to within 5 percent (Figure 13c and d). This provides further support for the view that lung deflation does not involve the skeletal muscle of the flanks, although it does not preclude the possibility that the tonic action of smooth muscle in the lung wall may assist elasticity in expelling lung gas.
Figure 13. (a) Pressure/volume loops from the buccal pump of an 80 g frog during 2 lung and 2 buccal ventilation cycles.
(b) Pressure/volume loop from the buccal cavity of the same frog with sealed nares, illustrating work performed against viscous forces in the pump.
(c) Pressure/volume loop recorded from the lungs during a lung inflation sequence.
(d) Pressure/volume loop obtained from the same frog by inflation and deflation of the lungs after curarisation.
(b) Mechanical work output of the buccal pump.

Figure 14a shows a typical lung inflation sequence recorded from the lungs, while 14b and c shows a typical sequence of P-V loops recorded from the buccal cavity. Pressure was measured in cm H$_2$O and volume in cm$^3$. The values of work calculated from the areas of the pressure volume loops were therefore in gram.cm. The mechanical work performed in the buccal cavity per hour was arrived at by calculating the mean amount of work performed in a sequence of lung ventilation cycles and multiplying by the frequency of such sequences then adding to this figure the mean mechanical work performed in a buccal ventilation loop, multiplied by the overall frequency of buccal ventilation. Measurement of mechanical work appearing in the lungs during a typical inflation sequence, multiplied by the frequency of such sequences enabled calculation of the amount of work appearing in lung inflation sequences per hour. The data obtained from these experiments was used to calculate the regression of mechanical work on body weight. The regression lines were fitted by the least squares method and were plotted by a computer.

Figure 15 illustrates the regression of mechanical work performed by the buccal pump per hour on body weight, for frogs ranging in weight from 24.5 to 86.5 g. For these frogs, if $M =$ Mechanical work performed per hour and $W =$ the body weight in grams, then:

$$\log M = \log k + b \log W$$

The exponent $b$ is equal to the slope of the plot of $\log_{10}$ mechanical work against $\log_{10}$ weight, while the $k$ value was obtained from the $y$ intercept. Therefore:

$$\log M = 2.47 + 1.199 \log W$$

or $$M = 3.39 \times 10^{-3.199}$$
Figure 14. (a) Pressure/volume loop recorded from the lungs during a typical lung inflation sequence.

(b) Sequence of pressure/volume loops recorded from the buccal cavity of a 24.5 g *Rana pipiens*.

(c) Simultaneous linear recording of pressure and volume. 1, event channel (7 lung ventilation loops occurring before event marker are shown in (b)); 2, buccal pressure; 3, buccal volume, up on the trace represents increase in volume; 4, time, 10 sec marker.
Figure 15. Regression of mechanical work output of the buccal pump on weight for *Rana pipiens* ranging in weight from 24.5 to 86.5 g. The interrupted lines indicate one standard error of the mean.
The regression of mean buccal volume change during ventilation on mass was also investigated (Figure 16) for frogs in the same size range. If $V =$ mean change in the buccal volume, then:

in lung ventilation,

$$\log V = 2.154 + 1.216 \log W$$

or

$$V = 7.015 \times 10^{-3} W^{1.216}$$

in buccal ventilation,

$$\log V = 2.253 + 1.175 \log W$$

or

$$V = 5.585 \times 10^{-3} W^{1.175}$$

Mean buccal pressure change in the lung ventilation cycles did not vary significantly between different sizes of frog. It therefore appears that for a given respiratory frequency, the mean volume change in lung ventilation cycles is the main factor in determining the mechanical work rate of the buccal pump in different weights of frog, and therefore the regressions of mechanical work and mean buccal volume change on weight have similar exponents.

For mechanical work appearing in the lungs per hour during lung inflation sequences, plotted against body weight (Figure 17):

$$\log M = 3.681 + 0.6451 \log W$$

or

$$M = 2.09 \times 10^{-4} W^{0.6451}$$

Thus only a fraction of the total work done on the lungs by the buccal force-pump appears in the lungs during sequences of lung inflation and deflation.
Figure 16. Regression of buccal volume change in lung and buccal ventilation cycles on weight for *Rana pipiens* at 25°C. Filled circles, lung ventilating cycles; open circles, buccal ventilating cycles.
Buccal volume change in ventilation movements

Diagram showing the relationship between buccal volume change and weight of frog.
Figure 17. Regression of work appearing in the lungs during lung inflation cycles on weight for *Rana pipiens* at 25°C. The interrupted lines indicate one standard error of the mean.
(c) The mechanical efficiency of the buccal pump.

Pressure-volume measurements of individual respiratory cycles obtained by the method described, produced the same type of pressure-volume loops as that obtained by X-Y plotting on the oscilloscope, or by the method of Jones (1970). Figure 18a illustrates a typical loop, the area of which gave the mechanical work done by the buccal pump in one lung ventilation; Figure 18b shows the calculated tension produced in the buccal floor for the same breathing cycle plotted against time, together with the corresponding buccal pressures. The muscles of the buccal floor are only active between A and B (Section 1) consequently only the area above A-B was used to determine the value of force.time. A good correlation was seen between tension in the buccal floor and buccal pressure at that time in all the frogs investigated, suggesting that the time integral of the pressure change over the appropriate part of the breathing cycle is a good index of the average respiratory force, as pointed out by McGregor and Becklake (1961).

Figure 19 illustrates the individual terms of the efficiency equation plotted against the peak pressure per cycle, which was considered to be an indication of muscular load, for the 12 cycles investigated. The heat of shortening of the buccal floor muscles a.x, is relatively constant in all cycles, the differing amounts of mechanical work performed in different cycles depending largely on the buccal pressure achieved through the cycle, which in turn depends upon force generated in the buccal floor. The heat of maintenance associated with force.time is relatively small compared to the heat of shortening of the respiratory muscles, although it increases with an increase in the maximum cycle pressure.

Figure 20 shows the calculated mechanical efficiencies of 9 lung and 3
Figure 18. (a) Pressure/volume loop measured from the buccal cavity of *Rana pipiens*. The loop cycles anti-clockwise from zero pressure.

(b) Calculated tension in the buccal floor, together with the corresponding buccal pressure (continuous line) for the same cycle.
Figure 19. Terms of the efficiency equation plotted against maximum cycle pressure for 12 individual ventilating cycles, together with calculated total energy input per cycle. Open circles, buccal ventilating cycles; filled circles, lung ventilating cycles.
Total energy

Mechanical work

Heat of maintenance

Heat of shortening

Maximum cycle pressure  cm H$_2$O
buccal ventilating cycles, plotted against the total mechanical work performed in each cycle. Efficiency of lung ventilation increased with mechanical work performed per cycle from 7.4 percent at 0.65 gram.cm to a maximum of 19.3 percent at 2.73 gram.cm. The mechanical efficiency of the three buccal ventilations investigated proved to be low, although efficiency increased in the high amplitude buccal movement in which a relatively large amount of mechanical work was performed. Calculated efficiency increased with increased mechanical work performed in ventilating cycles because total volume change varies very little in all lung ventilating cycles (Figure 14a and b), and hence the measured amount of shortening of the medial and lateral geniohyoids is virtually constant. This is reflected in the value of a.x, the heat of shortening, in the denominator of the efficiency equation, which is of relatively large magnitude compared to the values for mechanical work and the force.time equivalent. Therefore, if mechanical work is doubled, the numerator of the fraction is doubled, while the denominator is increased only slightly, and the value for efficiency almost doubles.

The calculated total energy, or mechanical equivalent of the oxygen consumed for each respiratory cycle studied is also plotted in Figures 19 and 20. Total energy per lung ventilating cycle increased steadily as the total mechanical work performed each cycle increased, in spite of the accompanying increase in mechanical efficiency. The low efficiency of the lung ventilation movement which generated the high peak pressure of 9.2 cm H₂O was due to the relatively large magnitude of the heat of maintenance in the denominator of the efficiency equation. Although the value for mechanical work in the low amplitude buccal ventilating cycles is low, mechanical efficiency is also low, 0.4 percent-0.5 percent, so that the calculated total energy for these
Figure 20. Calculated mechanical efficiency and total energy input plotted against mechanical work for 12 ventilating cycles. Open circles, efficiency of buccal ventilating cycles; filled circles, efficiency of lung ventilating cycles; triangles, total energy input per cycle.
cycles is only slightly lower than that calculated for low-amplitude lung-ventilatory cycles.

The mean mechanical efficiency of the respiratory cycles analysed was 10.6 percent, but this is almost certainly higher than the overall mechanical efficiency of ventilation, for in an undisturbed frog relatively inefficient low amplitude lung ventilation cycles and buccal cycles predominate. The overall mechanical efficiency was therefore taken to be 8 percent, the efficiency of low amplitude lung ventilation cycles. This value, together with a value for the mechanical work of ventilation (Section a) enables an estimate to be made of the oxygen cost of breathing if the data of Hutchinson et al (1968) are used for the total oxygen consumption and R.Q. of Rana pipiens at 25°C. Hutchinson et al. (1968) found an R.Q. of 0.73 for Rana pipiens at 25°C. This is equivalent to a calorific output of $4.71 \times 10^{-3}$ (Cal/ml $O_2$). This value was used in Table 1 in which the oxygen cost of ventilation is calculated for 20 and 50 g frogs at 25°C for maximum, minimum and mean, and assumed overall values of mechanical efficiency. The oxygen cost of ventilation for a resting frog proved to be approximately 5 percent, and all values are within the range 2-7 percent.

(d) Measurements of muscle mass and calculation of respiratory muscle metabolism.

Results of dissections of 3 Rana pipiens with a mean weight of 63.6 g (range, 60.5 to 68.2 g) gave an average of 0.92 percent (0.58 g), as the proportion of total body weight of the respiratory muscles, the range of the measurements being from 0.81 to 0.98 percent. The mechanical work of breathing of frogs in this weight range was found to be 0.50 Joules per hour (Section a). The oxygen consumption of the respiratory muscles, expressed in the standard way as ml $O_2/100$ g/min is calculated in Table 2 for the
### TABLE 1

For 50 g *Rana pipiens* at 25°C

<table>
<thead>
<tr>
<th>Mechanical work of breathing</th>
<th>Efficiency percent</th>
<th>Total energy input to buccal pump</th>
<th>Oxygen cost percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41 Joules per hour (Section a)</td>
<td>19.5%</td>
<td>2.1 Joules/hour</td>
<td>2.1%</td>
</tr>
<tr>
<td>Total oxygen consumption = 5.0 ml O₂ per hour (Hutchinson, Whitford and Kohl, 1968)</td>
<td>5.9%</td>
<td>6.9 Joules/hour</td>
<td>6.9%</td>
</tr>
<tr>
<td>= 100 Joules per hour</td>
<td>10.6%</td>
<td>3.9 Joules/hour</td>
<td>3.9%</td>
</tr>
<tr>
<td>Max. observed efficiency of lung ventilation</td>
<td>8.0%</td>
<td>5.1 Joules/hour</td>
<td>5.1%</td>
</tr>
<tr>
<td>Min. observed efficiency of lung ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean observed efficiency of lung ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall efficiency of lung and buccal ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For 20 g *Rana pipiens* at 25°C

<table>
<thead>
<tr>
<th>Mechanical work of breathing</th>
<th>Efficiency percent</th>
<th>Total energy input to buccal pump</th>
<th>Oxygen cost percent</th>
</tr>
</thead>
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<tr>
<td>0.22 Joules per hour (Section a)</td>
<td>19.5%</td>
<td>1.1 Joules/hour</td>
<td>1.9%</td>
</tr>
<tr>
<td>Total oxygen consumption = 2.8 ml O₂ per hour (Hutchinson, Whitford and Kohl, 1968)</td>
<td>5.9%</td>
<td>3.7 Joules/hour</td>
<td>6.8%</td>
</tr>
<tr>
<td>= 56 Joules per hour</td>
<td>10.6%</td>
<td>2.1 Joules/hour</td>
<td>3.7%</td>
</tr>
<tr>
<td>Max. observed efficiency of lung ventilation</td>
<td>8.0%</td>
<td>2.8 Joules/hour</td>
<td>4.9%</td>
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<tr>
<td>Min. observed efficiency of lung ventilation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean observed efficiency of lung ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall efficiency of lung and buccal ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 2

<table>
<thead>
<tr>
<th>Mechanical work output (Joules/hour)</th>
<th>Efficiency of buccal pump (percent)</th>
<th>Calculated work input (total energy) (Joules/hour)</th>
<th>Oxygen equivalent of work input (ml O₂/hour)</th>
<th>VO₂ respiratory muscle (ml O₂/100g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>19.5%</td>
<td>2.6</td>
<td>0.13</td>
<td>0.37</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.9%</td>
<td>8.5</td>
<td>0.42</td>
<td>1.21</td>
</tr>
<tr>
<td>* &quot;</td>
<td>8.0%</td>
<td>6.3</td>
<td>0.31</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*Overall efficiency*
maximum, minimum and overall efficiencies of ventilation, assuming 1 ml $O_2$ = 19.56 Joules (Weast, 1968).
DISCUSSION

No previous studies have been performed on the mechanical work output of the amphibian buccal pump, but it is possible to compare respiratory work in *Rana pipiens* and a mammal of similar size by using data of Crosfil and Widdicombe (1961) on the mouse, which indicates that 0.025 Joules per gram per hour is the work output in normal breathing. This is approximately 3 times the value of the respiratory work output in the frogs studied in the present investigation.

The method described for estimating the mechanical efficiency gives rise to a range of efficiencies of from 5.9 to 19.5 percent for lung ventilation with an overall efficiency value of 8 percent. There are no comparable figures for mechanical efficiency in amphibians although efficiency in fish is probably very low, about 1-4 percent at rest (Jones, 1971; Jones and Schwarzfeld, 1974). Mechanical efficiency in man has been found to range from 5.5 to 8.6 percent (Liljestrand, 1918; Otis, 1954; Campbell et al, 1957; Cherniak, 1959; Fritts and Cournand, 1956) although values of 19 to 25 percent have been found by comparing mechanical work, determined by pressure/flow measurement, with total energy required for breathing (Milic-Emili and Petit, 1960).

Hill's equation for muscular efficiency applies to an isotonic muscle contraction. How well is this condition fulfilled in the respiratory muscles of the buccal floor of a frog during a respiratory cycle? At the start of the cycle the buccal floor muscles cannot begin to shorten until the muscular force exceeds the load, which is a function of the pressure generated within the buccal cavity and this is therefore initially small. Load increases with muscular shortening, which reduces buccal volume, until at peak pressure
muscular force equals load on the muscles, and shortening stops. As the muscles shorten rapidly (0.2-0.3 seconds) the force exerted by them is probably never greatly in excess of load. The muscle contraction occurring in a respiratory cycle is therefore similar to an isometric contraction in which a muscle shortens under load, except in this case load increases through the contraction instead of remaining constant.

The values of maintenance heat given by Fales (1972) and used here were determined in amphibian muscle in isometric twitch and tetanus. For both conditions 1 gram.sec = 1 gram.cm at low values of force.time. The fundamental mechanism of contraction remains the same however in both isotonic and isometric contractions, so that it seems reasonable that the heat generated for a given value of force.time should not be significantly different in either condition. The estimation of muscle shortening by external measurement is probably a reasonable reflection of the shortening of the lateral and medial geniohyoids and the intermandibular and interhyoid muscles. It fails to take into account the shortening of the sternohyoids, omohyoids and petrohyoids which also contribute to the ventilation cycle. The calculated value of the heat of shortening is almost certainly therefore a conservative estimate of its true value, which would tend to result in high values for the mechanical efficiency of the buccal pump, in turn reducing the calculated energy cost of ventilation. Jones (1972) found that curarised, artificially ventilated Rana esculenta at 24°C showed an average reduction in oxygen consumption of 16 percent compared with normally breathing animals. Individual frogs showed reductions between 1 and 35 percent however, and in some frogs striated muscle other than respiratory muscle must have been curarised, so that it would be unwise to assume that 16 percent represented the energy cost of breathing.
The energy cost of 5 percent reported here is at least double that calculated for man at rest (Liljestrand, 1918; Cournand et al, 1954; Cherniak, 1959; Fritts et al, 1959; Milic-Emili and Petit, 1960; Campbell et al, 1970), although a good deal less than energy cost of ventilation in fish, which is generally reported to be 8 to 20 percent of the overall oxygen consumption at rest (Van Dam, 1938; Schumann and Piiper, 1966; Alexander, 1967; Cameron and Cech, 1970; Jones and Schwarzfeld, 1974). This large cost in fish appears to be due in part to the high viscosity and density of water (viscosity 100 times and density 1000 times that of air at 18°C) suggesting that the work done against viscous and elastic forces developed in the irrigating system, which is independent of the properties of the medium, is probably relatively unimportant in determining the oxygen cost of ventilation compared to the amount of work done against flow resistive forces, which depend on the viscosity and density of the medium. The amount of work performed against flow resistance in the frogs investigated proved to be small which is consistent with the favourable physical properties of air as a respiratory medium.

Only a small proportion of the total body weight in Rana pipiens is made up of respiratory muscle, and different estimates of the oxygen cost of breathing would obviously alter the calculated metabolic rate of these muscles. The calculated value of respiratory muscle oxygen consumption, \( \dot{V}O_2 \), 0.89 ml \( O_2 \)/100 g/min may be compared with the oxygen consumption of the whole frog at 25°C which is 0.17 ml \( O_2 \)/100 g/min (Hutchinson et al, 1968). The respiratory muscles at rest are therefore metabolising at 5 times the rate of overall metabolism. This estimate of respiratory muscle metabolism appears reasonable, although as yet there is no comparable data for other amphibians. Values of \( \dot{V}O_2 \) for some muscles in fish are somewhat higher, being 2.50 and 6.60 ml \( O_2 \)/100 g/min for Tuna red muscle and Carp white muscle, respectively.
at rest (Cameron and Cech, 1970). In man the basal metabolic rate for
muscle is estimated to be 0.2 ml O₂/100 g/min, while Landis and Pappenheimer
(1963) estimate the oxygen consumption of actively contracting muscle in
man at 10 ml O₂/100 g/min.
PART III. THE INITIATION OF DIVING APNOEA IN RANA PIPiens

INTRODUCTION

All air-breathing vertebrates are forced into an apnoeic condition on submersion in water. The initiation and maintenance of apnoea during submersion have not been greatly investigated, although there is a growing amount of evidence that sensory information from the area around the nostrils, the nasal cavity, and in some cases the region of the glottis and upper respiratory tract is involved (Huxley, 1913a; Lombroso, 1913; Andersen, 1963a; Cohn et al., 1968; Butler and Jones, 1968; Jones and Purves, 1970; Angell James and Daly, 1972a and b; Drummond and Jones, 1972).

In spite of the fact that amphibians are more completely adapted to a semiaquatic existence than other diving vertebrates, there is little information available concerning the stimulus which causes inhibition of ventilation during periods of submergence, or the sites sensitive to such stimulation. Willem (1920) thought that external narial closure and apnoea on submersion in Rana esculenta was reflex, due to the wetting of the snout, and that the occasional release of lung gas underwater through the nares involved the temporary overriding of this reflex inhibition. Spurway and Haldane (1953) considered that the presence of water at the snout provides an inhibitory stimulus to ventilation in newts and that resumption of ventilation observed on surfacing of the snout was due to "the cessation of inhibitory sensory stimuli rather than because of any positive sensory stimuli from the air". Certainly the presence of atmospheric O₂ or CO₂ does not appear to be involved
in stimulating the resumption of ventilation on surfacing in amphibians, for if frogs (*Rana temporaria*) emerge from water into an atmosphere of nitrogen they still resume ventilation (Jones, 1966).

The purpose of the present investigation was to determine the sensory areas important in the initiation and maintenance of diving apnoea in the frog *Rana pipiens*, to record from the nerves serving these areas during simulated dives, and to attempt to initiate apnoea in the frogs by electrical stimulation of these nerves, in order to establish their role during diving.
METHODS

The experiments were performed on 65 *Rana pipiens* with a weight range of from 50 to 85 g, although animals in the 75-85 g weight range were used exclusively in the nerve stimulation experiments. All the experiments were performed at an air temperature of 24°C.

Experiments involving submergence of normal animals and animals after section of the ophthalmic nerves, were carried out in a perspex tank of 3 litres capacity. The tank was connected to a large water reservoir so that the water level could be raised and lowered at will. Water temperature was maintained at 20°C except in those experiments in which a bead thermistor was placed in the nasal cavity of the frog, when it was maintained at 14°C. The animals were anaesthetised by immersion in MS 222 (Sandoz) (300-400 mg/l.), positioned on a cork board and restrained by pinning (Jones, 1970). During normal dives buccal pressure and volume were recorded by the methods described in Section 1. Struggling made this method impractical for recording respiration in frogs in which the ophthalmic nerves were cut. Consequently, in these frogs EMG activity was recorded from the posterior intermandibular muscle and the larynx in order to monitor ventilation.

Section of the ophthalmic branch of cranial nerve V at the level of the nasal cavity was accomplished under deep MS 222 anaesthesia. The frog was placed on its back, and the jaws were held apart. Incisions were then made in the floor of the nasal cavity, from the internal narial openings to the midline. The cartilages forming the floor were then reflected back to the midline, exposing the nasal cavities. The main branches of the ophthalmic nerve enter each nasal cavity through foramina in the sphenethmoid cartilage,
and then across the cavities between the cartilage and the olfactory epithelium (Figure 21a, b), before passing through the skull to supply the skin of the external narial region and the snout (Ecker, 1889). In the operated animals they were cut bilaterally close to the point of emergence from the sphenethmoid cartilage while for sham operations they were merely identified through slits made in the olfactory epithelium parallel to their courses. The nasal cartilages were then returned to position and, in the larger frogs, held in place by 2 silk stitches. Both experimental and sham operated animals were allowed to recover from the anaesthesia and left for 24 hours in a large holding tank before being used in an experiment, and only those frogs which appeared to respire normally after the operation were used. Blood loss during the operation was small and the survival was good in both operated and sham operated frogs.

Recordings of afferent nervous activity were made from the ophthalmic branches of V at the level of the nasal cavity. The nerves were sectioned centrally in double-pithed frogs and electroneurograms monitored using a pair of fine, silver wire hook electrodes under mineral oil. Those branches with their receptive fields round the external nares were chosen for the recordings. The response to a water meniscus moving over the nares and to water flow was investigated by placing the frogs in the 3 litre tank, in which the water level could be raised and lowered. The frogs were pinned on their backs with the lower jaw pinned back, and the external narial openings were plugged to prevent water entering them underneath the mineral oil and thus grounding the electrodes. An attempt was also made to subject the narial region to water pressure by sewing a membrane around the snout and connecting this to a water column. However, this proved impractical due to the difficulty of membrane attachment to the frog. Therefore, in order to determine the effects of
Figure 21a and b. Transverse sections of the snout of a 20 g frog, illustrating the course of the ophthalmic nerve. 1, nasal cartilage; 2, ophthalmic branches; 3, nasal mucous epithelium.
pressure some trials were carried out in which the narial region was submerged under 4-6 mm of mercury, which was retained around the nares by a modelling clay dam, while in others the entire frog was submerged under mineral oil (S.G. 0.87).

Recordings were made from the cutaneous branches of the second, third, and fourth spinal nerves in order to determine the responses of cutaneous nerves serving other areas of the skin to pressure and the movement of a water meniscus. In order to approximate the conditions obtaining at the snout, where the skin is firmly connected to the underlying cartilage, flaps of skin containing the receptive fields were placed on a ground glass disc to which hydrostatic pressure was applied. The fibres serving a receptive field were led out through a small hole in the centre of the disc.

Nervous activity was amplified and filtered by means of a Tetronix 122 preamplifier and displayed on a Tetronix 502A oscilloscope, being simultaneously recorded by means of a Hewlett-Packard 3900C tape recorder. Suitable signals were later photographed on playback by means of a Grass oscilloscope camera, or else played into a Brush penrecorder at reduced speed. In some experiments a ratemeter was used to determine firing rate, and some data was analysed as Time Interval Histograms by means of a Digital Lab 8E computer.

In those experiments which involved electrical stimulation of the ophthalmic branch of V, the nerves were exposed bilaterally at the level of the orbit. It was necessary to remove the eyes under deep MS 222 anaesthesia in order to accomplish nerve stimulation. Initially the nictitating membrane was removed and the eye muscles were cut close to the eyeball, and the optic nerve, artery and vein were tightly ligatured. The optic stalk was then cut distally to the ligature and the eye removed. The ophthalmic branch of V, which runs
between the cranium and the eyeball, below the superior rectus muscle but above all the other eye muscles (Ecker, 1889), was then carefully freed from its connective tissue sheath and associated blood vessels. Blood loss in the operation was negligible. Before complete recovery from the anaesthesia the frogs were secured in a head holder, which rigidly fixed the head in relation to the stimulating electrodes, but allowed respiratory movements to occur normally (Jones, 1970). Complete recovery from the anaesthesia usually occurred in 30-40 minutes and ventilation restarted spontaneously. Before the frog had completely recovered the nerves were placed on the stimulating electrodes under mineral oil, and the distal ends of the nerves were severed at the anterior end of the orbit. Fine hook electrodes were used, the stimulating pulses being provided from a Grass model S4G stimulator, and displayed on a Tetronix 502A oscilloscope. Unipolar stimulation was used at 50-1000 Hz 0.4-4 msec duration and 30 mv to 5 v intensity. Ventilation movements were monitored by recording buccal pressure, which was displayed on a Hewlett-Packard 4 channel pen recorder, while periods of nerve stimulation were indicated by means of the event channel. The position of the nares was observed by means of a binocular dissecting microscope. Care was taken to keep the skin of the animals moist throughout the course of these experiments.
RESULTS

(a) Preliminary experiments

Several types of preliminary experiments were performed on *Rana pipiens* to determine the site initiating apnoea on immersion in water. In the first of these the water level was gradually raised, and the effects on ventilation were noted. In a total of 30 trials on 5 frogs ventilation movements ceased when the water reached the level of the external nares in 27 tests. In three experiments temporary apnoea occurred when water came in contact with the buccal floor, but normal ventilation movements were resumed after 10-15 seconds, and continued until the water had risen to the level of the external nares when they stopped.

When the frogs were surfaced after periods of submergence which varied from 2 to 15 minutes, resumption of breathing occurred immediately the water level fell below the level of the external nares in 23 cases. In 3 trials breathing did not resume until after a lag of 10 to 30 seconds, while in the remaining 4 trials breathing did not restart until the water level had fallen below the level of the buccal floor. The rate of total submersion and emersion in the trials varied from 15 seconds to 2 minutes.

Three frogs, blinded by section of the optic nerves, performed in the same way as the sighted animals. Furthermore, no variations in ventilation rate could be induced in frogs which were placed in a large beaker in a tank in which the water level was raised and lowered. In these experiments the water surface passed across the frogs' visual field, although the frogs never came into contact with the water.

It was possible however, that the presence of water on the surface of the
eyes or tympanic membrane, both on a level with the external nares, could be important in the development of apnoea. To test this hypothesis 20 trials were made with three frogs in which the frogs were secured to a vertical cork block, head up, so that the tympanic membranes, tympanic membranes and eyes, and finally the tympanic membranes, eyes and external nares could be submerged by raising the water level. In no trial could apnoea be induced by wetting the tympanic membranes, or the tympanic membranes and eyes; only when the water meniscus was at the level of the external nares did apnoea occur.

These preliminary trials strongly suggested that water at the level of the external nares reflexly induced apnoea in *Rana pipiens*, water at the level of the eyes and tympanic membranes having no effect on ventilation.

(b) Diving in normal animals.

Figure 22a and b illustrates the pressure and volume changes recorded from the buccal cavity of frogs during the course of 2 dives of differing duration (the base line change in the volume trace on submersion and emersion is due to the water meniscus moving past the face of the photocell). The water surface reached the level of the external nares in 10-20 seconds, at which point respiratory movements ceased and the nares closed. In both cases the nares closed during a period of buccal ventilation when the lungs were inflated and isolated from the glottis. A few seconds after narial immersion in each dive bubbles of gas were released from the nares, bubble release being preceded by a rapid increase in buccal pressure (Figure 22a and b). In cases where animals were submerged with the lungs full this gas represented part of the lung contents, and its release was accompanied by contraction of the flanks, increasing endopulmonary pressure and decreasing lung volume. In others, however, it represented loss of some of the buccal gas (Figure 23a), and
Figure 22a and b. Two dives of differing duration. Top trace of each pair, buccal pressure; lower trace, buccal volume. Increase in volume is up on trace. The arrows indicate submersion and surfacing. 0 indicates loss of gas through the nares.
resulted in the buccal floor being pressed close to the roof of the buccal cavity during the dive due to its reduced gas volume. In the early part of the period of submersion both lung and buccal pressure slowly increased in response to the increase in hydrostatic pressure as the water rose, until both had increased by 2-3 cm H₂O which corresponded to the height of the water column above the frog.

Throughout the course of the dive two types of pressure events were recorded from the buccal cavity and lungs. In the first (Figure 23b), glottal opening resulted in a simultaneous fall in lung pressure and increase in buccal pressure, followed by equilibration of lung and buccal pressures. The buccal pressure rose faster than in the corresponding phase A-B of a lung ventilation cycle (Section 1) and the equilibration pressure was higher, because in this case there was no loss of gas through the nares, which were closed underwater. Buccal volume increased in this phase due to the influx of lung gas into the buccal cavity through the open glottis (Figure 23b). After equilibration, decrease in buccal volume raised the pressure in the system until the original lung pressure was attained, at which point the glottis closed, isolating the lungs once more. The posterior intermandibular muscle was active during this phase, as presumably were the other muscles involved in lung ventilation cycles. The cessation of muscular activity in the buccal floor muscles then allowed buccal pressure to fall back to the pressure due to the column of water above the buccal cavity. The frequency of these underwater lung ventilation cycles was very variable, ranging from 1-10 per minute in individual frogs and often their frequency increased during the course of the period of submergence (Figure 22b). Lung pressure was maintained at a fairly constant level throughout the period of submergence,
Figure 23. Pressures recorded from the buccal cavity and lungs during a dive. a, submersion; b, 10 minutes after submersion; c, surfacing. Top trace, event marker. Middle trace, lung pressure. Lower trace, buccal pressure.
although buccal pressure often fell slightly, together with a slight increase in buccal volume throughout the course of the longer dives (Figure 22b), suggesting that it must have initially been held slightly higher than the hydrostatic pressure by tone in the muscles of the buccal floor. The period of apnoea caused by submersion ended when the water level fell past the level of the external nares. Emersion initiated a burst of lung ventilation cycles of high pressure, followed by an increased frequency of ventilation (Figure 23c), most of the cycles being of the lung ventilation type (Jones and Shelton, 1964).

A second type of pressure event was recorded from the lungs, and sometimes the buccal cavity, during dives. This consisted of a low amplitude fluctuation in the pressure record which occurred at a frequency of 40-50 per minute (Figure 23a, b, c), and was probably due to the volume changes of the cardiac cycle being transmitted via the thoraco-abdominal cavity to the closed buccal cavity and lungs. This was never noted during periods of air breathing when it was masked by the pressure changes associated with buccal ventilation and the indirect transmission of these pressure changes to the lungs. The appearance of this fluctuation in the buccal pressure trace appeared to depend on buccal volume. It was frequently observed in those dives in which part of the buccal gas was initially lost through the nares and buccal volume was low, in which case the volume changes of the cardiac cycle presumably produced significant pressure fluctuations in the buccal cavity.

No water could be found on inspection of the buccal and nasal cavities of normal frogs on emergence. Figure 24 illustrates the response of a bead thermistor in the nasal cavity of a frog during immersion in water 10°C colder than the ambient temperature. No temperature drop was observed on
Figure 24. Output from a thermistor located in the narial cavity.

Arrow indicates the point of narial submergence.
2°C
ex.

30 sec.

nares submerged
narial submergence, the deflection of the trace after submergence being due to the release of gas bubbles through the nares.

(c) Denervation experiments

Rhythmic electrical activity associated with ventilation recorded from the larynx and the posterior intermandibular muscles ceased immediately water covered the external nares in normal and sham operated frogs, while resumption of ventilation rapidly followed narial emergence (Figure 25a). In the course of these experiments, during which the frogs underwent repetitive 15-minute dives, evidence was found for laryngeal constrictor muscle activity, in the form of a spindle of low amplitude muscle spikes occurring immediately after the burst of activity in the laryngeal dilator muscles. On some occasions laryngeal constrictor activity occurred, accompanied by a short burst of activity in the posterior intermandibular muscle a few seconds after submergence in both normal and sham operated frogs (Figure 25b). This muscular activity was associated with an elevation of the buccal floor, and the release of buccal gas through the nares.

Bilateral section of the ophthalmic branch of the trigeminal nerve at the level of the nasal cavity resulted in an altered response to submergence and emergence. Instead of a cessation of electrical activity, sustained tonic activity started in both the laryngeal constrictor muscles and also in the posterior intermandibular muscles one to two seconds after submergence of the nares (Figure 26a). This was accompanied by intense struggling on the part of frog, and elevation of the floor of the buccal cavity to the roof of the buccal cavity, giving the buccal floor a concave appearance. At the same time air was lost from the nares, and in some frogs the mouth gaped and gas was lost from the side of the mouth. The tonic electrical activity recorded
Figure 25. EMGs recorded from the posterior intermandibular and laryngeal dilator muscles on submergence and surfacing. 

a, normal animal; b, sham operated animal.
Figure 26. EMGs recorded from the posterior intermandibular and laryngeal dilator muscles.

a) denervated frog. Arrow indicates narial submergence.

b) normal frog. Arrow indicates injection of 0.2 ml water into buccal cavity.
from the muscles died down in one to two minutes, and the frogs became quiescent. Narial emergence did not initiate the resumption of normal respiration in these denervated frogs. Respiration in two of the five frogs restarted in the second and third minute respectively after emergence, while in the remaining three frogs, respiration had not resumed by the fifth minute. On investigation, 0.02 to 0.1 ml of water was removed by means of a 1 ml hypodermic from the buccal cavity of 4 of the five frogs, while water was found in the lungs of the three frogs that did not resume respiration. Water was never found in the buccal cavities of normal frogs after dives.

Injection of 0.2 ml of water into the buccal cavity via a cannula in the tympanic membrane in normal frogs produced tonic activity in both sets of muscles and struggling similar to that observed in denervated frogs (Figure 26b), suggesting that this is a response to the presence of water in the buccal cavity.

(d) Recordings from the external branch of the ophthalmic nerve.

When the external nares were submerged in water at room temperature, mechanoreceptor activity in branches of the ophthalmic nerve serving the skin around the nares was initiated. Activity was greatest during the time when the water surface was moving over the external nares, and gradually adapted afterwards (Figure 27a). Emersion of the nares resulted in a similar burst of spike activity, the units involved being those of large and intermediate spike height (Figure 27b). The closeness of the recording sites to the nares limited the depth of water that could be used to 5-6 mm, and under these conditions adaptation occurred in approximately five seconds on submersion, and in approximately ten seconds on emersion. Once adaptation had occurred after water submersion, activity could be restarted by causing water flow over
Figure 27. Afferent nervous activity in a narial branch of the ophthalmic nerve.

a) Narial submersion. Meniscus starts to travel across external nares at arrow.

b) Narial emersion. Meniscus starts to travel across external nares at arrow.
the nares. Figure 28a (i) and (ii) illustrates the response of the nerve to two rates of water flow. Water was delivered from 1 mm I.D. tubing positioned 1 cm from the external nares which were at a depth of 6 mm. The flow rate was 0.2 ml per second in Figure 28a (i) and 0.5 ml per second in Figure 28a (ii). In order to simulate the effect of pressure at greater depths mercury or mineral oil was used. Figure 28b illustrates a trial using mercury. Under a pressure of 1.4 cm H₂O the nerve fired at a frequency of 20 Hz after 15 seconds stimulation and had completely adapted after 60 seconds (Figure 28b (i)). At a simulated depth of 5.6 cm H₂O the adaptation rate of the nerve was considerably slower, and the nerve still fired at an impulse frequency of 20 Hz after 4 minutes stimulation (Figure 28b (ii), (iii), (iv)). The response obtained when a mineral oil meniscus moved across the external nares was generally not as great as the response obtained with a water meniscus, possibly because of the lower density of the oil (Figure 29b).

The units involved were mechanoreceptors of intermediate spike height (Figure 29a and c), which adapted rapidly once the meniscus had risen above the level of the nares. Tonic activity in the nerve had generally increased over the control level in air by the time the nares were under 1 cm oil (0.87 cm H₂O pressure), but no further increase in frequency occurred under applied pressures of 2 and 3 cm H₂O (Figure 30a, b, c and d). The tonically active fibres were those of small and intermediate spike height.

Control recordings of mechanoreceptors serving the cutaneous branches of the second, third and fourth spinal nerves showed that they responded to suddenly applied steady (rectangular) stimulation of their receptive fields in a similar way to those serving the ophthalmic nerve (Figure 31a, b, c), but no response to the passage of a water meniscus was obtained in 12 preparations. Response to an increase in hydrostatic pressures to 10 cm H₂O
Figure 28.  

a. Response of a narial branch of the ophthalmic nerve to water flow over the nares after adaptation to submersion has occurred.  
   i, 0.2 ml/sec; ii, 0.5 ml/sec. Top trace, event marker.

b. Tonic activity in a narial branch of the ophthalmic nerve in response to pressure applied at the nares.  
   i, 1.4 cm H₂O pressure, 20 sec after application; ii, 5.6 cm H₂O, 20 sec; iii, 5.6 cm H₂O, 90 sec; iv, 5.6 cm H₂O, 4 min.
Figure 29.  

a. Response of a narial branch of the ophthalmic nerve to punctate stimulation in air, with time interval histogram of discharge frequency.  

b. Response of same branch to the passage of a mineral oil meniscus across the nares.  

c. Response to punctate stimulation after 10 min under 1 cm mineral oil.  

Top trace, event marker; middle trace, neurogram; bottom trace, time.
Figure 30. a. Ongoing activity in a fine narial branch of the ophthalmic nerve in air.

b. Tonic activity under 1 cm oil (S.G. 0.87).

c. Tonic activity under 2 cm oil.

d. Tonic activity under 3 cm oil.
Time 0-23 sec

a

b

c

d

0.25 sec

0.25 sec

0.25 sec

0.25 sec

Total 12

APs

0 25 sec

30

Total 114

APs

0 25 sec

30

Total 119

APs

0 25 sec

30

Total 109

APs

0 25 sec

30
Figure 31. Recordings of mechanoreceptors serving the dorsal cutaneous branch of the 3rd spinal nerve.

a. Rectangular punctate stimulation in air.

b. Rectangular punctate stimulation under a hydrostatic pressure of 10 cm H₂O.

c. Few fibre preparation in air.

Top trace, event marker; middle trace, neurogram; bottom trace, time.
in 2 cm H$_2$O increments followed by a sudden fall to atmospheric pressure was limited to a few fibres responding to the initial increment and to the fall in pressure (Figure 32), with no increase in tonic activity at any pressure level, and no response to the water meniscus.

(e) Nerve stimulation

Bilateral stimulation of the ophthalmic nerves at the level of the orbit with 1-4 msec pulses proved effective in initiating periods of apnoea with a latency of less than 30 msec in most frogs. The most effective frequency of stimulation was from 250-500 Hz while the threshold voltage was 30-300 mv. Frogs in apnoea became very quiescent and virtually no struggling occurred. The apnoeic periods did not continue indefinitely. Short periods of apnoea of 10 to 20 seconds duration, produced by stimulation at a voltage just above the threshold voltage were terminated by the resumption of ventilation, even though the stimulation still continued. The nares remained open in these short periods of apnoea, and buccal pressure remained at atmospheric (Figure 33a and b). A slight increase in the stimulating voltage resulted in narial closure occurring at the start of the period of apnoea, buccal pressure being held slightly above atmospheric until the nares opened once more, often midway through the apnoeic period (Figure 33c and d). Further increase in the stimulating voltage produced longer periods of apnoea with closed nares. On narial closure buccal pressure was initially maintained above atmospheric, presumably due to tone in the buccal floor muscles, although it fell slowly through the apnoeic period (Figures 34, 35). Buccal volume was low during the apnoeic periods, the buccal floor being elevated, and often small pressure fluctuations reflecting the heartbeat were present in the buccal pressure trace (Figures 34, 35).
Figure 32. Response of mechanoreceptors serving the dorsal cutaneous branch of the 3rd spinal nerve to hydrostatic pressure.

Top trace, event marker; middle trace, neurogram; bottom trace, time.
Figure 33. Bilateral electrical stimulation of the ophthalmic nerves in the orbit. 200 pps, 4 m sec pulses.

a and b, 30 mv.
c, 60 mv.
d, 150 mv.

Top trace, event marker; middle trace, buccal pressure; lower trace, time (sec).
Figure 34. Bilateral electrical stimulation of the ophthalmic nerves. 300 mv, 200 pps, 4 m sec pulses. Top trace, event marker; middle trace, buccal pressure; lower trace, time (sec).
Figure 35. Bilateral electrical stimulation of the ophthalmic nerves. 300 mv, 200 pps, 4 m sec pulses. At arrow stimulating frequency was changed. Top trace, event marker; middle trace, buccal pressure; lower trace, time (sec).
Pressure events resembling underwater lung ventilation cycles occurred with increasing frequency throughout the longer periods of apnoea, often threatening to "break through" the apnoea (Figure 35). Narial opening did not occur during these cycles and if buccal pressure was above atmospheric it never fell to the atmospheric value. These cycles appeared similar in every respect to those occurring during submersion.

Narial opening and the resumption of normal ventilation cycles at the end of a stimulation period was not immediate (Figure 34) but occurred after a lag of about 10 seconds. Even after periods of apnoea as short as 3-5 minutes the frequency of ventilation increased greatly over the pre-apnoeic frequency and initially consisted entirely of lung ventilation cycles. Periods of apnoea of "indefinite" duration were obtained in 2 frogs. Apnoea was considered to be indefinite if a period of apnoea, broken only by dive-type ventilation movements, was induced for longer than 15 minutes, and only ended after the stimulation stopped. Figure 36 illustrates one such trial, which continued for 21 minutes before the stimulation was stopped. The normal respiratory pattern was regained 2 minutes after the end of stimulation.

In an attempt to simulate the situation during a normal dive, where presumably impulse frequency in the trigeminal nerve peaks on submersion and emersion while adaptation occurs during the dive, the stimulating frequency was reduced by an order of magnitude shortly after the initiation of apnoea, then briefly pulsed at the original frequency just before the end of stimulation in some trials. This method of stimulation proved to be as effective in producing apnoea as maintaining the original frequency throughout the course of the "dive" (Figure 35). Efforts were also made to simulate the
Figure 36. Bilateral electrical stimulation of the ophthalmic nerves.

2 V, 300 pps, 2 m sec pulses. a, 0 min; b, 7 min; c, 21 min.
At arrow stimulating frequency was changed. Top trace, event marker; middle trace, buccal pressure; lower trace, time (sec).
Figure 37. Relationship of time in apnoea to stimulus voltage. 200 pps, 4 m sec pulses.

a. open bars, frog in air; solid bars, submerged to nares.
b. open bars, bilateral ophthalmic stimulation; solid bars, unilateral stimulation. Standard errors are shown where appropriate.
effects of water flow past the external nares by gating the stimulating pulses so that their frequency varied randomly between 0 and the maximum frequency of stimulation throughout the period of stimulation. This method, however, proved less effective than the above and was discontinued.

In order to test whether sites other than the external nares were important for the initiation and maintenance of apnoea, the duration of apnoea was compared in trials in which the frog was submerged to the level of the external nares with that induced when the frog was completely out of water. No consistent difference in the length of the apnoeic period was discernible in the two conditions (Figure 37a), although stimulation of one ophthalmic nerve was less effective in maintaining apnoea than bilateral stimulation (Figure 37b). Bilateral stimulation of the cutaneous branches of the dorsal branches of the second, third and fourth spinal nerves performed as a control produced no changes in the respiratory pattern until the voltage was increased to the point where struggling occurred. Bilateral stimulation of the abdominal cutaneous branch of the ventral branch of the third spinal nerve at 1-2 v, 250 Hz, 3 msec pulses inhibited lung ventilation cycles in 2 of the 5 frogs, although they reappeared before the end of the period of stimulation. Sato (1954) produced a similar effect in *Rana nigromaculata* by lightly clipping the abdomen.
DISCUSSION

The results indicate that diving apnoea in *Rana pipiens* is reflexly initiated by the contact of water with the external nares, and that water does not normally enter the nasal cavities or the buccal cavity. The nares are closed during a dive, but occasional respiratory movements occur in some frogs in which gas is moved from the lungs to the buccal cavity and back again. These results agree with Lombroso's (1913) early observations that apnoea is induced by contact of the nostrils with water, and that there is no intake or exhalation of water during the dive, although movements of the buccal floor may occur at intervals.

Bilateral section of the ophthalmic branch of the trigeminal nerve whose sensory fibres serve the narial region, resulted in a failure to close the nares on submergence and the entry of water into the buccal cavity, and in some cases the lungs. The entry of water into the buccal cavity caused sustained tonic activity in the laryngeal constrictor muscles, the posterior intermandibular muscle of the buccal floor, and probably other buccal floor muscles, as well as struggling on the part of the frog. Zotterman (1949) suggested that the tongue water receptor of the frog may "reflexly contribute in keeping the mouth of the frog closed as well as to inhibit the respiratory movements when under water". Although water does not normally enter the mouth on immersion it seems likely that sensory information from tongue water receptors is responsible for the responses observed in denervated frogs. These appear to be designed to clear the buccal cavity of water by reducing buccal volume, and to prevent water entering the airways and lungs by laryngeal constrictor muscle activity.
Bilateral electrical stimulation of the ophthalmic branches of the trigeminal nerve caused periods of apnoea in *Rana pipiens*. Stimulation near the threshold voltage resulted in brief periods of apnoea during which the external nares remained open and buccal pressure was at atmospheric. Increase in voltage induced longer periods of apnoea with closed nares. Electrical, mechanical or chemical stimulation of the nose were early known to cause reflex apnoea and bradycardia in several mammalian species (Brodie and Russel, 1900; Lombroso, 1913; Allen, 1928a). More recently, Angell James and Daly (1972a) produced periods of apnoea of 10 to 40 seconds duration in dogs by drawing tap water or saline solution over the nasal mucosa. The liquid had to be in motion to initiate apnoea, suggesting that information from nasal mechanoreceptors was of prime importance.

Recordings made from the ophthalmic branch of the trigeminal nerve show that skin mechanoreceptors in the region of the external nares are able to respond to the movement of a water meniscus across their receptive fields in a simulated dive. Adaptation to pressures of 5 to 6 cm H\textsubscript{2}O occurred in a matter of minutes, but once adapted the mechanoreceptors still responded to water flow. Gregory (1973), working on ducks, could find no response from beak mechanoreceptors served by the ophthalmic nerve to simulated diving. In the units he investigated there was no response even when the hydrostatic pressure at the surface of the beak was raised and lowered between 0 and 50 cm H\textsubscript{2}O, although he points out that they may not have been the most sensitive units present. The units involved in the frog appear to be similar to Catton's (1958) type a and b fibres, producing respectively large fast adapting spikes, and smaller relatively slowly adapting spikes with a lower threshold. According to Catton (1958), these spikes are propagated in myelinated fibres, while the receptors appear to be free nerve endings. The snouts of two frogs
were serially sectioned, and stained in Glee's silver stain, but no specialised endings could be found in the region of the external nares.

In some preparations studied, complete adaptation to a stimulus of a few cm $H_2O$ occurred in a matter of minutes, while in others a pressure of less than 1 cm $H_2O$ produced tonic activity, although the frequency did not increase with increased pressure. If trigeminal input is an important factor in inhibiting rhythmic activity in the respiratory centre during immersion, overriding chemoreceptive input for example, it is probably maintained throughout the dive. How then do frogs maintain apnoea in dives lasting an hour or more? It is feasible that in the field movement of the frog, or currents in the body of water could bring about continuing spike activity in response to flow. Furthermore increase in pressure, due to deep diving would provide a greater stimulus intensity than those investigated, possibly slowing adaptation and causing the recruitment of less sensitive units. However, frogs secured on boards and submerged in a few centimetres of water for periods of an hour maintain apnoea and spontaneously resume ventilation when emerged (Jones, 1967). It is possible that the mechanoreceptors could become sensitised during the course of a free dive, enhancing the afferent input to the C.N.S. It has been demonstrated in Rana pipiens that stimulation of the first sympathetic ganglion results in a sympathetic efferent response in cutaneous branches of the trigeminal nerve which elicits an afferent mechanoreceptor discharge (Chernetski, 1964a). In cats, injection of strychnine into the spinal trigeminal nucleus makes head regions hypersensitive to touch (King and Barnett, 1957). The adjustment of mechanoreceptor excitability appears to be under sympathetic adrenergic control, application of epinephrine to Pacinian corpuscles increasing the amplitude and rise rate of the generator potential.
(Lowenstein and Altamirano-Orrego, 1956). The possibility of such a sympathetic enhancement of trigeminal receptor input on immersion does not seem unreasonable in frogs, where there are close morphological relations between the cranial nerves and the sympathetic system (Chernetski, 1964b), although it was not observable in the double-pithed preparations used in these experiments.

Hyperpnoea occurred after short dives and also after short periods of apnoea induced by trigeminal stimulation. In mammals hyperpnoea is probably a response to the increase in arterial $P_{CO_2}$ which occurs in the apnoeic period (Angell James and Daly, 1969), while in the frog the fall in blood pressure which occurs during long periods of submersion may also help stimulate hyperpnoea on surfacing (Jones, 1967). De Marneffe-Foulon (1962) found that endopulmonary pressure can control the ventilation rate in the frog, a fall in pressure stimulating ventilation, and it has been suggested that this may explain post-dive hyperpnoea if lung pressure falls on submersion due to the release of gas bubbles at the start of the dive (Jones, 1966). However, lung pressure invariably rises when a frog is submerged even though lung volume may be low, the lungs acting as simple closed hydrostats. It seems more likely then that the increase in ventilatory drive after short periods of apnoea is due to the effect of the absence of rhythmic input from lung mechanoreceptors during the apnoeic period, and to the direct influence of the release of trigeminal inhibition on the respiratory centre, although nothing is known of the central mechanisms involved.
Although it appears that modern Anurans have probably reverted to a more aquatic mode of life than their Palaeozoic ancestors (Foxon, 1964), use of a force-pump for air ventilation apparently arose early in phylogeny. The modern lung fish *Protopterus aethiopicus* follows a similar sequence during air breathing in which the buccal cavity expands and air enters through the mouth into the buccal cavity before the lungs are deflated (McMahon, 1969). Mixed buccal gas is then forced into the lungs by buccal force-pumping in order to refill them. It has been suggested that aspiration of air into the buccal cavity could serve to clear it of water in lung fish, or alternatively, that preliminary inflation of the buccal cavity could compensate for changes in specific gravity on lung deflation and so prevent sinking when the animal is at the water surface.

Gans (1970) considered that the buccal force-pump was retained and perfected in frogs in order that vocalization, an important factor in modern anuran behaviour and social organisation, be facilitated, arguing that such an important mechanism is unlikely to be restructured. It seems, however, that the vocal cords could be vibrated as efficiently by an aspiration pump as by a buccal pressure pump. A more likely explanation for the retention of the buccal pump in modern anurans may be related to their reversion to an aquatic habitat. Early amphibia were presumably more terrestrial in habit, showing well developed ribs, which could have been used in suction-pumping but their reversion to an aquatic habit, and the subsequent reduction in rib size may have led to the renewed dominance of the buccal pump in modern forms (Romer, 1972).
The need for apnoea to be reliably induced in a diving vertebrate such as the frog is obvious. Many mammalian physiologists have deduced a voluntary component in the responses to submergence (Irving et al., 1941), but this is hardly likely to be true for the Amphibia, and recent work has suggested that even in the harbor seal the responses may in fact be conditioned (Jones et al., 1973). Angell James and Daly (1972b) noted that receptors situated in the nasal passages themselves are hardly likely to be involved in the respiratory and cardiovascular adjustments to diving in the seal, as the seal, like the frog, closes its nostrils on submergence. Jones et al. (1973) pointed out that there is nothing in the literature to suggest that the seal in fact possesses such receptors, although the recent studies of Dykes (pers. comm.) suggest that mechanoreceptors on the skin of the head and particularly at the base of the vibrassae may have a role to play, particularly in the cardiovascular responses to submersion. Although it is clear that stimulation of trigeminal mechanoreceptors can bring about apnoea in Rana pipiens it is unlikely that there is a direct chronotropic effect on the heart from peripheral receptors, as the generation of diving bradycardia is a slow process in the frog, the two main factors influencing heart rate during periods of submersion being the shortage of oxygen and the cessation of ventilation (Jones and Shelton, 1964).

Jones (1967) demonstrated that to evaluate the true oxygen debt at the end of a dive it is necessary to know the oxygen cost of ventilation in the frog, because hyperpnoea follows periods of submergence in anurans, and the work output of the buccal pump must be above the pre-dive level, the increased oxygen consumption of the pump being included in the increase in oxygen consumption which follows a dive. In R. pipiens lung ventilation rate was
double the pre-dive rate in the first ten minutes of a 60 min dive in aerated water, while the total oxygen consumption was 26% more than pre-dive. Assuming that the oxygen cost of ventilation pre-dive is 5% of the resting metabolism (Part II), the combination of switching to high amplitude lung ventilation cycles requiring perhaps double the energy input per cycle, and the increased respiratory frequency could well cause the oxygen cost of hyperventilation to rise to 15-20% of the total pre-dive oxygen consumption. In *Rana pipiens* no oxygen debt appears to build up during a dive, as much extra oxygen being consumed on emergence from 100% oxygenated water as on emergence from aerated water. In both cases hyperpnoea occurs after the dive and it appears feasible that the cost of hyperpnoea could be a major factor in the extra post-dive oxygen consumption shown by frogs.
1. The lungs are ventilated in _Rana pipiens_ by means of buccal force pump mechanism. Pressure in the buccal cavity fluctuated around atmospheric, but lung pressure was never allowed to fall to atmospheric pressure.

2. Two distinct types of pressure events occurred in the buccal cavity; buccal ventilation cycles in which the glottis remained closed and the external nares were open, and lung ventilation cycles which involved the sequential participation of the nares and glottis as well as the respiratory muscles of the buccal floor. No evidence was found for a substantial differential pressure across the glottis, or for the active participation of the flank muscles during lung ventilation cycles.

3. Lung and buccal ventilation cycles were not randomly interspersed. Lung ventilation cycles occurred in regular sequences towards the end of which each cycle reached a pressure peak some 10-20 percent higher than the preceding cycle, until the lungs were fully inflated. These sequences were separated by periods during which the filled lungs were isolated from the buccal cavity by the closed glottis. During these periods only buccal ventilation cycles occurred. Lung deflation was accomplished during the first few cycles of the subsequent lung ventilation sequence.

4. Gas flow recorded at the nares was found to be biphasic during buccal ventilation cycles, but to consist of 4 phases during lung ventilation cycles.

5. The suggestion is made that the initial emptying of lung gas into the buccal cavity on lung ventilation and the reaspiration of mixed gas may be significant in the maintenance of blood $P_{O_2}$ in _Rana pipiens_.
6. An analysis was made of the mechanical work done by the buccal pump during one lung ventilation cycle, and the proportion of this work available for inflation of the lungs after various losses against viscous and flow resistive forces in the pump itself.

7. The mechanical work of ventilation was estimated in restrained but unaanaesthetised *Rana pipiens* by recording the areas of representative sequences of pressure/volume loops recorded from the buccal pump, together with the respiration frequency. Mechanical work of ventilation proved to be 0.5 Joules per hour for frogs with a mean weight of 64 grams.

8. The mean mechanical efficiency of the buccal pump was calculated at 10.6 percent. The calculated efficiencies of individual lung ventilation cycles increased as the mechanical work done in a cycle increased from 7.4 percent at 0.65 gram.cm per cycle to 19.3 percent at 2.73 gram.cm per cycle, after which efficiency fell.

9. By combining data on the mechanical work of ventilation and efficiency with data in the literature on the oxygen consumption of *Rana pipiens* it was possible to estimate the oxygen cost of ventilation at 5 percent. The respiratory muscles make up 0.92 percent of body weight. The oxygen consumption of these muscles, $V_{O_2}$, was calculated at 0.89 ml $O_2$ per 100 gram per minute.

10. Upon submersion, apnoea in *Rana pipiens* was not induced until the water surface had reached the level of the external nares. During the period of submersion the nares were closed and buccal pressure was elevated due to the hydrostatic pressure of the head of water above the buccal cavity. The period of apnoea was punctuated in some frogs by ventilation movements in which lung gas entered the buccal cavity. The nares remained closed. Ventilation spontaneously restarted at the end of a period of submersion as soon as
the water level fell below the external nares.

11. Water did not normally enter the buccal cavity during periods of submersion, and there was no tonic electrical activity in the buccal floor muscles during the dive. Denervation of the region of the external nares, by bilateral section of the ophthalmic branch of the trigeminal nerve (cranial nerve V) resulted in intake of water into the buccal cavity and in some cases the lungs, together with intense tonic activity in the muscles of the buccal floor, which elevated it towards the roof of the buccal cavity. Surfacing did not result in resumption of ventilation in these frogs.

12. Mechanoreceptors in the region of the external nares served by the ophthalmic branch of the trigeminal were found to be capable of responding to the movement of a water meniscus across the snout. Adaptation to pressures of 6 cm H\textsubscript{2}O occurred in a matter of 4-5 minutes in some preparations, while in others a long term increase in tonic activity occurred. Mechanoreceptors still responded to water flow after complete adaptation to pressure had occurred.

13. Bilateral stimulation of the ophthalmic branch at the level of the orbit caused periods of apnoea in Rana pipiens. Thresholds varied from 30 to 300 mv. at 200 p.p.s., 4 msec. pulses, in individual frogs. At voltages near the threshold the apnoeic periods occurred with the nares open. Increase in voltage resulted in longer periods of apnoea in which the nares were closed and buccal pressure was independent of atmospheric pressure. Nerve adaptation was simulated in some experiments by dividing the stimulating frequency by a factor of ten shortly after the initiation of apnoea. This proved to be as effective in bringing about apnoea as constant stimulation at the initial frequency.


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