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HYPERCAPNIA AND ACETYLCHOLINE RELEASE FROM THE CEREBRAL CORTEX AND MEDULLA

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SUMMARY

1. The cerebral cortex and medulla of fifty-eight anaesthetized dogs released ACh spontaneously through push-pull cannulae after perfusion with the anticholinesterase, sarin. Hypercapnia (12 % CO_2) evoked a significant release of ACh above the basic spontaneous level, from the medullary and cortical areas. Hypercapnia + hypoxia (12 % CO_2 + 8 % O_2), in combination, produced an ACh release comparable to hypercapnia; hypoxia (8 % O_2) had no effect in any region.

2. Areas in the medullary reticular formation responsive to injections of CO_2-bicarbonate solutions ('respiratory responsive areas') produced a significant increase of ACh after exposure to hypercapnia or hypercapnia + hypoxia, over that obtained from either the 'non-respiratory responsive areas' of the medulla or the cerebral cortex.

3. The evidence supports the concept that ACh may participate as a neurotransmitter within the cerebral cortex and medulla. Also the results would suggest but do not prove, that a cholinergic factor may be a component in respiratory control under certain circumstances, such as exposure to hypercapnia.

INTRODUCTION

There is an accumulation of evidence that acetylcholine (ACh) may be a neurotransmitter within the mammalian central nervous system (Feldberg, 1950; MacIntosh & Oborin, 1953; Mitchell, 1963; Curtis, 1963; Curtis, Ryall & Watkins, 1965). Recent methods utilizing micro-electrophoresis have revealed the presence of ACh-sensitive neurones within areas such as the cerebral cortex (Krnjević & Phillis, 1963; Randić, Siminoff & Straughan, 1964), and the medulla (Bradley & Wolstencroft, 1962, 1965; Salmoiraghi & Steiner, 1963). While many neurones within the C.N.S. have been found to be ACh-sensitive, this is not conclusive evidence that their innervation is cholinergic in nature, but one might reasonably assume so, in

view of the fact that the results from these micro-electrophoretic studies correlate well in most instances with other histochemical and neurochemical evidence.

Although there is abundant evidence supporting the hypothesis for the presence of cholinoreceptive cells in the medulla (Bradley & Wolstencroft 1962, 1965; Salmoiraghi & Steiner, 1963), there are conflicting interpretations and experimental data as to whether ACh is a major neurohumoral transmitter for the stimulation of the respiratory neurones within the medullary tissue (Gesell & Hansen, 1945; Metz, 1961, 1962, 1964; Salmoiraghi, 1962; Salmoiraghi & Steiner, 1963).

Therefore, the present investigations are primarily concerned with re-evaluating or reconciling the various contradictions with respect to the role of cholinergic mechanisms with relation to central respiratory control, utilizing the push-pull cannulae technique developed by Gaddum (1960), which might furnish additional information on this complex problem.

A brief account of this work has been reported (Metz, 1966).

METHODS

Fifty-eight mongrel dogs of both sexes ranging in weight from 8·3 to 11·6 kg, were anaesthetized with morphine sulphate (2·5 mg/kg, subcutaneously) followed by urethane (1 g/kg, intraperitoneally). Tracheotomies were performed on the dogs; they were then connected to rebreathing tanks and spirometer plus accessories for recording respiration.

The head of each dog was secured in a stereotaxic instrument (Baltimore Instrument Co.), and portions of the occipital bones and cerebellum were removed to expose the floor of the fourth ventricle. Similarly, holes were trephined in the skull to make available for examination the coronal gyrus of the cerebral cortex. Push-pull cannulae (Gaddum, 1960) were fashioned from a 27 gauge needle, 3 in. (7·6 cm) in length, inserted through polyethylene tubing (0·023 in. (0·058 cm) i.d., 0·038 in. (0·097 cm) o.d.), and held together by an epoxy adhesive. These cannulae were stereotaxically placed in the obex region of the medulla and into its reticular formation (at depths ranging from 2·5 to 4·5 mm) and into the coronal gyrus of the cerebral cortex at depths from 1·2 to 1·5 mm.

Comroe (1943) described a method for achieving respiratory stimulation by injecting CO_2 buffered bicarbonate solutions through stereotaxically placed micropipettes within the medulla. In order to obtain a qualitative estimate of regions within the medulla which may or may not participate in the neural regulation of the respiratory process, 1·5 μl. of a buffered bicarbonate solution (1·3 g $NaHCO_3$/100 ml. solution, buffered by CO_2 so as to have a P_{CO_2} of 250 mm Hg, at pH 7·4) was introduced through the push-pull cannulae. Before these micro-injections, the push-pull cannulae had been completely filled with the CO_2 buffered bicarbonate solutions. An area which produced a respiratory response (i.e. a stimulation or depression in ventilation) was designated as a 'respiratory responsive area', whereas an area in which no change in ventilation occurred was referred to as a 'non-respiratory responsive area'.

Four such areas were located in the medulla of each dog, i.e. two which were 'respiratory responsive' and two which were 'non-respiratory responsive'. Each point was perfused with Locke solution (NaCl 9·00, KCl 0·42, $CaCl_2$ 0·24, $NaHCO_3$ 0·20, glucose 1·00 g./l.; pH 7, P_{CO_2} 0·3 mm Hg) containing the potent anticholinesterase, sarin (1×10^{-5} g./ml.) at a rate of 0·2 ml./min. Following this procedure, each region was perfused with Locke

solution containing 1 μg/ml. eserine sulphate for the remainder of the experiment at the constant rate of 0·2 ml./min. The effluents were collected at 10 min intervals for the duration of the experiment, during the control, experimental gaseous exposures, and recovery periods.

After collecting the perfusates for 30 min, the dogs were subjected to the following stimuli: hypercapnia (12·0 % CO_2 + 30 % O_2 + 58 % N_2), twenty-five dogs; hypoxia (8·0 % O_2 + 0·2 % CO_2 + 91·8 % N_2), ten dogs; hypercapnia + hypoxia (12·0 % CO_2 + 8 % O_2 + 80 % N_2), ten dogs, for 30 min, followed by a 30 min recovery period. The gas mixtures were analysed by means of a Lloyd-Gallenkamp Haldane Apparatus. Similar experiments were performed on the coronal gyrus of the cerebral cortex in thirteen dogs. This area according to Hamuy, Bromiley & Woolsey (1956) is the sensory field (SI) of the head of the dog. Since this sensory area is not directly involved in respiratory regulation, no apparent change in the respiratory pattern occurred after perfusion of four loci within the coronal gyrus of each dog with CO_2 buffered $NaHCO_3$ solution. Consequently there is no distinction between 'respiratory-responsive areas' and 'non-respiratory responsive areas' within the coronal gyrus. The effluents were collected for each 10 min interval as above, with seven animals being exposed to hypercapnia and six to hypoxia, respectively.

The ACh content of the samples was assayed on the longitudinal strip of the dorsal muscle of the leech, *Macrobdella decora*, according to the method described by Szerb (1961). The samples were tested in a constant temperature microbath similar to that described by Gaddum & Stephenson (1958), with slight modifications, e.g. using a Grass Force Displacement Transducer (Model FT.03) coupled to a Brush Analyser and Recorder for measuring changes in the isometric muscular contractions.

RESULTS

The administration of 1·5 μl. of the CO_2 buffered bicarbonate solution produced in the medullary regions investigated (circumscribed areas, 4 mm caudal and 2 mm rostral to the obex) a variety of respiratory patterns. These were, (*a*) no change in the respiratory response; (*b*) a marked hyperpnoea, the most frequent type of response (Fig. 1); (*c*) an expiratory arrest followed by a hyperpnoea; (*d*) an expiratory arrest followed by depressed breathing. The duration of these responses varied from approximately 0·5 to 2 min; (*a*) was designated as a 'non-respiratory responsive area' whereas *b*, *c* and *d* were considered as 'respiratory responsive areas'. The administration of the CO_2-bicarbonate solution into the coronal gyrus of the cerebral cortex produced no change in ventilation.

Medulla–hypercapnia series (12 % CO_2 + 30 % O_2 + 58 % N_2)

In the 'non-respiratory responsive areas'. A spontaneous release of ACh (range 1·5–2·8 ng/10 min) was evident following the perfusion of the anticholinesterase, sarin, through the push-pull cannulae. The exposure of the twenty-five animals to the 12 % CO_2 mixture resulted in a marked and significant increase in ACh release ($P < 0·01$), followed by a decline when the hypercapnic stimulus was removed (Fig. 2).

In the 'respiratory responsive areas'. The control level of spontaneous ACh release was similar to that found in the 'non-respiratory responsive

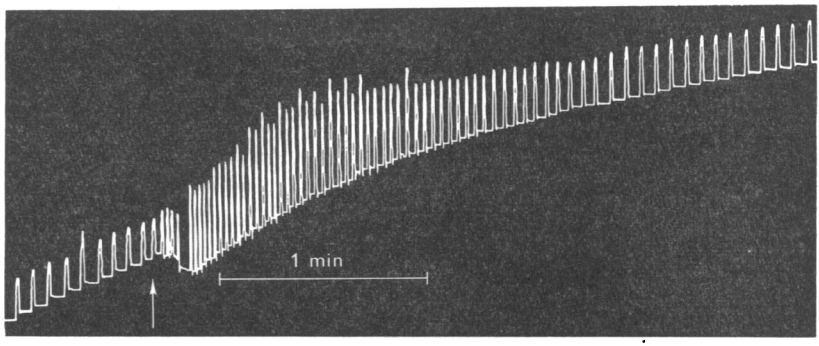

Fig. 1. Respiration recording in dog. At arrow, 1·5 μl. CO_2-bicarbonate solution was introduced through the push-pull cannulae into the reticular tissue of the medulla; now designated as a 'respiratory responsive area'.

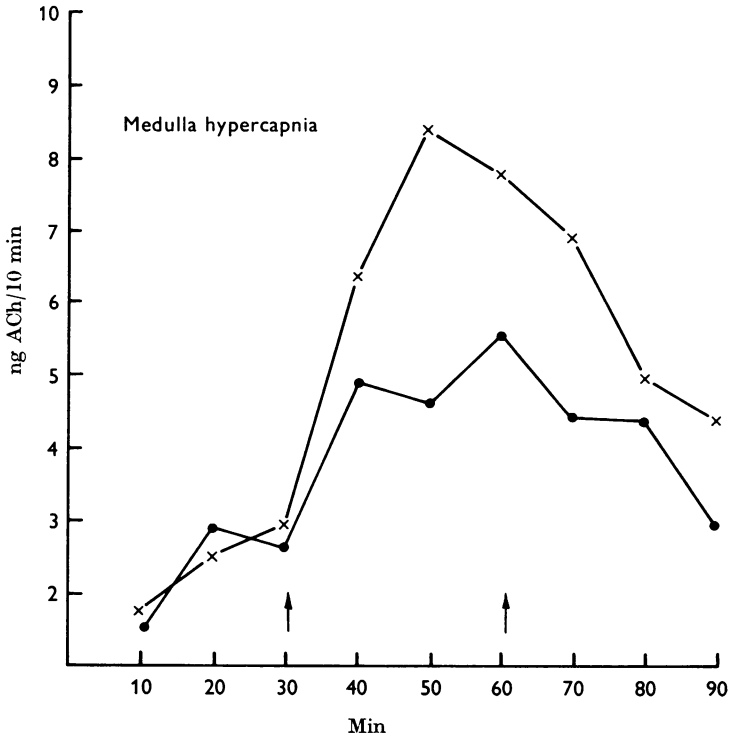

Fig. 2. Curve (– × – × –) indicates the release of ACh from the 'respiratory responsive areas' of the medulla. Curve (–●–●–) indicates the release of ACh from the 'non-respiratory responsive areas' of the medulla. Dogs were exposed to hypercapnia (12 % CO_2 + 30 % O_2) during the time period between the two arrows. Each point on the curve represents fifty samples.

areas' of the medulla, but after the 12 % CO_2 mixture was introduced, there was a sharp rise in the evoked ACh release, which was significantly greater ($P < 0{\cdot}01$) than that found in the 'non-respiratory responsive areas' with a corresponding decline when the CO_2 stimulus was removed (Fig. 2). The maximal release of ACh occurred after 20 min of exposure to hypercapnia.

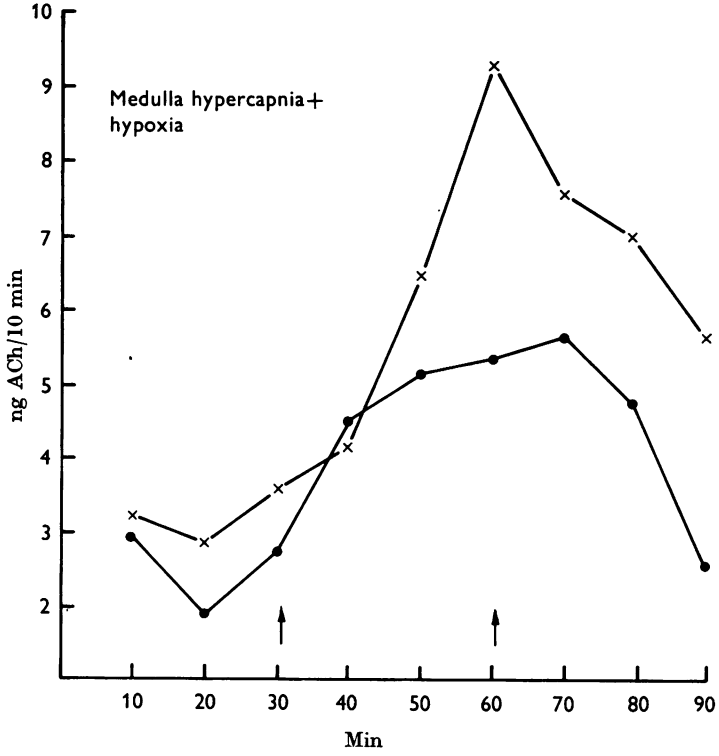

Fig. 3. Curve (– × – × –) indicates the release of ACh from the 'respiratory responsive areas' of the medulla. Curve (–●–●–) indicates the release of ACh from the 'non-respiratory responsive areas' of the medulla. Dogs were exposed to hypercapnia + hypoxia in combination (12 % CO_2 + 8 % O_2) during the time period between the two arrows. Each point represents twenty samples.

Medulla–hypercapnia + hypoxia combination series (12 % CO_2 + 8 % O_2 + 80 % N_2)

In the 'non-respiratory responsive areas'. The ACh release, both spontaneous and evoked, were comparable to the responses obtained with hypercapnia alone from the 'non-respiratory responsive areas' (Fig. 3).

In the 'respiratory responsive areas'. The control level of spontaneous ACh release was similar to that found in the 'non-respiratory responsive

areas' of the medulla. After the 12 % CO_2 – 8 % O_2 mixture was introduced, there was a sharp rise in the evoked ACh release, which was significantly greater ($P < 0{\cdot}01$) than that found in the 'non-respiratory responsive areas', with a decline when the hypercapnic-hypoxic stimulus was removed. The peak release of ACh occurred 30 min after exposure to the 12 % CO_2 – 8 % O_2 mixture (Fig. 3).

Although the time of maximal ACh release differs, there is no statistically significant difference between the responses obtained during hypercapnia and those during hypercapnia + hypoxia, from the 'respiratory responsive areas' (Figs. 2, 3).

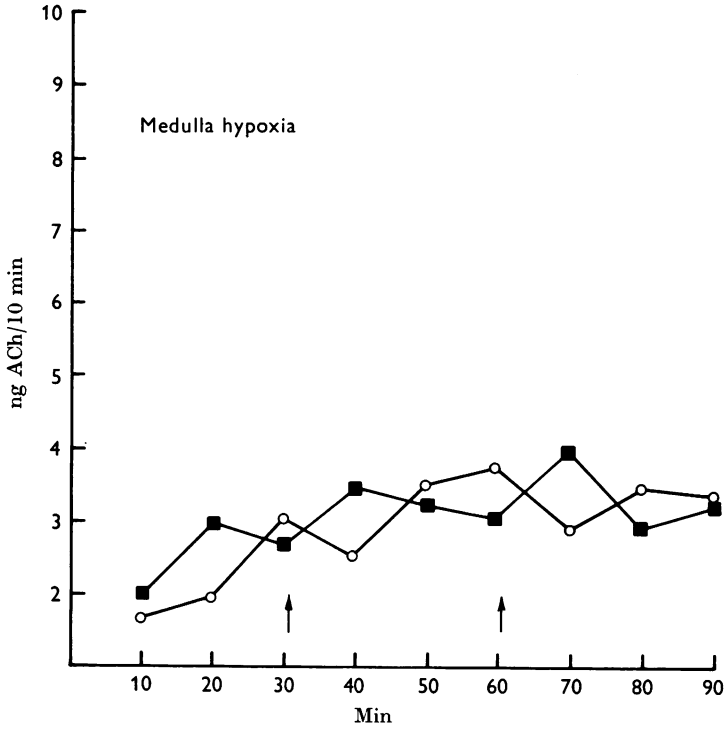

Fig. 4. Curve (–○–○–) indicates the release of ACh from 'respiratory responsive areas' of the medulla. Curve (–■–■–) indicates the release of ACh from 'non-respiratory responsive areas' of the medulla. Dogs were exposed to hypoxia (8 % O_2 + 0·2 % CO_2) during the time period between the two arrows. Each point on the curves represents twenty samples.

Medulla–hypoxia (0·2 % CO_2 + 8 % O_2 + 91·8 % N_2)

No significant change was apparent after the exposure to hypoxia, as compared with the basic spontaneous release of ACh; this was true for the 'respiratory responsive areas' and the 'non-respiratory responsive areas' (Fig. 4).

Cerebral cortex (coronal gyrus)—hypercapnia
(12 % CO_2 + 30 % O_2 + 58 % N_2)

The basic spontaneous release of ACh from the coronal gyrus was similar to that seen in the medulla, and a small but significant increase in ACh release from this area occurred with hypercapnia. This change resembled that seen in the 'non-respiratory responsive areas' of the medulla (Fig. 5).

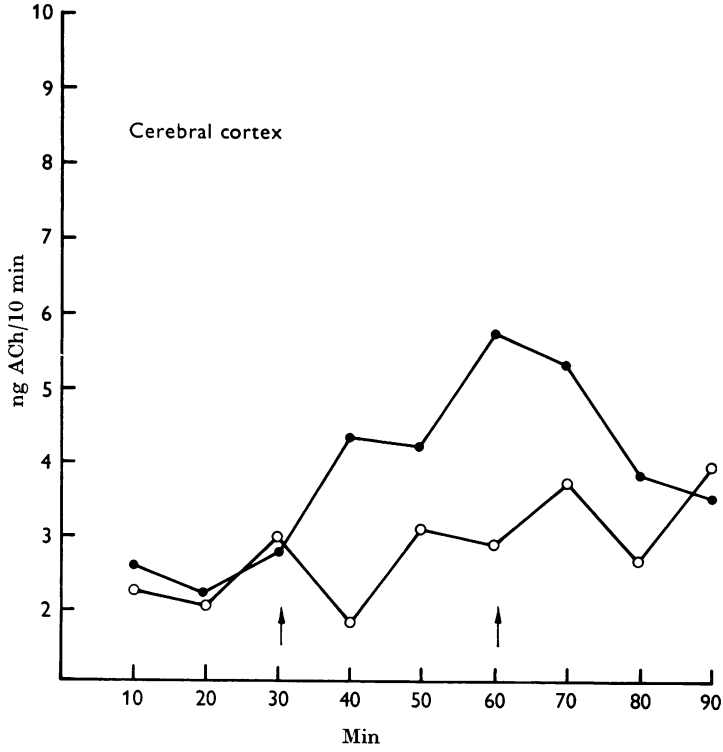

Fig. 5. Release of ACh from coronal gyrus of cerebral cortex of dogs. Curve –●–●– illustrates experiment where animal was exposed to hypercapnia (12 % CO_2 and 30 % O_2) during the period marked with arrows. Each point is mean of twenty-eight samples. Curve (–O–O–) illustrates results with hypoxia (8 % O_2 and 0·2 % CO_2) in period between arrows. Each point is mean of twenty-four samples.

Cerebral cortex (coronal gyrus)—hypoxia
(0·2 % CO_2 + 8 % O_2 + 91·8 % N_2)

No significant change in ACh release was in evidence above that of the control level of spontaneous release, paralleling the data obtained from the 'respiratory responsive areas' and the 'non-respiratory responsive areas' in the medulla when exposed to the same hypoxic stimulus (Fig. 5).

Exposure to hypercapnia or hypercapnia + hypoxia produced the characteristic hyperpnoea associated with breathing high CO_2 concentrations; hypoxia caused a considerably smaller increase in the minute ventilation as contrasted with the hypercapnic stimuli.

DISCUSSION

The role of ACh as a chemical transmitter within the C.N.S. has been a matter of controversy for many years (Feldberg, 1950; Salmoiraghi, 1962; Curtis, 1963; Curtis *et al.* 1965). Earlier investigations, largely indirect, and based substantially on histochemical and neurochemical evidence, presented an impressive array of evidence for the presence of a cholinergic mechanism within the C.N.S. Further support for this concept was generated by the micro-electrophoretic techniques which demonstrated the existence of ACh-sensitive nerve cells within the cerebral cortex (Krnjević & Phillis, 1963; Randić *et al.* 1964) and medulla (Bradley & Wolstencroft, 1962, 1965; Salmoiraghi & Steiner, 1963). Comparable studies with the application of plastic cups or cylinders (MacIntosh & Oborin, 1953; Mitchell, 1963; Szerb, 1964), and push-pull cannulae (Mitchell & Szerb, 1962; Mitchell, 1963; Szerb, 1963; McLennan, 1964) gave additional backing for the possible role of ACh as a neurotransmitter within the mammalian C.N.S. The present investigations tend to support this concept, as there is a spontaneous and increased release of ACh as a result of hypercapnia, after local inhibition by an anti-cholinesterase, in the medulla and coronal gyrus of the cerebral cortex in the dog.

However, the effect of hypercapnia upon the synaptic systems in the cerebral cortex and in various regions of the brain stem may not be physiologically equivalent. For example, concentrations of CO_2 that stimulate respiration may depress cortical excitability (Wyke, 1963). Similarly, Metz & Bernthal (1953) demonstrated that hypercapnia can depress a respiratory reflex drive whilst simultaneously stimulating breathing. In addition, ACh can excite some neurones, inhibit others, and have no detectable effect on a great majority of the remainder in the medulla, pons and cerebral cortex (Salmoiraghi & Steiner, 1963; Randić *et al.* 1964; Bradley & Wolstencroft, 1965). Thus, although the release of ACh from the cerebral cortex and the 'non-respiratory responsive areas' of the medulla are of comparable magnitude, it would indeed be hazardous to conclude that the physiological action or excitability of the aggregates of neurones in those areas are also equal.

Interpretation of the statistically significant release of ACh after hypercapnia in the 'respiratory responsive areas' over that of the 'non-respiratory responsive areas' in the medulla is similarly fraught with difficulty.

Kellogg (1964), Kim & Carpenter (1961), Dehaven & Carpenter (1964) all seriously question the significance of the chemical stereotaxic micro-injection of bicarbonate and other solutions relative to the medullary chemical control of breathing, and suggest that the respiratory responses to such micro-injections are primarily non-specific in nature. However, the present studies indicate that the 'respiratory responsive areas' in the medulla which respond to micro-injections of CO_2-bicarbonate solutions, by causing depression or excitation, does evoke a release of ACh significantly greater than that of a region in which no change in respiration was apparent, i.e. the 'non-respiratory responsive areas'. One cannot definitely claim that this 'respiratory responsive area' is discretely pinpointing respiratory neurones of the so-called 'respiratory centre' as described by Pitts (1946) for the size of the push-pull cannulae in enclosing a rather extensive number of neurones precludes such a conclusion, but it might indicate that this region contains aggregates of respiratory neurones, or be adjacent to such nerve cells which may influence the firing or sensitivity of the latter cells. On this basis one may cautiously conclude that cholinergic synapses are involved in some manner in the neural control of breathing, which would tend to give supporting evidence to the earlier findings of Gesell & Hansen (1945) and their acid-humoral concept that CO_2 and other acids by changing the pH of the respiratory neurones produce their effects by inhibiting the destruction of ACh. Similarly, Metz (1961, 1962, 1964) has presented suggestive evidence for the existence of correlations between the cholinergic system and respiratory reflexes of central origin. On the other hand, Salmoiraghi & Steiner (1963) have provided micro-electrophoretic evidence that ACh is an improbable candidate for the role of a major neurotransmitter of medullary respiratory neurones.

The results also suggest that particular regions within the reticular formation of the medulla evoke a significantly greater release of ACh than others close by, indicating a probable greater sensitivity of certain neuronal areas to high CO_2 and/or increased H^+ concentrations. Certain observations are apparently not compatible with this hypothesis, for the reflex centre of the medulla is considered to be anatomically separate from the central chemoreceptors. Studies by Mitchell, Loeschcke, Severinghaus, Richardson & Maisson (1963) claim that the medullary CO_2 chemoreceptors lie within or just below the pia on the ventrolateral surface of the medulla and respond to cerebrospinal fluid H^+ concentration, whereas in the present experiments the areas scrutinized were located principally within the reticular formation in the more dorsal and medial regions near the obex. However, Pappenheimer, Fencl, Heisey & Held (1965) question the hypothesis of the presence of special H^+ chemoreceptors on the surface of the medulla and they present evidence which tends to support the

earlier concepts that the central chemical control of breathing may be intimately related to the acidity of the fluid in contact with respiratory neurones within the brain tissue, located at some distance from the cerebrospinal fluid. Since inhalation of high concentrations of CO_2 do cause an increase in the arterial and brain tissue P_{CO_2}, with a corresponding decline in the arterial and brain tissue pH (Meyer, Gotoh & Tazaki, 1961), the significant ACh increase noted after hypercapnia may occur by virtue of an increase in the H^+ concentration. Unfortunately, the entire relation of CO_2 to pH within the C.N.S., with particular reference to the central regulation of respiration, still remains obscure.

The ACh content of the brain rises during anaesthesia (Elliott, Swank & Henderson, 1950; Crossland, 1956) as well as during morphine administration (Giarman & Pepeu, 1962). Confirmation of the increased ACh content of various areas of the brain is reported by Metz (1961) utilizing the identical dosage and combination of morphine–urethane as in the present studies. Beleslin & Polak (1965) perfused the lateral ventricle of the brain with morphine and observed a diminution of ACh release. They attributed the inhibition of ACh release as being responsible for the concomitant rise in the ACh content of the brain, noted by other workers after the administration of a variety of anaesthetics and morphine. Thus, in the present studies, the level of ACh release, both spontaneous as well as the evoked release after hypercapnia, presumably is at a lower level than would be the case in an unanaesthetized animal.

The failure of hypoxia to cause any significant change in the ACh level raises several perplexing questions. Since the hypoxic stimulation presumably acts through the peripheral chemoreceptors of the aortic and carotid bodies which are still intact, through central respiratory neuronal pathways, how does one account for the fact that there was no discernible change in the ACh level, although there was an increase in ventilation as a consequence of exposure to hypoxia? Reinforcement for the lack of any influence of hypoxia on ACh release is evidenced by the failure of any augmentation of ACh release as a result of the addition of hypoxia to the hypercapnia, for it has been repeatedly demonstrated that interaction between low O_2 and high CO_2 at the peripheral chemoreceptors (i.e. the carotid bodies) produces a response that is significantly greater than the sum of the individual responses (Otey & Bernthal, 1960; Joels & Neil, 1961).

Several hypotheses may be considered to explain this action of hypoxia. First, is the unlikely proposition that there are separate sets of central neurones for different stimuli, e.g. CO_2 sensitive, O_2 sensitive, etc. Secondly, hypercapnia may indeed affect central respiratory neuronal components through a pathway in which cholinergic neurones participate,

whereas the hypoxia stimulates these same central neurones by a pathway devoid of cholinergic neurones, stimulated by a neurotransmitter, as yet unidentified. That is, ACh may be involved in the central respiratory control mechanism, but only under particular circumstances, as may be the case when subjected to specific stimuli such as hypercapnia, and may conceivably not be included when exposed to a stimulus such as hypoxia.

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