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EFFECTS OF HYPOXIA, HYPERCAPNIA, AND pH ON THE CHEMORECEPTOR ACTIVITY OF THE CAROTID BODY *IN VITRO*

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There is little doubt that chemoreceptor discharges in the carotid nerve are elicited by environmental changes which act on the carotid body (cf. Heymans & Neil, 1958). However, the mechanism responsible for the initiation of these discharges has remained obscure since many apparently unrelated agents activate the chemoreceptors. Low O_2, high CO_2, pH changes, metabolic products, etc., are all highly effective stimuli. A common factor responsible for these effects has not been found.

The essence of the problem is to know whether or not different stimuli act directly on the nerve endings or through an intermediary process. From a physiological point of view it is difficult to explain chemoreceptor excitation by hypoxia, hypercapnia or acidity merely as direct effects on the sensory terminals. Truly, hypoxia may depolarize the endings since it depolarizes nerve fibres (Lorente de No, 1947). But hypercapnia usually increases the membrane potential of nerve (Lorente de No, 1947) and it is unlikely that chemoreceptor endings would behave in an opposite manner. Furthermore, chemoreceptors are highly sensitive to pH changes while nerve fibres require extremes of either acidity or alkalinity before alterations in function are detected (Lorente de No, 1947; Spyropolous, 1960). It is attractive, therefore, to assume an indirect generation of impulses elicited by glomus cell activity which, in turn, would activate the sensory endings. This idea, which implies non-specific nerve terminals, has been advanced in different reports on carotid body function (Heymans & Neil, 1958; Lever, Lewis & Boyd, 1959; Joels & Neil, 1962, 1963; Biscoe & Taylor, 1963). It has received experimental support mainly from the work of de Castro (1951); he could re-innervate in the cat a carotid body (previously deprived of its sensory innervation) with vagal fibres. The new pathway showed activity during carotid body stimulation by CO_2

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and acidity. De Castro interpreted this effect as being produced by activation of carotid body cells and not by direct stimulation of the sensory endings. His assumption was that the newly formed terminals came from fibres which normally had a completely different function. However, the precise nature of the fibres which re-innervated the carotid body was not established by de Castro, and the vagus of the cat contains some fibres from aortic chemoreceptors (Neil, Redwood & Schweitzer, 1949). Consequently, de Castro's interpretation is not beyond criticism.

A first approximation in any attempt to solve this problem requires the determination of whether or not different stimuli act *directly* on the chemoreceptor nerve endings and this has not been done properly. In fact, the majority of studies dealing with carotid body function have been performed on preparations supplied by blood or by saline perfusions. In the first type of carotid body preparation it is difficult to eliminate completely secondary systemic effects produced by different stimuli which also act on the carotid body. In perfusates, local vascular effects within the glomus are unavoidable in some cases, e.g. during perfusion with saline equilibrated with CO_2 (de Castro, 1951; Neil & Joels, 1963). Consequently, these methods do not allow the study of direct effects of various stimuli on the chemoreceptor nerve endings. This being the case, it is desirable to study these effects *in vitro* where both systemic and local vascular effects are absent (cf. Eyzaguirre & Lewin, 1961*b*).

The study that follows will show that carotid body chemoreceptors respond *in vitro* to direct stimulation by different agents such as changes in flow, P_{O_2}, P_{CO_2}, and pH. In this and succeeding papers, since the P_{O_2} in the centre of the isolated carotid body is not known but must be substantially lower than that of most of the bathing solutions, the term hypoxia is used whenever the organ is bathed with solutions equilibrated with any gas mixture containing 50% O_2 or less. These observations complement and enlarge previous ones where the chemoreceptors *in vitro* were readily activated by low P_{O_2}, high temperature and interruption of flow (Eyzaguirre & Lewin, 1961*b*). Analysis of chemoreceptor fibre discharges indicates that, in all probability, the sensory impulses are elicited by a chemical released from the glomus cells. The nature of this substance will be discussed in the following papers (Eyzaguirre & Koyano, 1965*a*, *b*; Eyzaguirre, Koyano & Taylor, 1965).

METHODS

The methods employed were a modification of those used by Eyzaguirre & Lewin (1961*b*). The carotid body and its own nerve were removed from cats anaesthetized with 40 mg/kg of sodium pentobarbital (Nembutal, Abbott). The preparation was placed in oxygenated Locke's solution and cleaned from surrounding connective tissues. It was then mounted in

a small Perspex chamber. The chmaber was modified several times through the course of the work. The final version consisted of a small Perspex channel (0·8 ml. capacity) through which Locke's solution (at different pH and equilibrated with the required gas mixtures) flowed under a layer of paraffin oil. In the early phase of this work flow was controlled by adjusting a fine needle valve. Later, an automatic device operated by a solenoid driven by a stimulator was employed. The latter system allowed more rigorous control of very slow flows (less than 1·5 ml./min). The saline was brought into contact with the carotid body through stainless-steel tubing to prevent excessive gas losses (cf. Joels, Neil & Vaughan Hodgson, 1960). The dead space of the system was 0·6 ml. Saline was drained from the system as shown previously (Eyzaguirre & Lewin, 1961*b*). The composition of the Locke's solution was the following: NaCl, 6·0; KCl, 0·42; $CaCl_2$, 0·24; Tris buffer (Sigma 121), 6·0 g/l. In the absence of CO_2, the pH was adjusted by adding different amounts of 1 N-HCl. When the gas mixtures contained CO_2, both 1 N-HCl and $NaHCO_3$ were added (after 1–2 hr of vigorous CO_2 bubbling) and pH was adjusted as desired. In all cases 1·0 g/l. of dextrose was added immediately before the experiment. The total molarity of the solutions was 310–318 mM/l. Temperature was kept at or below 37° C (unless specifically noted) by a Tecam circulator and stirrer. The barometric pressure was between 635 and 643 mm Hg in all experiments.

The carotid nerve was lifted into the oil to record action potentials from either the whole nerve or small filaments which often contained single active fibres. Nerve discharges were fed to an oscilloscope, counter and printer system (Computer Measurements Corp.) through a stage of pre-amplification. The action potential frequency per second was measured by the counter once every 2 or every 12 sec (cf. Eyzaguirre & Lewin, 1961*a*, *b*). Impulse intervals were measured from single-fibre preparations in order to construct histograms of interval distribution frequency. For that purpose, the nerve discharge was recorded on moving film at relatively high speed from a Grass kymograph camera. Afterwards, the developed film was fed into the same camera and replayed at 10–20 × slower speed. A microscope lamp fastened to the camera window allowed the projection of nerve spikes on a photocell covered by insulating tape except for a narrow slit. The output of the cell, which consisted of a sharp spike, stopped the counter (running at a fixed frequency) whenever an action potential crossed the slit. The value obtained from the counter was automatically registered by the printer which had a print-out time of 200 msec. One millisecond later, a pulse from an external source triggered the counter again and counting was stopped when the next nerve spike crossed the photocell slit. Misses occurred occasionally, but when a histogram was constructed they were lumped together with the shortest intervals counted. Misses were avoided when care was taken not to 'crowd' spikes in the original film. The delay in the counter-printer system (201 msec) was well below the duration of the replayed mean interval since the film was moving quite slowly. The time delay of the system did not alter the shape of the histogram.

RESULTS

The sensory discharge of carotid body chemoreceptors was influenced by changes in P_{O_2}, P_{CO_2} and pH. The effect of these agents was additive.

Since the preparations remained many hours in flowing saline it was important to know the effects of saline flow on sensory discharge frequency. It was shown previously that interruption of flow greatly increased the sensory discharges although a quantitative study of this effect was not done (Eyzaguirre & Lewin, 1961*b*).

The chemoreceptor discharge was clearly influenced by changes in flow of the bathing solution. Sensitivity to flow varied in different experiments

for no apparent reason. Occasionally, it was difficult to maintain a stable chemoreceptor discharge when saline flow was less than 1·0 ml./min since the receptor frequency tended to increase continuously. When flow was interrupted, the discharge attained a high frequency in confirmation of previous observations (Eyzaguirre & Lewin, 1961*b*). The experiment illustrated in Fig. 1 shows the effects of a stepwise reduction in flow on the chemoreceptor discharge obtained from a carotid nerve filament. Locke's solution (pH 7·49 at 35·5° C) equilibrated with 50% O_2 in N_2 flowed at 4·4 ml./min, and the discharge averaged 28/sec. Flow was reduced and sufficient time allowed for the discharge to reach a new steady level.

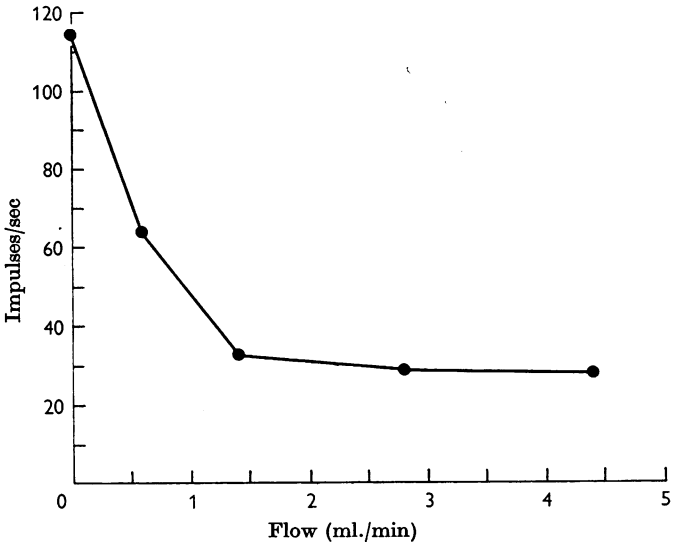

Fig. 1. Chemoreceptor impulse frequency recorded from carotid nerve filament. Locke's solution (pH 7·49 at 35·5° C) equilibrated with 50 % O_2 in N_2. Flow reduced in steps from 4·4 ml./min with concomitant increase in average sensory discharge frequency. Note steep incline of curve when flow was reduced from 1·4 to 0·6 ml./min.

When flow was decreased to 1·4 ml./min the sensory fibres discharged at an average frequency of 33/sec. A reduction in flow to 0·6 ml./min increased the sensory frequency to 64/sec. Discharges reached a maximum of 115/sec when flow was interrupted.

The experiment illustrated and similar ones showed that it was better to work at flow rates fast enough to avoid abrupt changes in sensory discharge frequency produced by inadvertent variations in flow. This critical flow value varied in different experiments. An automatic device (see Methods) had to be employed to keep flow constant when, of necessity,

flows slower than 1·5 ml./min were used. The importance of flow and its effects on chemosensory discharges in perfused preparations has been stressed by Joels & Neil (1963).

Effects of hypoxia and hypercapnia on chemoreceptor discharges

In carotid-body preparations supplied by blood (Hornbein, Griffo & Roos, 1961; Eyzaguirre & Lewin, 1961*a*), or by saline perfusions (Neil & Joels, 1963), or *in vitro* (Eyzaguirre & Lewin, 1961*b*) the chemosensory

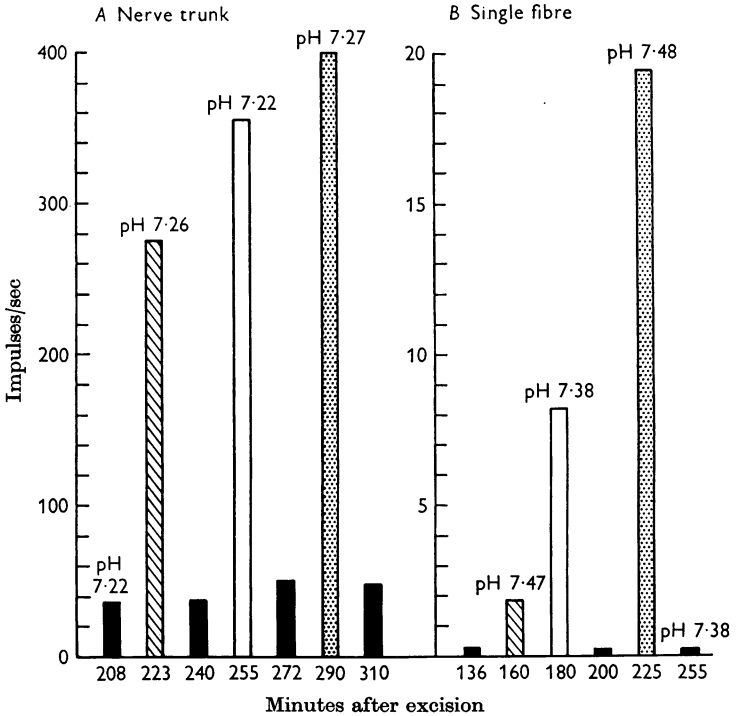

Fig. 2. Effects of equilibrating bathing solutions with different gas mixtures on the discharge of chemoreceptors. Black bars, 100 % O_2; striped bars, 6 % CO_2 in O_2; clear bars, air; stippled bars, 6 % CO_2 in air. pH values above bars. *A*, recording from whole carotid nerve. Temperature, 36·5° C; flow, 4·5 ml./min. *B*, recording from single chemoreceptor fibre in different experiment. Temperature, 34·6° C; flow, 3·2 ml./min.

discharge is modified by hypoxia, hypercapnia or a combination of both. The following series of experiments will present an analysis of chemoreceptor discharges during *in vitro* exposure of the carotid body to different gas mixtures.

Representative experiments are illustrated in Fig. 2. In *A*, discharges

were recorded from the whole carotid nerve; the pH of the bathing solutions which contained either 100% O_2 or air was 7·22; the solutions equilibrated with CO_2 had a pH of 7·26–7·27. The carotid nerve discharged at average frequencies of 36–50/sec when pure oxygen was employed (black bars). The discharge increased to 276/sec during exposure to 6% CO_2 in O_2 (striped bar), and to 357/sec during application of air (clear bar). It reached a maximum of 400/sec when 6% CO_2 in air was used (stippled bar). *B* was taken from a different experiment where recording was done from a single chemoreceptor fibre. The same gas mixtures used in the experiment illustrated in *A* were employed. In this case the pH of the bathing solution was also slightly more alkaline when CO_2 was employed. The receptor discharged at 0·2/sec during exposure to 100% O_2 (black bars) and its discharge increased to 1·8/sec (striped bar) and to 8·4/sec (clear bar) when 6% CO_2 in O_2 and air without CO_2 respectively, were used. A much larger increase in sensory discharge frequency occurred when 6% CO_2 in air (stippled bar) was employed since the discharge reached an average frequency of 19·5/sec.

Perfusion of the carotid body with saline equilibrated with high CO_2 at constant pH elicits an increase in the activity of carotid body chemoreceptors (Neil & Joels, 1963). This effect has been interpreted as being due to a possible reduction of flow through the carotid body vascular bed because of an increase in patency of the arterio-venous shunts at the expense of the glomus circulation (de Castro, 1951; Neil & Joels, 1963). This being the case, direct effects of CO_2 on carotid body chemoreceptors have been open to question. The experiments just presented have shown that *in vitro* (where vascular effects are absent) it is possible to elicit appreciable chemoreceptor discharge when the bathing solution contains high CO_2 and its pH is kept within physiological limits.

The discharge of single chemoreceptor units was analysed in twenty-one preparations bathed in solutions equilibrated with different gases. In general, the receptors were silent or showed very few discharges when bathed with 100% O_2. They showed increased activity when the bathing solution contained less oxygen and/or more CO_2. When a gas mixture was changed, enough time was allowed (at least 15 min) for stabilization of the sensory discharge before the discharges were photographed on moving film. Figure 3 illustrates sample records obtained in this series during the discharge of a single chemosensory fibre while the preparation was exposed to different gases. The receptor was quiet under 100% O_2. During exposure to 50% O_2 in N_2 (*A*) discharges occurred at an average frequency of 2·1/sec. When the gas mixture was replaced by another which contained 5% CO_2 in O_2 (*B*) the discharge decreased to 1·7/sec. *C* shows the effect of saline equilibrated with air on this receptor. The sensory

discharge frequency increased to an average of 7·1/sec. These and similar records were used in the measurement of impulse intervals as indicated in Methods. The results obtained are presented immediately below.

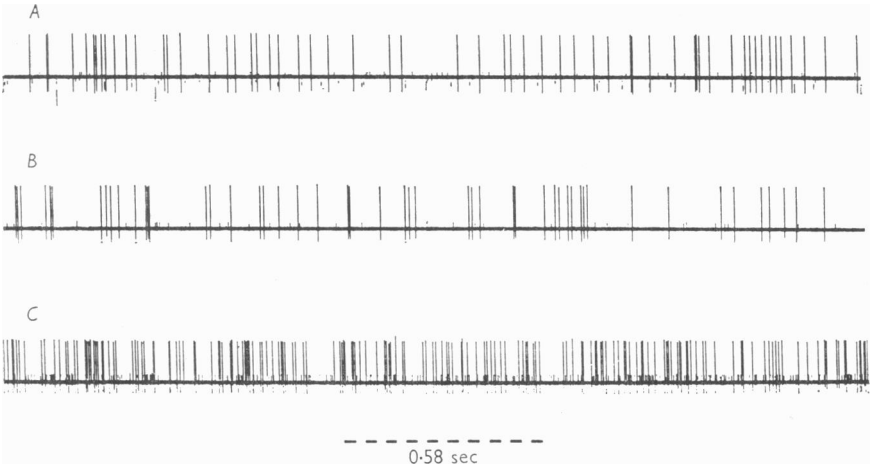

Fig. 3. Effects of different gas mixtures on discharge of single chemosensory fibre. Receptor silent under 100 % O_2 (not shown). *A*, 50 % O_2 in N_2, pH 7·32; average fibre discharge, 2·10/sec. *B*, 5 % CO_2 in O_2, pH 7·34; average discharge, 1·71/sec. *C*, air, pH 7·32; average discharge frequency, 7·06/sec. In all cases Locke's solution flowed at 6·0 ml./min. Temperature, 35° C.

Interval analysis during hypoxia. In total, 12,152 intervals were measured from discharges of twenty single fibres. The bathing solutions had a pH of 7·3–7·5 at temperatures of from 35·0 to 37·0° C. Records were obtained from preparations during periods ranging from 95 to 510 min after excision. In two instances intervals could be measured from fibres which discharged in solutions equilibrated with 100 % O_2. The mean interval ranged from 2133 to 2849 msec. When 50 % O_2 was applied in 11 instances the mean interval was 538 msec (S.E. ± 69·5). Air was used in twelve instances and the duration of the mean interval was reduced to 137 msec (S.E. ± 20·9). These results are not surprising but they serve to illustrate the general tendency of chemoreceptor responses to a given stimulus in different experiments.

Histograms of the frequency distribution of impulse intervals were constructed in all cases studied and representative samples are shown in Fig. 4. *A* shows the distribution of impulse intervals constructed from measurements obtained during exposure of the carotid body to 100 % O_2. The frequency of interval distribution approaches the curve suggested for random events (interrupted line in the illustration) and given by the formula:

$$n = N(\Delta t/T) \exp(-t/T),$$

where n is the number of occurrences of any interval between t and $t+\Delta t$; N is the total number of intervals and T is the duration of the mean interval; for accuracy Δt had to be considered as 1/20 or less of the longest interval measured (Feller, 1950). B was taken from the same preparation while the carotid body was exposed to 50 % O_2 in N_2. The mean interval decreased but the interval distribution curve still approached the distribution suggested for random events (interrupted line), although it had a clear peak at the first class interval (0–200 msec). C, also from the same

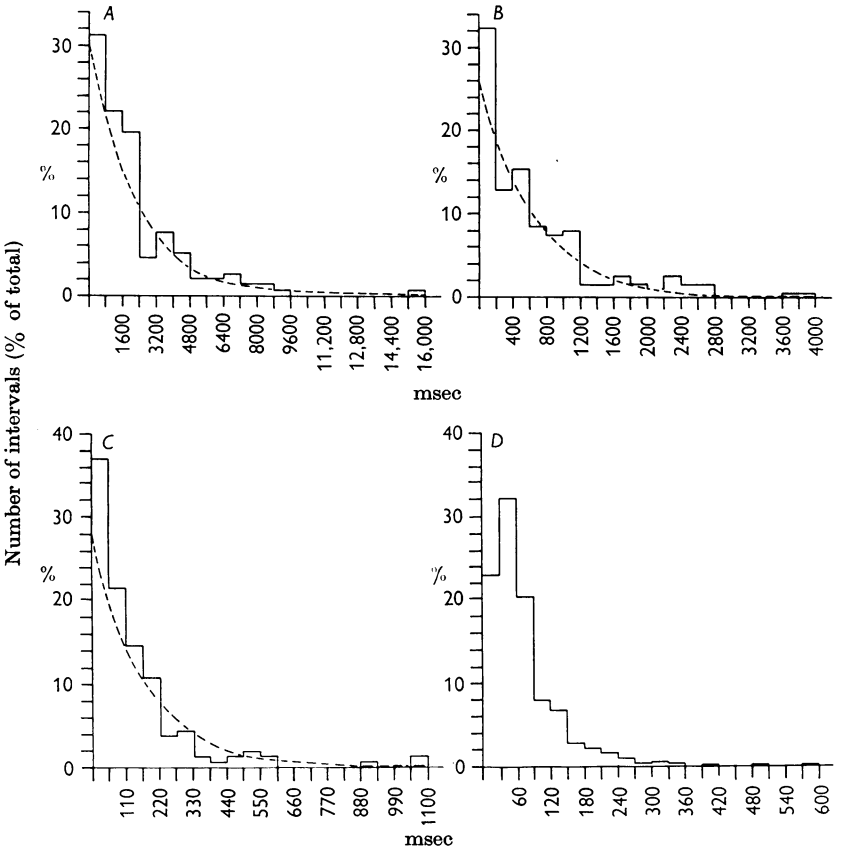

Fig. 4. Histograms of frequency distribution of impulse intervals at different P_{O_2}. Single chemoreceptor fibres. *A*, *B* and *C* from same preparation. Flow 4·7 ml./min, pH 7·39 at 37·0° C. *A*, interval distribution during exposure to 100 % O_2. Mean interval 2138 msec obtained from measurements of 259 intervals. *B*, constructed from 201 intervals (mean value 647 msec) during exposure to 50 % O_2 in N_2. *C*, constructed from 258 intervals (mean value 169 msec) during exposure of organ to air. *D*, from different experiment. Organ exposed to air. Flow 4·0 ml./min, pH 7·34 at 36·3° C. Mean interval 77 msec obtained from measurements of 300 intervals. Interrupted lines, expected distributions of intervals for random events (see text).

preparation after exposure to air, shows an interval distribution similar to that presented in *B*, although the mean interval was still further reduced and the peak occurred at 0–55 msec. *D* illustrates the interval distribution obtained from a different preparation exposed to air. In this case, *T* was 76·62 msec and there was a clear peak at the second class interval (30–60 msec).

Interval analyses during hypercapnia alone and combined with hypoxia

The discharges from ten single chemosensory fibres which responded to an increase of CO_2 tension alone or combined with hypoxia were analysed statistically. In total, 6811 intervals were measured (see Methods). When 5–6% CO_2 in O_2 was employed in seventeen trials the mean interval was 630 msec ± 74·7 S.E. This value is similar to that obtained with 50% O_2 in N_2. In four fibres, when 6% CO_2 in air was used, the mean interval fluctuated between 40 and 60 msec. This value was considerably less than that obtained during application of air alone.

Histograms of the distribution of impulse intervals were constructed in all cases studied. Representative samples are presented in Fig. 5. *A* and *B* show the interval distribution of impulses recorded from single fibres in two different preparations. In both cases 5% CO_2 in O_2 was used. In *A* the interval distribution curve (constructed from 406 intervals) followed closely the distribution suggested for random events (interrupted line). In *B* (constructed from 305 intervals) there is a clear peak and deviation from the 'random curve' at the 0–140 msec interval. These two histograms are representative since in about 60% of the cases peaks at the first or subsequent class intervals were observed. In other cases the interval distribution curves followed more closely a random distribution. There was no significant correlation between the presence of peaks and the value of the mean intervals. *C* and *D* were constructed from a different preparation. In *C*, 6% CO_2 in O_2 was applied and the distribution curve (from 212 intervals) shows a slight peak at the first class interval (0–140 msec). *D* shows the change in the histogram (constructed from 1041 intervals) produced by switching to Locke's solution equilibrated with 6% CO_2 in air. The mean interval was reduced to 40·23 msec and there was a clear peak between 10·5 and 21·0 msec. The presence of noticeable peaks, such as those in *D*, occurred in all instances when 6% CO_2 in air was employed.

A fairly extensive statistical treatment of the discharge of single chemoreceptor fibres has been done recently by Biscoe & Taylor (1963). The results obtained by these authors on preparations supplied by blood and perfused with saline are in good agreement with those presented here.

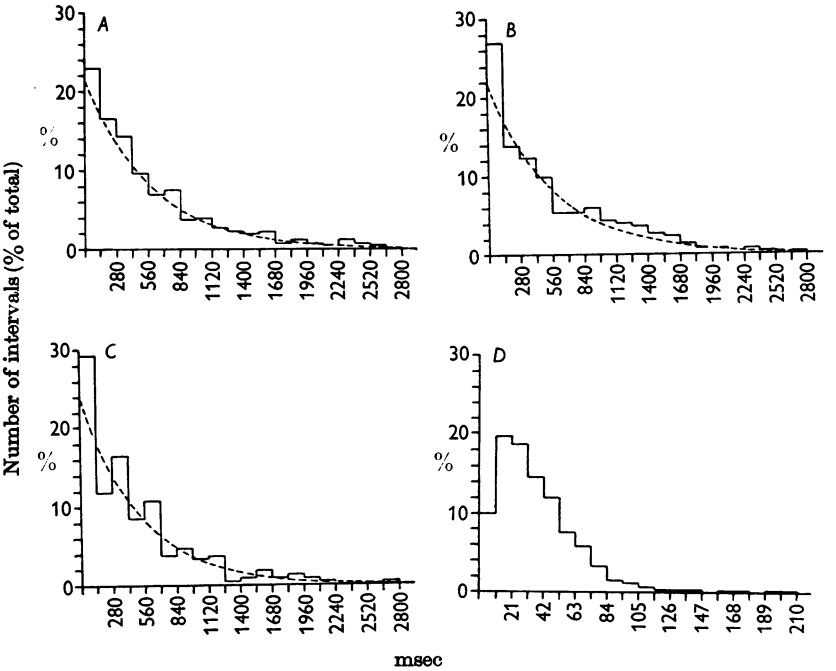

Fig. 5. Histograms of frequency distribution of impulse intervals during hypercapnia and during hypercapnia combined with hypoxia. Single chemoreceptor fibres. *A*, 5% CO_2 in O_2 in Locke's solution at 35·6° C and pH 7·32. Flow, 6·0 ml./min. Mean interval 585 msec. *B*, different preparation. 5% CO_2 in O_2 in Locke's solution flowing at 3·5 ml./min. Temperature, 35·5° C and pH 7·59. Mean interval 564 msec. *C*, different preparation. 6% CO_2 in O_2 in Locke's solution allowed to flow at 3·1 ml./min. Temperature, 34·5° C and pH 7·48. Mean interval 525 msec. *D*, same fibre as in *C*. Locke's solution equilibrated with 6% CO_2 in air flowing at 3·4 ml./min at 34·5° C and pH 7·48. Mean interval 40 msec.

Chemoreceptor activity during and after prolonged anoxia

The preceding series showed that the discharge of a single chemoreceptor fibre was modified both by hypoxia and by hypercapnia (cf. also Joels & Neil, 1960*b*; Eyzaguirre & Lewin, 1961*a*). Since one fibre branches to end on different glomus cells (de Castro, 1951), the possibility arose that some terminals could be recording activity from loci particularly sensitive to CO_2 and other terminals from receptors especially sensitive to O_2 lack. In order to determine whether or not one, or more than one, chemoreceptor sites were involved the following experiments were designed.

In one series, single chemoreceptor fibres were dissected from the carotid nerve while the receptor was bathed in Locke's solution equilibrated with 30% O_2 in N_2. The receptor response to anoxia was tested by injections

into the stream of small amounts of NaCN (Mallinckrodt), in order to induce histotoxic anoxia. The response to hypercapnia was tested by replacing the 30% O_2 Locke's solution with saline which contained 6% CO_2, 30% O_2 and 64% N_2. After these tests the preparation was exposed to 100% N_2 for 74–110 min in order to fatigue possible oxygen-sensitive systems. The response to NaCN was also tested a few times during exposure to N_2. Afterwards, the N_2-Locke's solution was replaced with 30% O_2-Locke's solution and the responses to both CO_2 and NaCN were tested again as done before the application of N_2. Such an experiment is illustrated in Fig. 6.

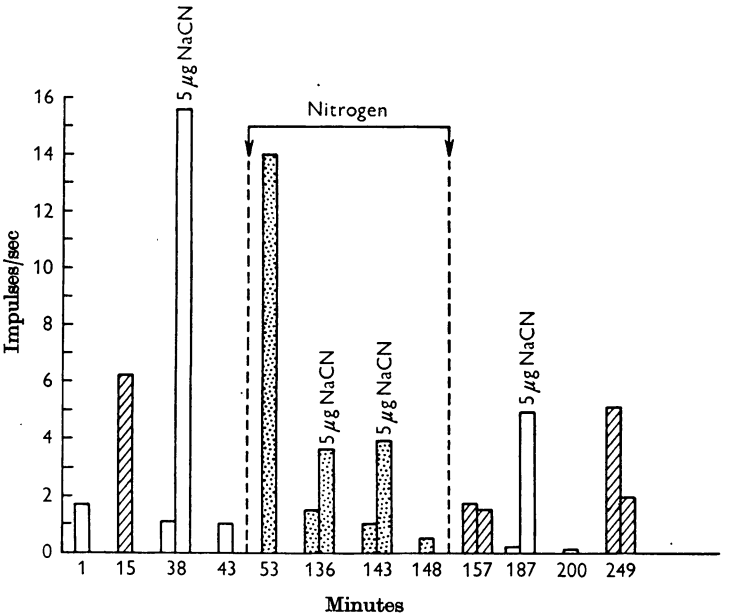

Fig. 6. Chemoreceptor depression produced by prolonged exposure to nitrogen. Single chemosensory fibre. Clear bars, response obtained during exposure to 30% O_2 in N_2. Striped bars, response during application of 6% CO_2 in 30% O_2 and 64% N_2. Stippled bars, response during application of 100% N_2 (between vertical interrupted lines) for a period of 110 min. Responses to 5 μg NaCN indicated over bars. Locke's solution at 36·0° C flowed at 1·0 ml./min throughout the experiment. pH of solutions which contained CO_2, 7·43; pH of other solution, 7·41.

A single chemoreceptor fibre preparation discharged at 1·7/sec when bathed with 30% O_2 in N_2 (clear bars). The discharge increased to 6·2/sec when the bathing saline contained 6% CO_2, 30% O_2, and 64% N_2 (striped bars). At 38 min, when the preparation was again exposed to 30% O_2, 5 μg of NaCN were injected into the stream. The chemosensory discharge increased to 15·6/sec and remained at that level for a few minutes. Sub-

sequently, the discharge returned to base line as NaCN was washed out. After this test, Locke's solution equilibrated with 100% N_2 was made to flow over the preparation. The sensory discharge increased to 14·0/sec, but as nitrogen exposure continued the discharge declined steadily (stippled bars). At 136 and 143 min 5μg of NaCN were again injected into the stream. The sensory discharge increased from a base line of 1·0/sec (at 143 min) to a peak of 3·9/sec. After 110 min of exposure to N_2 the solution was replaced with one equilibrated with 6% CO_2 in 30% O_2. This time the discharge reached a peak of 1·7/sec but very soon afterwards it declined to an average of 1·5/sec (at 157 min). At 187 min, 30% O_2-Locke's solution was used again and discharges decreased to 0·2/sec. An injection of NaCN increased the discharge to a peak of 4·9/sec. Application of CO_2 at 249 min elicited a discharge peak of 5·05/sec which declined to a steady level of 1·95/sec.

The experiment just described shows that during prolonged exposure to anoxic anoxia (induced by N_2) the response of the carotid body chemoreceptors to NaCN (histotoxic anoxia) was depressed. This observation is interesting since Lorente de No (1947) has reported that prolonged exposure to nitrogen depolarizes the nerve membrane which remains depolarized for the duration of the exposure to N_2. In such a situation one would expect that the response to NaCN would be increased and not depressed during N_2 application if both agents acted solely by depolarizing the nerve terminals. However, if both N_2 and NaCN produced a large enough depolarization they may have induced cathodal block (cf. Eyzaguirre & Kuffler, 1955).

Soon after N_2 application was discontinued the receptors remained depressed in their response to NaCN and to moderate hypoxia (produced by 30% O_2). It is more interesting, however, that the response to CO_2 was also depressed (at 157 min). As recovery proceeded the receptor seemed unable to maintain a relatively high level of discharge during CO_2 stimulation since there was a clear decline in sensory frequency after a peak was reached (at 249 min). These observations seemed to indicate that only one receptor site (for low O_2 and high CO_2) was involved. Such an assumption would be valid only if severe anoxia did not alter the response of some chemosensory endings. However, Lorente de No (1947) has reported that nerve depolarization induced by anoxia is followed by marked hyperpolarization once oxygen is reintroduced into the nerve chamber. If this was the case with the chemoreceptor endings one would expect an increased receptor threshold to all forms of stimulation, hence the depression observed after nitrogen application.

In order to test whether or not under our experimental conditions anoxia changed the response of the chemosensory endings, the receptors

were exposed to pure nitrogen for several hours. The response to applied ACh (acetylcholine; Matheson, Coleman & Bell), which stimulates sensory endings (Brown & Gray, 1948; Douglas & Gray, 1953; Landgren, Skouby & Zotterman, 1953; Diamond, 1955), was tested before, during and after exposure to N_2. In these experiments prolonged exposure of the carotid body to N_2 did not depress the response of the chemosensory endings to 10^{-6} (wt./vol.) ACh (see Fig. 4 in Eyzaguirre & Koyano, 1965*a*). As a further control, discharges from a single pressoreceptor fibre were recorded in the same preparations. Nitrogen application did not alter the pressoreceptor discharge. Possibly, there was either some entry of oxygen through the oil covering the preparation, or the cylinder N_2 contained a small percentage of oxygen (cf. Lorente de No, 1947). However, the fact remains that under the conditions employed here the sensory endings were not appreciably depressed by N_2.

A logical conclusion of the experiments just described is that the increased discharge and ensuing receptor depression produced by prolonged anoxia are not due primarily to an action of anoxia on the nerve endings. Furthermore, there appear to be receptor sites in the carotid body which respond both to high CO_2 and to low O_2.

In order further to explore these problems a different set of experiments was designed since Joels & Neil (1962) have reported that perfusion of the carotid body with NaCN (1:50,000) for some minutes exhausted the response of the preparation to asphyxia but not to applied ACh (10^{-4}). Their contention was that asphyxial solutions acted through the glomus cells and, once the latter were exhausted by NaCN, the nerve endings remained intact as shown by their response to applied ACh. As shown below, Joels & Neil's results could not be confirmed in the *in vitro* preparation since NaCN (10^{-5}, wt./vol.) depressed the sensory nerve endings, although this drug may have also altered other mechanisms responsible for chemoreceptor impulse initiation (see Discussion).

The response of the carotid body to ACh and to interruption of flow (which produces strong chemosensory stimulation) was tested before, during and after prolonged exposure to NaCN (Fig. 7). The organ was bathed with Locke's solution equilibrated with 30% O_2 in N_2. Recording from a few-fibre carotid nerve filament revealed a discharge of 2·8/sec 10 min after the beginning of the series. During ACh flow (10^{-6}, wt./vol.) the discharge increased to 23/sec at 24 min. When ACh was washed out the impulse frequency decreased (at 52 min). Then flow was interrupted (IF) and the discharge reached a peak of 88/sec (at 60 min). Resumption of flow reduced the discharge to 1·7 impulse/sec (at 80 min). Subsequently, the preparation was bathed with Locke's solution (also equilibrated with 30% O_2) which contained NaCN (10^{-5}, wt./vol.). The discharges increased

to a peak of 58/sec at 96 min, remained at that level for a few minutes, and then declined, to reach 2·0/sec at 120 min. At 135 min the NaCN-Locke's solution flow was interrupted and the discharge increased to 10·2/sec. When the NaCN-Locke's solution flow was resumed the chemosensory discharge decayed to 1·2/sec (at 140 min). After this measurement, the

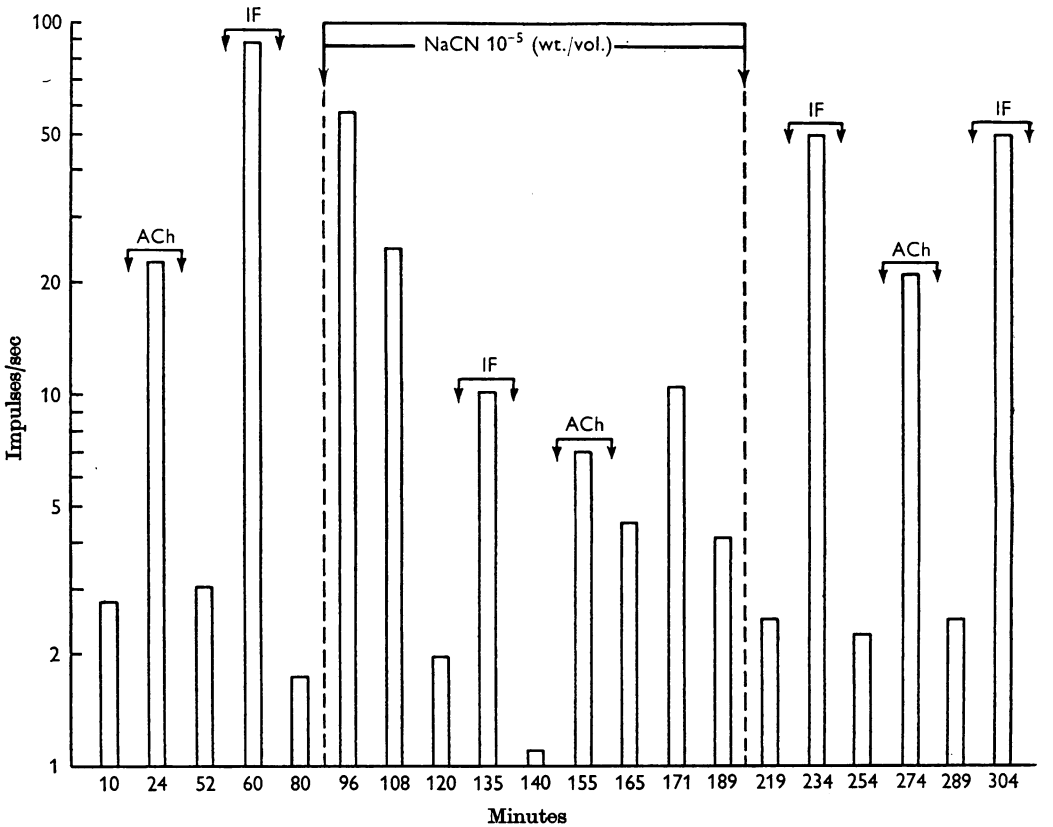

Fig. 7. Effects of ACh and interruption of flow (IF) on chemosensory discharges before, during and after exposure of receptor to NaCN. Recording from small carotid nerve filament which contained few active fibres. Locke's solution, equilibrated with 30 % O_2 in N_2, flowed at 0·6 ml./min. Temperature, 37·0° C. IF, interruption of flow. ACh, application of ACh (10^{-6}, wt./vol.). Between vertical interrupted lines, NaCN in Locke's solution (10^{-5}, wt./vol.) applied for 90 min.

addition of ACh (10^{-6}, wt./vol.) to the flowing NaCN-Locke's solution elicited a discharge of 7·0/sec at 155 min. When ACh was washed out with NaCN-Locke's solution the chemosensory discharge remained at a relatively high level and even increased to 10·6/sec at 171 min before the impulses began to decline (cf. also Eyzaguirre & Koyano, 1965*a*). At

200 min the NaCN-Locke's solution was replaced with normal Locke's solution (also equilibrated with 30% O_2) and the discharge became stable at 2·5/sec. At 234 min the flow of normal Locke's solution was interrupted and the impulse frequency rose to 51/sec. Flow was resumed and discharges declined to base-line levels. When ACh (10^{-6}, wt./vol.) was added to the normal Locke's solution the impulses reached a peak of 21/sec at 274 min. Near the end of this series flow was interrupted again and the discharge reached 51/sec at 304 min.

After the series already described a single pressoreceptor fibre was isolated from the carotid nerve in the same preparation. The pressoreceptor (located by punctate stimulation at the origin of the occipito-ascending pharyngeal artery) discharged at 18–20/sec while in normal Locke's solution. Exposure of the pressoreceptor to NaCN-Locke's solution produced complete block of the discharge in a few minutes. Pressoreceptor block was relieved after washing out the NaCN with normal Locke's solution (not illustrated).

The results just presented show that prolonged exposure to NaCN may block nerve terminals as shown by the block of pressoreceptor discharges induced by the drug. Consequently, NaCN may have depressed chemosensory discharges mainly by blocking some sensory endings. This is emphasized by the fact that before the NaCN solution was allowed to flow over the preparation, ACh increased the sensory discharges by a factor of 8·14. During NaCN application (where the base-line discharge was lower than the control) ACh increased the discharge by a factor of 5·8. This reduced response to ACh is a good index of sensory ending depression because, as will be shown in the following paper (Eyzaguirre & Koyano, 1965*a*), the ratio of chemosensory discharge increase should be higher when the base-line frequency is lower. Furthermore, it is interesting to note that chemoreceptor depression induced by prolonged exposure to NaCN was relieved *after* exposure to ACh (see 165–189 min). This decreased receptor threshold after washing a dose of ACh was also observed during depression induced by exposure to pure nitrogen (see Fig. 4 of Eyzaguirre & Koyano, 1965*a*). After washing out NaCN, there was a better recovery of the response to ACh than that produced by interruption of flow; this difference may not be significant.

The receptor depression obtained after prolonged exposure to NaCN *in vitro* is similar to that reported by Anichkov & Belen'kii (1948) and by Krylov (1956) both in preparations with intact circulations and in those perfused with saline. These authors found that large doses of KCN induced (after a period of chemosensory stimulation) a complete loss of chemoreceptor response both to natural stimulation and to injected ACh or nicotine. KCN block was relieved after washing out the drug.

Effects of pH on chemoreceptor discharges

Heymans, Bouckaert & Dautrebande (1930) have demonstrated that acidity stimulates the chemoreflex respiratory drive while alkalinity has the opposite effect. Furthermore, Zotterman (1935), von Euler, Liljestrand & Zotterman (1939) and Bartels & Witzleb (1956) have indicated that chemoreceptor discharges are dependent on the pH of the blood. More recently Joels & Neil (1960*a*) have shown in perfusion studies that variations in the pH of the perfusing fluid change the chemosensory discharge. However, Neil (1963) has indicated that pH effects on the sensory discharge in perfused carotid bodies may be due to local vascular changes.

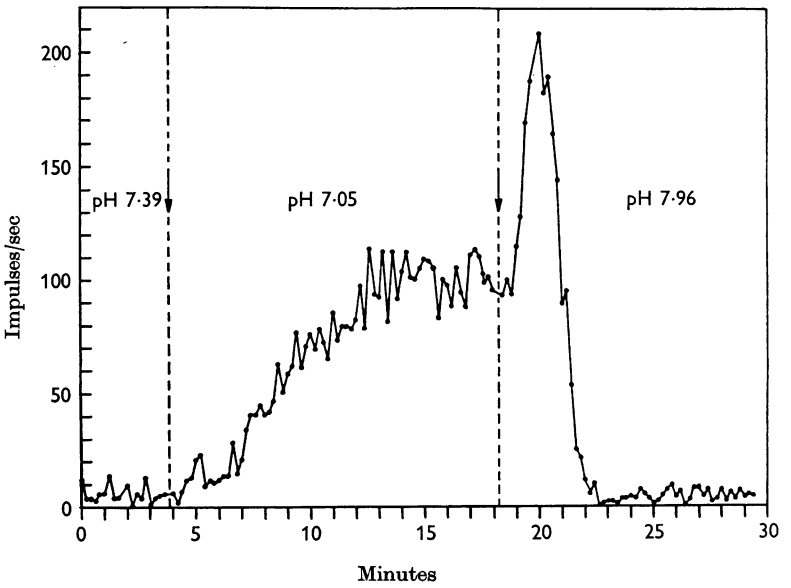

Fig. 8. Effects of pH changes on chemoreceptor discharges. Recording from whole carotid nerve. Carotid body bathed in Locke's solution equilibrated with 50 % O_2 in N_2. Locke's solution flowed at 5·0 ml./min at 37·2° C. Zero time up to first downward arrow, pH 7·39; between arrows, pH 7·05; from second downward arrow to end of experiment, pH 7·96.

In our experiments the pH of the bathing solution was changed by modifying the HCl concentration in the bathing saline while the amount of Tris buffer was kept constant (see Methods). The sensitivity to pH varied appreciably in different preparations and, as will be shown later, pH effects were dependent on temperature as well as on the P_{O_2} of the solutions. Figure 8 illustrates the chemoreceptor discharge obtained under 50 % O_2 in N_2. At the beginning of the series, exposure to Locke's solution

at pH 7·39 elicited an average discharge of 6·0/sec. When initial solution was replaced by Locke's solution at pH 7·05 the sensory discharge increased to an average of 100/sec in about 10 min. When Locke's solution at pH 7·96 was applied, there was an early stimulation presumably due to the alkalinity of the fluid and the discharge reached 209/sec. Subsequently, the discharge subsided and reached a steady level of 5·4/sec.

The experiment illustrated shows that a reduction in pH (e.g. from 7·39 to 7·05) elicited the expected increase in sensory discharge frequency. The early stimulation by alkalinity was not due to an extreme change in pH (e.g. from 7·05 to 7·96) since in other cases it was observed when pH was changed from 7·4 to 7·6. Early stimulation by alkalinity was present in the majority of instances when the pH of the bathing solution increased. At present the meaning of this effect is obscure. The fact that the steady discharge was about the same at pH 7·39 and 7·96 was not uncommonly observed (see below).

Figure 9*A* illustrates the effects of pH on the steady discharge of carotid body chemoreceptors obtained from three different preparations. Each point represents the average discharge obtained at a certain pH, once any phasic effects (such as early stimulation by alkalinity) had subsided. The illustration shows that pH–sensory discharge curves varied in different experiments, although the general tendency was similar, i.e. low pH tended to increase the chemosensory discharge while alkalinity had the opposite effect. However, the slopes of the curves were quite different. Thus, curve *a* shows a steep pH–sensory discharge response when pH varied from 7·10 to 6·98. From pH 7·65–7·10 the curve was fairly flat. The other two curves (*b* and *c*) showed a much steeper pH–sensory discharge relation when pH was varied from 7·44 to 6·89 (*b*) or from 7·78 to 7·46 (*c*).

As will be shown later, variations in the slope of the pH–sensory frequency curves may have been due to different oxygenation of the tissues in spite of the fact that in all cases 50% O_2 in N_2 was employed. Different oxygenation may be produced by the relative amount of connective tissue surrounding the glomus (Eyzaguirre & Lewin, 1961*b*). Finally, one may not ignore the possibility that different flow rates (5·2 ml./min in *a*, 3·4 ml./min in *b*, and 3·3 ml./min in *c*) may have changed the response of the chemoreceptors to pH changes, since flow was an important variable in these experiments (see above).

Figure 9*B* shows that pH effects on the chemosensory discharge may be altered by temperature. In the experiment illustrated the temperature of the bathing solutions (equilibrated with 50% O_2 in N_2) was changed in steps from 35·7 to 37·8° C. At a given temperature (e.g. 35·7° C) Locke's solution at pH 7·1 was allowed to flow over the preparation. The sensory

discharge reached a steady level of 84/sec. Subsequently, the bathing solution was replaced by another at pH 7·3 (at the same temperature) and the discharge declined to 37/sec. When the solution had a pH of 7·49 the average discharge frequency was 33/sec and finally at pH 7·75 the discharge declined to 24–25/sec. Similar pH–sensory curves were then constructed by varying the pH at 36·2 and 37·8° C, respectively.

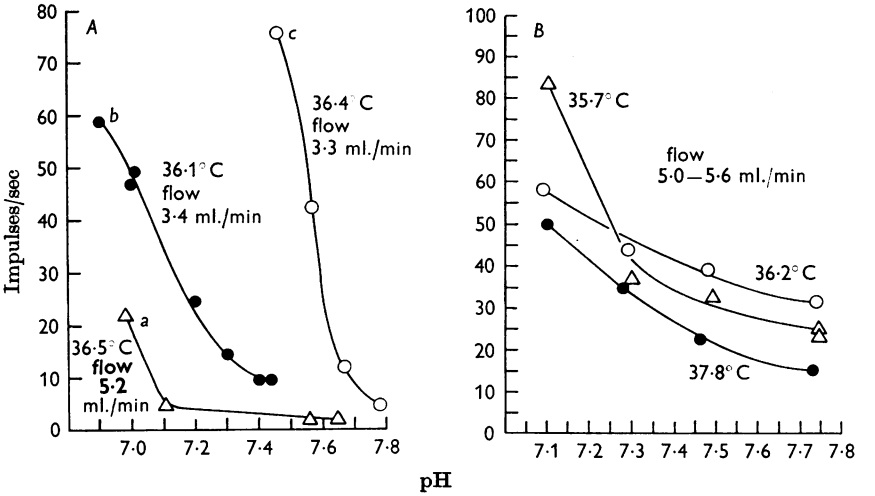

Fig. 9. *A*, effects of pH on chemosensory discharges. Recording from whole carotid nerve. *a*, *b* and *c*, three different experiments. Each point on the curves represents average frequency of discharges during exposure to Locke's solutions at different pH and equilibrated with 50 % O_2 in N_2. Flow and temperature indicated in illustration. *B*, three curves obtained from a different experiment showing combined effects of pH and temperature on chemoreceptor discharges. Locke's solution equilibrated with 50 % O_2 in N_2. Flow and temperatures indicated in illustration.

The three curves presented in Fig. 9*B* show that at pH of from 7·25 to 7·75 the receptors discharged at higher frequency at 36·2° C. The discharge was lower at 35·7° C and even lower at 37·8° C. At pH of from 7·09 to 7·25 the same relation persisted between the points obtained at 36·2 and at 37·8° C. However, the point obtained at pH 7·1 and 35·7° C was considerably higher. These results indicate that the combined effects of temperature and pH on the chemosensory discharge are complex.

Combined effects of pH and hypoxia. Joels & Neil (1960*a*) have shown that low *pH* stimulates chemosensory discharges whether the P_{O_2} of a perfusate is high or low. However, quantitative studies on the combined effects of oxygen want and acidity on these receptors have not appeared. The ensuing series will show some interactions of the hypoxic and pH effects on the sensory discharges recorded from the whole carotid nerve.

The carotid body was exposed to Locke's solutions at four values of pH at each P_{O_2}. A pH–sensory discharge curve was constructed at a single P_{O_2} and then the gas mixture equilibrating the bathing solution was changed. One hour was allowed for the new gas to come into equilibrium with the physiological solutions and then a pH–sensory discharge curve was constructed at the new P_{O_2}. These procedures were repeated several times. Figure 10 illustrates an isometric projection of experimental points obtained in one of these experiments.

At the beginning of this series the solutions were equilibrated with 100% O_2. At pH 7·93 the receptor discharged at 7·5/sec. The discharge reached 13·0/sec at pH 7·73 and 3·3/sec at pH 7·39. Sensory frequency increased to 42/sec at pH 7·06. When the bathing solution contained 50% O_2 in N_2 the pH–sensory discharge curve showed a flat response of about 5/sec when pH was varied from 7·93 to 7·39. However, when pH was reduced to 7·06 the sensory discharge increased steeply to 100/sec. When the gas mixture contained 20% O_2 in N_2 the sensory fibres discharged at 26/sec at pH 7·93. Subsequent reductions in pH produced a steeply ascending curve which reached a maximum of 504/sec at pH 7·39. Further reduction in pH brought the discharges down to 467/sec at pH 7·06. When the oxygen mixture was reduced to 10% O_2 in N_2, pH 7·93 induced a discharge of 221/sec which declined to 121/sec when pH decreased to 7·73. Reduction in pH to 7·39 and 7·06 increased the discharge to 306 and 387/sec respectively (points linked by interrupted line).

Careful examination of the curves already described revealed that pH effects were negligible when pH was varied from 7·93 to 7·39 and the bathing solution contained either 50 or 100% O_2. These effects were appreciably more marked when pH was reduced to 7·06. However, when Locke's solution contained 20% O_2 there was a considerable increase in sensory discharge produced by reduction in pH of the bathing solution until a maximum was reached at pH 7·39. Further increases in acidity tended to depress the sensory discharges. pH effects obtained during exposure of the organ to 10% O_2 were important but less marked than those obtained during exposure to 20% O_2. It is interesting to notice, however, that at 10% O_2 when the saline was alkaline (pH 7·93) there was a tendency to receptor stimulation. This tendency was frequently observed when alkaline solutions were used together with low O_2 mixtures.

Examination of the O_2–sensory discharge curves revealed that at pH 7·93 there was little effect of O_2 lack on the sensory discharge until O_2 was reduced to 10% O_2. At pH 7·39 there was not much difference between the discharge obtained at either 100 or 50% O_2. But, when 20% O_2 was employed the discharge increased greatly. Further reduction in O_2 (to 10% O_2) depressed sensory activity. The O_2–impulse curve obtained at

pH 7·06 was similar to that obtained at pH 7·39, except that there was a clear increase in discharge from 42/sec to 100/sec when the gas mixture was changed from 100 to 50% O_2.

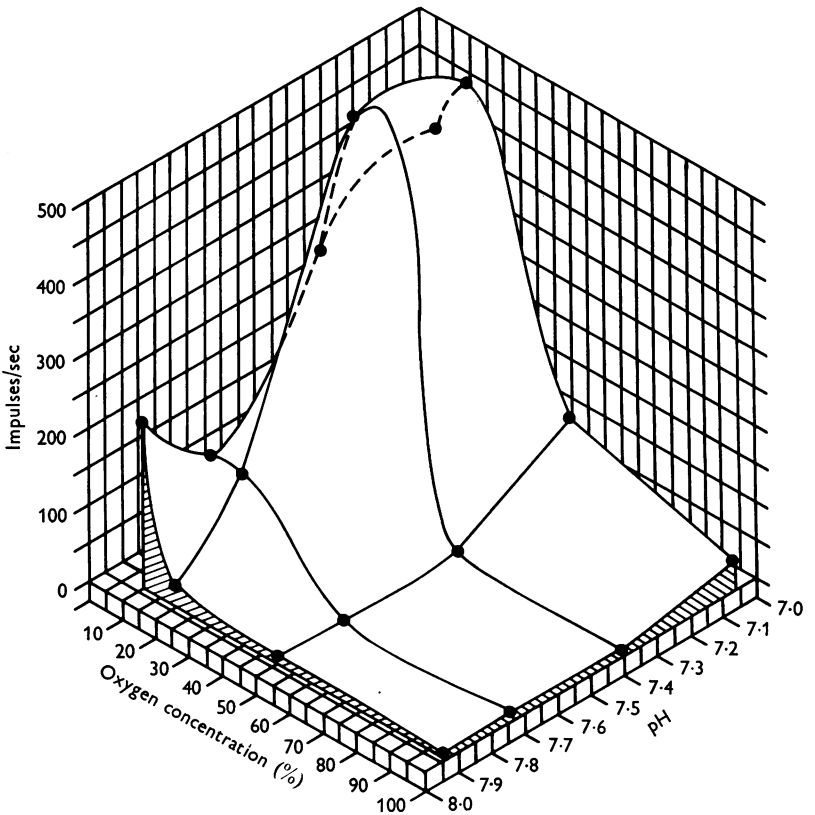

Fig. 10. Three-dimensional projection showing interaction of pH and P_{O_2} on chemosensory discharge. Recording from whole carotid nerve. Locke's solution flowing at 4·5–5·0 ml./min at 36·5–37·0° C. Saline was equilibrated with different gas mixtures (100, 50, 20 and 10% O_2 in N_2). pH of different solutions was 7·93, 7·73, 7·39 and 7·06. *x*-axis, oxygen concentration (%) in saline; *y*-axis, pH units; *z*-axis, frequency/sec of sensory impulses. For detailed explanation see text.

The isometric projection shows an interaction between low O_2 and acidity. pH effects are more effective when there is some degree of hypoxia. Stronger hypoxia tends to depress the effects of acidity on the sensory discharge. Similarly, hypoxia is more effective in changing sensory frequency at lower pH.

Interval analysis. Discharges from four single chemoreceptor fibres were analysed in three experiments. A total of 2754 intervals was measured over a pH range of 6·89 to 7·74 in solutions equilibrated with

50% O_2 in N_2. The number of observations was not enough for a quantitative analysis of the discharge. However, the general tendency was clear: the mean interval decreased as pH was lowered.

Histograms of the distribution frequency of impulse intervals were constructed in all cases studied. The general shape of the histograms was similar to that of those obtained under either hypoxia or hypercapnia and already presented in Figs. 4 and 5; the interval distribution curve followed relatively closely the curve suggested for random events. Small peaks usually appeared at the first or second class intervals. Prominent peaks suggesting a relatively regular distribution of impulse intervals were not observed.

DISCUSSION

Oxygen lack is a potent stimulus to the carotid body chemoreceptors. Hypoxia does not seem to act merely through depolarization of the non-myelinated nerve endings (although anoxia depolarizes nerve fibres), since Lorente de No (1947) has indicated that the membrane potential of nerve kept in a nitrogen environment is reduced and remains steadily depolarized for hours. The observations reported in Fig. 6 show that exposure to nitrogen produces initially a great increase in sensory discharge frequency followed by decreased receptor activity if exposure to nitrogen continues. Receptor depression is produced, in all probability, by exhaustion of some mechanism not linked exclusively with the membrane potential of nerve endings since nitrogen does not alter significantly the ACh response of the sensory endings.

The fact that hypercapnia stimulates the chemoreceptors directly and not through vascular effects is a good indication that, in this case, generation of chemosensory discharges originates at a site other than the nerve endings. In fact, one could hardly expect an agent such as CO_2, which is well known for its hyperpolarizing effects on nerve (Lorente de No, 1947), to depolarize the sensory endings. Similarly, the effect of relatively moderate changes in acidity of the bathing solutions on the chemosensory discharges suggests an indirect action. Small, environmental, pH changes are usually without effect on nerve or muscle membrane potential and conduction (Lorente de No, 1947; Caldwell, 1958; Spyropolous, 1960).

It could be argued that chemoreceptor endings have very special properties and that one should not expect them to behave either like nerve or like any other type of sensory ending. The fact remains, however, that these receptors are stimulated both by low O_2 and by high CO_2 and that these agents act on excitable membranes in an opposite manner. It seems, therefore, more reasonable to assume that the chemoreceptor endings are not specific to either form of stimulation and that a common factor, viz. a 'transmitter' or 'generator' substance, is released during

activity. This substance, in turn, would be responsible for the activation of the sensory terminals.

The marked effects of saline flow on the chemosensory discharges may result from accumulation of generator substance in the extracellular spaces with the consequent stimulation of the sensory terminals. However, this may not be the only cause of chemosensory stimulation in these circumstances since the carotid body cells have a high metabolic rate (Daly, Lambertsen & Schweitzer, 1954) and slow flow may produce conditions leading to poor oxygenation and asphyxia of the tissues. Indirect evidence for the presence of a generator substance is provided by the fact that even during stimulation impulse intervals are distributed in a manner which approaches that suggested for random events (Feller, 1950; Fatt & Katz, 1952). Deviations from the random curve are usually small, as indicated by the presence of small peaks, unless very strong stimulation is employed. Similar results have been obtained by Biscoe & Taylor (1963) and these authors have suggested also that a chemical may be released during chemoreceptor activity. In slowly adapting mechanoreceptors (where release of a chemical is unlikely) intervals may be distributed 'at random' while the receptor is either 'at rest' or weakly stimulated. During stronger stimulation the distribution of impulse intervals usually becomes regular and clear distribution peaks are present. A known exception to this is the first order neurone of auditory receptors which shows a near random distribution of impulse intervals during stimulation (cf. Viernstein & Grossman, 1961). A regular receptor discharge is probably due to the presence of a steady generator potential (Katz, 1950; Eyzaguirre & Kuffler, 1955).

The sites for storage and release of the generator substance should be the glomus cells of the carotid body. Two types of such cells have been identified with the electron microscope. One type is a spherical cell (6–10μ in diameter) which is equivalent to the epithelioid cells of de Castro (Garner & Duncan, 1958; Lever *et al.* 1959; Ross, 1959; Eyzaguirre & Uchizono, 1961). The other type of cell is an elongated one which has been described by Ross (1959) as a 'sustentacular' cell mainly because it envelops the terminal nerve branches and most of the epithelioid cells. At present the function of these cells is totally unknown. It is tempting to assume that the epithelioid cells are the elements responsible for storage and liberation of the generator substance. These cells are numerous and contain a large number of vesicles and other inclusions such as electron-dense granules, etc. (Lever *et al.* 1959; Ross, 1959; also A. Hess, unpublished).

If one assumes that the epithelioid cells are responsible for the initiation of chemosensory discharges a study with intracellular micro-electrodes is in order. These studies have been started and results have been incon-

clusive in terms of membrane potential changes under different forms of stimulation (C. Eyzaguirre & H. Koyano, unpublished). However, more information on the behaviour of these cells is needed before conclusions are reached.

The behaviour of carotid body chemoreceptors seems to parallel that of the neuromuscular junction where 'at rest' miniature end-plate potentials are recorded from the post-synaptic membrane. The miniature potentials are considered to be produced by release of packets of transmitter substance (ACh in the case of the vertebrate junction) at random (Fatt & Katz, 1952; Boyd & Martin, 1956; Dudel & Kuffler, 1961). Subthreshold depolarization of the motor nerve endings increases the miniature end-plate potential frequency (Castillo & Katz, 1954), although statistical studies of their interval distribution during stimulation do not seem to be available. However, when an action potential reaches the motor endings there is a synchronous discharge of transmitter packets as evidenced by the presence of a post-synaptic junctional potential (cf. Katz, 1962).

If an analogy with the neuromuscular junction is legitimate, one may visualize the production of a 'generator' substance and its release during activity of the chemoreceptors. This substance may set up a short-lasting local depolarization at the nerve endings (similar to a miniature e.p.p.) and when this local depolarization attains a critical amplitude it may trigger the all-or-none activity of the sensory nerve fibre. This idea is consistent with the irregular increase in discharge observed during carotid body stimulation if more packets of substance are released per unit time, provided that a 'generator flood' does not occur. It is possible that in this receptor a massive liberation of transmitter may not occur rapidly enough to elicit a high frequency and regular discharge.

More direct evidence for the presence and release of a transmitter or generator substance will be presented in the ensuing papers (Eyzaguirre & Koyano, 1965*b*; Eyzaguirre *et al.* 1965).

SUMMARY

1. The chemoreceptors of the cat's carotid body *in vitro* were readily activated by hypoxia, hypercapnia and acidity, as well as by reduction in flow of the bathing solution.

2. Single-fibre analysis showed that chemoreceptor fibres were activated by either form of stimulation. Few units responded only to one type of stimulus. Histograms of the frequency distribution of intervals showed that in a number of instances the intervals were distributed following closely the distribution suggested for random events. In many instances, however, small peaks were superimposed on curves which

otherwise would be random. Under strong stimulation the discharge became more periodic and clear distribution peaks were observed.

3. On prolonged exposure to nitrogen the chemoreceptor discharge declined progressively and there was a depression of the response to NaCN and to CO_2. The response to ACh was unaltered by exposure to nitrogen. Prolonged exposure to NaCN depressed the response to interruption of flow and to ACh.

4. The response of the chemoreceptors to acidity was influenced both by temperature and by the oxygen concentration of the environment.

5. It is concluded that, in all likelihood, chemoreceptor discharges are produced by liberation of a chemical (presumably from the glomus cells) which, in turn, is capable of triggering the all-or-none response of the sensory fibres.

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