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An Investigation of the Effects of External Acidification on Sodium Transport, Internal pH and Membrane Potential in Barnacle Muscle Fibers

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Summary. Radiosodium efflux from barnacle muscle fibers is a function of pH_e, the threshold pH_e for stimulation of Na efflux into HCO_3^--artificial sea water (ASW) being 6.8 and the 'fixed' threshold pCO_2 (in an open CO_2 system) being approximately 30 mm Hg. Acidification of ASW containing non-HCO_3^- buffer is without effect on the Na efflux. The Na efflux following stimulation by reducing the pH of 10 mM HCO_3^--ASW from 7.8 to 6.3 is reduced by 17.3% as the result of microinjecting 100 mM EGTA, and increased by 32.6% as the result of microinjecting 0.5 M ATP. The Na efflux into K-free HCO_3^--ASW is markedly stimulated by external acidification. Ouabain-poisoned fibers are more responsive to a low pH_e than unpoisoned fibers. Applying the 2-^{14}C-DMO technique, it is found that fibers bathed in 10 mM HCO_3^--ASW at pH 7.8 have an internal pH of 7.09 ± 0.106 (mean $\pm$ SD), whereas fibers bathed in 25 mM TRIS-ASW at pH 7.8 have a pH_i of 7.28 ± 0.112. The relationship between pH_i and pH_e as external pH is varied by adding H^+ is linear. Measurements of the resting membrane potential indicate that external acidification in the presence of HCO_3^- as buffer is accompanied by a fall in E_m, the threshold pH_e being 7.3 both at 24 and 0 °C. This sensitivity amounts to 8.2 mV per pH unit (at 24 °C) over a wide range of pH_e. Membrane resistance following external acidification remains unchanged. Microinjection of the protein inhibitor of Walsh before external acidification fails to stop depolarization from occurring. Cooling to 0 °C also fails to abolish depolarization following acidification. Whereas external ouabain and ethacrynic acid reduce E_m in the absence or presence of acidification, DPH hyperpolarizes the membrane or arrests depolarization both at 24 and 0 °C. This effect of DPH at 0 °C is seen in the absence or presence of acidification. It is suggested that depolarization following acidification of a HCO_3^--containing medium is due to activation of a Cl^-- and/or HCO_3^--pump and that ouabain and ethacrynic acid reduces E_m by abolishing uncoupled Na transport.

The work described in this paper is a continuation of the study of Na efflux in single muscle fibers from the barnacle *Balanus nubilus,* reported earlier by Bittar, Chen, Danielson, Hartmann and Tong (1972) and Bittar, Hift, Huddart and Tong (1974). Its first objective was to obtain information concerning the behavior of the Na efflux in the presence of a raised pCO_2. Unlike frog muscle, barnacle fibers show a large rise in the Na efflux following acidification of a bicarbonate-containing

medium, the pH threshold for stimulation being in the region of 6.8 when the external HCO_3^- concentration is 10 mM (Bittar & Tong, 1971; Danielson, Bittar, Chen & Tong, 1971). The onset of the stimulatory response to external acidification is rapid and the evidence indicates that the active species is CO_2 rather than H^+. However, the possible effects of changes in extracellular pCO_2 on intracellular pCO_2 and pH have not been examined. It therefore seemed of special interest to investigate the effect of varying the pCO_2 on the relationship between external and internal pH. To this end the internal pH of fibers exposed to a range of bicarbonate concentrations was estimated by the DMO method and compared with that of fibers bathed in a medium in which the bicarbonate buffer had been replaced by TRIS.

A second objective of this study was to investigate the influence of external pH and pCO_2 on the resting membrane potential. This was done by recording E_m while changing the external pH and HCO_3^- concentration in an open (i.e. exposed to the atmosphere) system. The effect of external acidification on E_m was also studied in a closed system in order to separate more definitively the possible effects of HCO_3^- concentration *per se* and pCO_2 on E_m. The closed system allowed large changes to be made in extracellular pCO_2 without making concomitantly large changes in the magnitude of extracellular bicarbonate concentration.

Materials and Methods

Specimens of *Balanus nubilus* and *B. aquila* were purchased from a supplier at Friday Harbor, Seattle, and kept in an Instant Ocean aquarium (150-gallon capactiy) containing aerated artificial sea water (ASW). The temperature of the ASW was maintained at 12 °C. The composition of the ASW was the same as that given by Bittar, Chen, Danielson, Hartmann and Tong (1972). Single muscle fibers were isolated by dissection from the three pairs of depressor muscle bundles under ASW and cannulated in the same way as crab muscle fibers (Caldwell & Walster, 1963). The fluids used as external bathing medium in experiments involving radiosodium, E_m and pH_i measurements had the following composition (mM): NaCl 465, KCl 10, $CaCl_2$ 10, $MgCl_2$ 10, and $NaHCO_3$ 10. Other buffers used in place of HCO_3^- included HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pK 7.55), MES (2-(N-morpholino) ethanesulfonic acid, pK 6.15), citrate, disodium monohydrogen phosphate, Na·H phthalate and TRIS (Tris (hydroxymethyl) aminomethane, pK 8.3). Adjustment of the pH of ASW was carried out by the addition of HCl or NaOH.

The Microinjector

The microinjector employed in most of the flux experiments was of the type devised by Hodgkin and Keynes (1956) as modified by Caldwell and Walster (1963). In some experiments, it was of the type described by Bittar and Tallitsch (1975).

Radiosodium Measurement

The method used for counting ^{22}Na in the effluent and fiber was essentially that described by Bittar (1966) and Bittar, Caldwell and Lowe (1967). The results were treated in two ways: 1) by plotting the efflux in cts/min/min against time on semilog paper, and 2) by plotting the fractional rate constant for ^{22}Na loss against time on linear paper. The fractional rate constant is given by the expression

$$k(\text{Time}^{-1}) = \frac{\text{Efflux}}{\text{Fiber count during collecting period}}.$$

Stimulation of the Na efflux was estimated on the basis of the difference between the rate constant immediately before the onset of stimulation and the maximum rate constant following the onset of stimulation.

Measurement of Internal pH

The overall internal pH of uncannulated fibers was estimated by the ^{14}C-DMO method. This was done by suspending fibers in HCO_3^--ASW or TRIS-ASW containing 2-^{14}C-DMO [5,5-dimethyloxazolidine(2,4-dione-2-^{14}C] (Radiochemical Center, Amersham) for 60–120 min. In the case where TRIS-ASW containing ^{14}C-DMO was used, the fibers were allowed to bath for 90–120 min. Each fiber was then weighed before transfer to scintillation vials containing *soluene-100* (Packard Instruments). Control vials included 1 ml soluene plus 100 µl ASW. Blanks included an untreated fiber, with 1 ml soluene and 100 µl ASW. All vials were left to stand overnight for digestion. Within 12 hr the fibers were found to have dissolved. This was followed by the addition of 10 ml dimilume (Packard Instruments) scintillation solution and shaking of the vials. Counting was carried out in the evening when the background count had already fallen. The samples however were left to stand for an additional 2 hr so as to reduce the error due to chemiluminescence.

The extrafiber fluid volume was determined by using ^{35}S-sulfate as indicator. ^{35}S-sulfate was obtained from the Radiochemical Center at Amersham. Briefly, fibers were bathed in 10 mM HCO_3^--ASW for $1^1/_2$–2 hr, then blotted gently with filter paper and weighed on a Cahn microbalance. Each fiber, as well as a 100-µl sample of the ASW containing ^{35}S-sulfate were analyzed for ^{35}S activity by adding 1 ml soluence-100 to each vial. The vials were then left overnight to stand at room temperature. Following digestion, 10 ml dimilume were added to each vial.

Counting of ^{35}S-sulfate and ^{14}C-DMO was carried out in a Beckman scintillation counter, model LS250. Each sample was counted thrice. Quenching of the samples was checked by using the external standard method. No discernible difference could be found in quenching between the vials containing the fibers and the ASW.

The total water content of barnacle fibers was estimated by blotting gently each fiber with filter paper and then weighing. The weight was recorded 1, 2 and 3 min after blotting. The values obtained were plotted on semilog paper against time and then the line drawn was extrapolated back to zero time. After drying overnight in an oven at 60 °C, the weight of each fiber was recorded and subtracted from the wet weight.

The internal pH of the fiber was calculated from the equation

$$\text{pH}_i = \text{pK}'_a + \log\left\{\left[\frac{C_t}{C_e}\left(1+\frac{V_e}{V_i}\right)-\frac{V_e}{V_i}\right][1+10^{(\text{pH}_e-\text{pK}'_a)}]-1\right\}$$

where pK'_a is the negative logarithm of the apparent ionization constant of DMO, C_t is

the intrafiber activity of DMO, C_e the extrafiber activity of DMO, V_e and V_i the extra- and intrafiber fluid volumes and pH_e the extrafiber pH (Waddell&Butler, 1959). The pK'_a of DMO at 25 °C and ionic strength of 0.5 was taken as 6.2.

Measurement of pCO_2 and E_m

The pCO_2 was measured in an open and closed CO_2 system by using a Radiometer pCO_2 electrode (type E5036) maintained at 24 °C and a Beckman physiological gas analyzer, model 160. The DMO experiments were carried out with an open system in which the vials used were covered with parafilm. This unavoidably led to some escape of CO_2 during manipulation of the vials in the course of the experiments.

To achieve a closed CO_2 system, the cannulated fiber was placed in a sealed chamber (Fig. 1), with the cut-end of the fiber sealed with petroleum jelly. The ASW inside the chamber was changed by allowing the solution to flow in from a reservoir under gravity. After passage through the chamber the solution flowed through a small chamber containing a pCO_2 electrode. E_m was measured by inserting an electrode longitudinally down the cannula a distance of 8.5–10 mm into the fiber. The reference electrode was inserted through a second hole in the top of the chamber. Silicon grease was applied as a seal to the inlet holes at the top of the chamber.

Two different designs of microelectrodes were used throughout the course of the experiments with the closed CO_2 system. One was supplied by Transidyne General Corporation (Ann Arbor, Michigan) and had a tip diameter of <1 μm and a shank which enlarged to >100 μm within about 3 mm of the tip. Electrodes of this design were also used for measuring E_m in an open CO_2 system. The other was hand-drawn and had an external diameter of about 20 μm for about 1 cm along the shank from the tip. The external tip diameter ranged between 10 and 20 μm, and the internal tip diameter between 3 and 8 μm. The electrodes were filled by boiling in 3 M KCl. In some experiments the tip and lower half of the reference electrode were filled by pressure with 3M KCl in 3% agar.

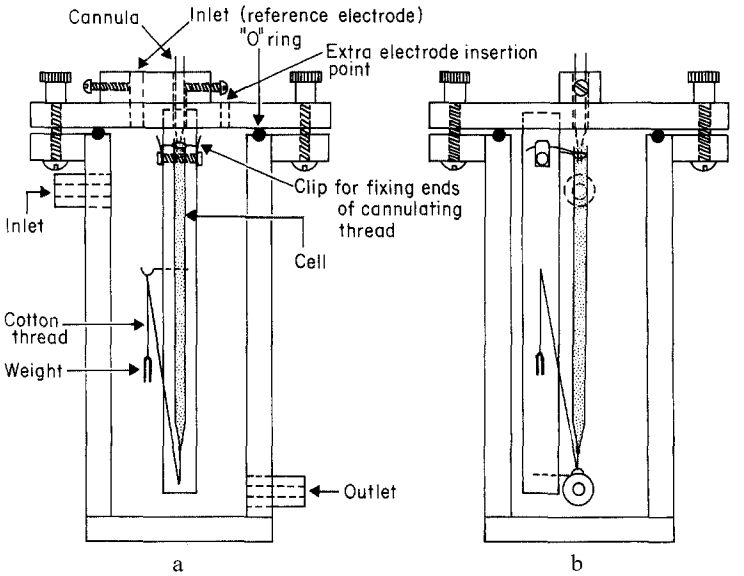

Fig. 1. Schematic diagram of the closed system for investigating the effects of CO_2 on E_m. (*a*) Front view; (*b*) side view

The electrodes were connected by Ag/AgCl wires to the input of a Microprobe System-model M-701 (W.P. Instruments Inc., Hamden, Connecticut) for recording the membrane potential. This solid-state electrometer-amplifier is a high input impedance (20,000 MΩ) system with current injection circuitry which allows current pulses to be passed through the potential measuring electrode. Current pulses of 1 sec duration were supplied by a modified Electronic Stimulator-model 751-B (American Electronics Laboratorium, Inc). Continuous recordings of membrane potential, the applied current pulses and pCO_2 were made using three channels of a Watanabe chart recorder.

Electrode tip potentials were measured prior to intrafiber electrode insertion by placing the tip of the electrode in the external medium via the small additional hole in the top of the chamber containing the cannulated fiber. During the course of the experiments, this small hole was sealed with adhesive tape. Electrodes with tip potentials >5 mV or resistances >5 MΩ were rejected. Electrode resistances of $\leqq 5$ MΩ were balanced by means of the d-c balance controls on the Microprobe system. Before each experiment the pCO_2 electrode was calibrated by bubbling external medium (without buffer) in each of two reservoirs with one of two calibration gases and allowing the medium to flow through the chamber containing the pCO_2 electrode. The compositions of the calibration gases were as follows: 20% O_2; 78.5% N_2 and 1.5% CO_2; and 20% O_2, 72% N_2 and 8% CO_2. Equilibration of the one liter volume in each of the two reservoirs was achieved by bubbling each vigorously for 5 min with its respective calibration gas followed by a further 2 min of vigorous bubbling prior to the measurement of pCO_2. Slow bubbling with the calibration gas was continued during the course of the calibration. Calibration was carried out in the absence of a fiber in the chamber.

Unless stated otherwise, all experiments were done at a room temperature of 22–25 °C. The protein inhibitor (from rabbit muscle) was a gift from Dr. Donal Walsh of the Department of Biological Chemistry, University of California at Davis.

Results

The experiments described here fall into three sections: (*i*) measurement of Na efflux at various external pH's, (*ii*) determination of internal pH, and (*iii*) measurement of E_m and pCO_2 under a variety of conditions.

Na Efflux into HCO_3^--ASW as a Function of pH_e

Stimulation of the Na efflux by external acidification is completely dependent on the presence of HCO_3^- in the bathing medium. Replacement of HCO_3^- by TRIS, HEPES, citrate, phthalate or Na_2HPO_4 as buffer followed by acidification fails to lead to stimulation. The kinetics of the response indicate that it is rapid in onset and reaches a peak within 15–20 min (Bittar & Tong, 1971). Moreover, it is completely reversible and unaccompanied by contraction. Only when pH_e is reduced below 5 are the fibers found to become opaque in appearance. Summarized in Fig. 2 are the experiments in which the effect of external acidification

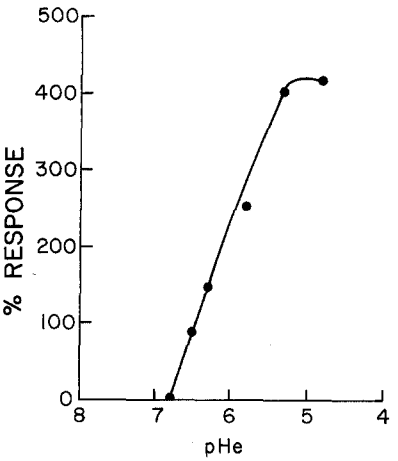

Fig. 2

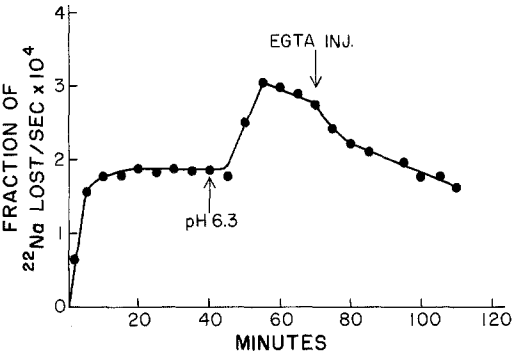

Fig. 3

Fig. 2. Na efflux as a function of external pH. Each point is the mean of data obtained from six fibers. The fibers used were isolated from different barnacle specimens

Fig. 3. Effect on the Na efflux of microinjecting 100 mM EGTA (pH 7.0) following reduction of pH_e from 7.8 to 6.3

on Na efflux was studied over a wide range of pH_e. The results indicate a curve which consists initially of a linear component whose intercept value is 6.8. This agrees with the experimental value itself and with the earlier observation that this is the threshold pH for stimulation of the Na efflux, whenever ASW containing 10 mM HCO_3^- is used at the start of the experiment.

The possibility that part of the stimulatory response is due to a fall in myoplasmic *p*Ca caused by the resulting internal acidity was examined by microinjecting 100 mM EGTA after reducing pH_e from 7.8 to 6.3. Fig. 3 shows that injected EGTA caused a fall in the stimulated Na efflux. The magnitude of the stimulation averaged $105.2 \pm 31.3\%$ (SEM) while the EGTA effect averaged $17.3 \pm 2.2\%$ ($n=4$) of the stimulated Na efflux. By comparison, control fibers bathed in ASW at pH 7.8 showed a fall in Na efflux following an injection of 100 mM EGTA in the order of $25.1 \pm 5.6\%$ ($n=4$). This result is in line with the concept that the CO_2- and Ca^{2+}-sensitive components of the Na efflux overlap to some extent (Bittar *et al.*, 1974) and that the rise in myoplasmic cAMP resulting from activation by external acidification of the adenyl cyclase system leads in part to a fall in myoplasmic *p*Ca (Bittar, Chambers & Schultz, 1976; Bittar & Schultz, *unpublished* experiments with aequorin). However, this explanation is inadequate because EGTA is also

an effective pH buffer and because the resulting internal acidosis influences the chelation process. Thus, one would expect the formation of $[EGTAH_2]^{2-}$, thereby leading to a rise in pH_i. This argument has force in view of recent evidence showing that injection of HCO_3^- into barnacle fibers causes partial reversal of the stimulatory response of the Na efflux to external acidification. At first sight it would seem to follow that a fall in pH_i caused by a raised pCO_2 leads to stimulation of the Na efflux, but this theory is not borne out by experiment, since injection of HCl fails to bring about an appreciable stimulation of the Na efflux (Bittar, Schultz & Pennington, 1977). The new view then to which these observations lead is that the internal acidosis is partly responsible for the maintenance of the stimulatory response (e.g. acidosis is known to stimulate respiration) and that pH_i in barnacle fibers might not return to normal as rapidly as it does in crab muscle or squid axon (Aickin & Thomas, 1975; Boron & DeWeer, 1976).

Since ATP is the substrate for the membrane adenyl cyclase system, it seemed worthwhile to check whether microinjection of ATP following external acidification modifies the stimulated Na efflux. The results showed that injection of 0.5 M $ATPNa_2$ at pH 7 raised the Na efflux into ASW at pH 6.3. Estimates of the magnitude of the ATP effect gave a value of $32.6 \pm 21.7\%$ ($n=5$). One plausible explanation of the smallness of the observed effect is that ATP not only acts as a substrate but also as a pH buffer (the pK for the reaction: $[ATP]^{4-} + H + \rightleftharpoons [ATPH]^{3-}$ being 6.9). Evidence that the overriding effect, that of stimulation, is related to the catalytic breakdown of $[ATPH]^{3-}$, and hence to cAMP formation, has been produced on the basis of experiments with the protein inhibitor of Walsh and of Corbin (Bittar & Schultz, 1977).

In the last group of experiments an attempt was made to see whether stimulation of the Na efflux by external acidification does or does not involve the membrane $(Na^+ + K^+)$-ATPase system. The first way in which to check this was to measure the Na efflux into K-free ASW before and after acidification. The results showed that a sudden reduction in pH_e from 7.8 to 6.3 and 5.8 caused a rise in the Na efflux in the order of $817.8 \pm 130.6\%$ ($n=6$) and $775.3 \pm 59.4\%$ ($n=4$), respectively. In further experiments the Na efflux into K-free ASW at pH 6.8 was measured. It was found that the stimulatory response was of the order of $22.8 \pm 6.3\%$ ($n=3$). Since the Na efflux into 10 mM K-ASW at pH 6.8 shows only a change in slope of the log efflux plot rather than a clear-cut step-up, the results with K-free ASW can be taken to mean that the pH threshold following uncoupling of the Na:K pump was somewhat

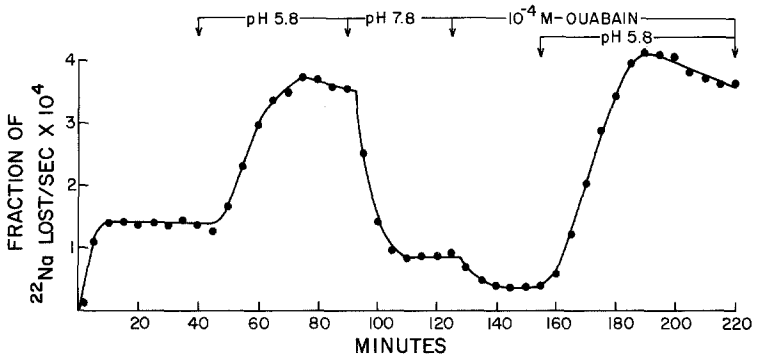

Fig. 4. The behavior of the Na efflux into ASW at pH 7.8, 5.8, and again, 7.8, and then into ASW at pH 7.8, and 5.8 in the presence of 10^{-4} M ouabain

above 6.8. That this is so is shown by the fact that reduction of the external pH from 7.8 to 6.8 following application of 5×10^{-5} M ouabain led to stimulation of the Na efflux ($n=2$). However, the simplest way of demonstrating increased sensitivity in ouabain-poisoned fibers was to have each fiber serve as its own control. Fig. 4 illustrates this. It can be seen that the Na efflux rose rather markedly following a reduction in external pH from 7.8 to 5.8 and that this was completely reversed by restoring pH_e to 7.8. It is also seen that a similar reduction in pH_e from 7.8 to 5.8 following external application of 10^{-4} M ouabain led to a much greater rise in the Na efflux (an average effect of $542.1 \pm 232.1\%$ *vs.* $174.0 \pm 16.9\%$, $n=3$). Taken together, these results are of special interest partly because they support the view that the Na efflux consists of a ouabain-insensitive component, which is sensitive to external acidification, and partly because they suggest that the magnitude of the response to external acidification is partly governed by the internal Na concentration.

Estimation of pH_i

The results of the experiments with ^{35}S-sulfate are given in Table 1. They show a mean value for the extrafiber fluid volume of 0.07 ml/g wet weight, with a range of 0.06–0.10 ml/g wet muscle based on 29 determinations. Also shown in this Table is the water content of 26 fibers, this being an average value of 0.77 g/g wet muscle. It will be noticed that the extrafiber fluid volume varied very little despite the fact that in this type of experiment the fibers used were isolated from different

Table 1. Determination of ^{35}S-sulfate space (ml) per 100 g wet tissue and the total water content

Extra-fiber fluid volume		Total H_2O (%)
Mean	7.12	76.6
Range	5.55–10.20	72–79
SD	±1.17	±1.36
n	28	26

bundles of several barnacle specimens. Hence, when calculating pH_i the mean value just mentioned was employed.

The experiments carried out with ^{14}C-DMO were of two kinds. In the first series, fibers were soaked in ASW containing bicarbonate as buffer, and ^{14}C-DMO for 90–120 min. Fig. 5*a* and *b* presents the pCO_2 values obtained for ASW at various pH's and at almost zero time, 60 and 120 min following external acidification. The values found at almost zero time after acidification agree with those obtained from the equation

$$pCO_2 = \frac{C + (H^+) - K_w/(H^+)}{K_1' q/(H^+) + 2K_1' K_2' q/(H^+)^2}$$

where $K_1 = 10^{-6}$, $K_2 = 10^{-10}$, $q = 0.0373/760 = 10^{-4.3}$ and $pK_w = 14.167$ (*see* Edsall & Wyman, 1958). It will, however, be appreciated that the measurement of pCO_2 in the range of 5–15 mm Hg was somewhat unreliable mainly because a reference gas mixture with a low CO_2 tension was unavailable.

In the second series of experiments, fibers were soaked in 25 mM TRIS-ASW containing ^{14}C-DMO for 90–120 min. The fibers were isolated from bundles bathed in 10 mM HCO_3-ASW at pH 7.8 and not TRIS-ASW because TRIS pre-exposed fibers were found to be hypersensitive to mechanical stimulation. Table 2 summarizes the results obtained in both series of experiments. Using 10 mM HCO_3^- as buffer, pH_i was found to be 7.09 ($n=62$) at pH_e 7.8, whereas with 25 mM TRIS pH_e was 7.28 ($n=46$). The relationship between pH_i and pH_e is given in Fig. 6. The difference ($p<0.01$) is accounted for by supposing that TRIS penetrates the fiber membrane and hence is able to buffer the internal environment. Whether this is really so or not cannot yet be decided partly because the available evidence is contradictory (Robin, Wilson & Bromberg, 1961; Aickin & Thomas, 1975). As for the results with HCO_3^--ASW, they show a negligible fall in pH_i when pH_e is 7.4. Only

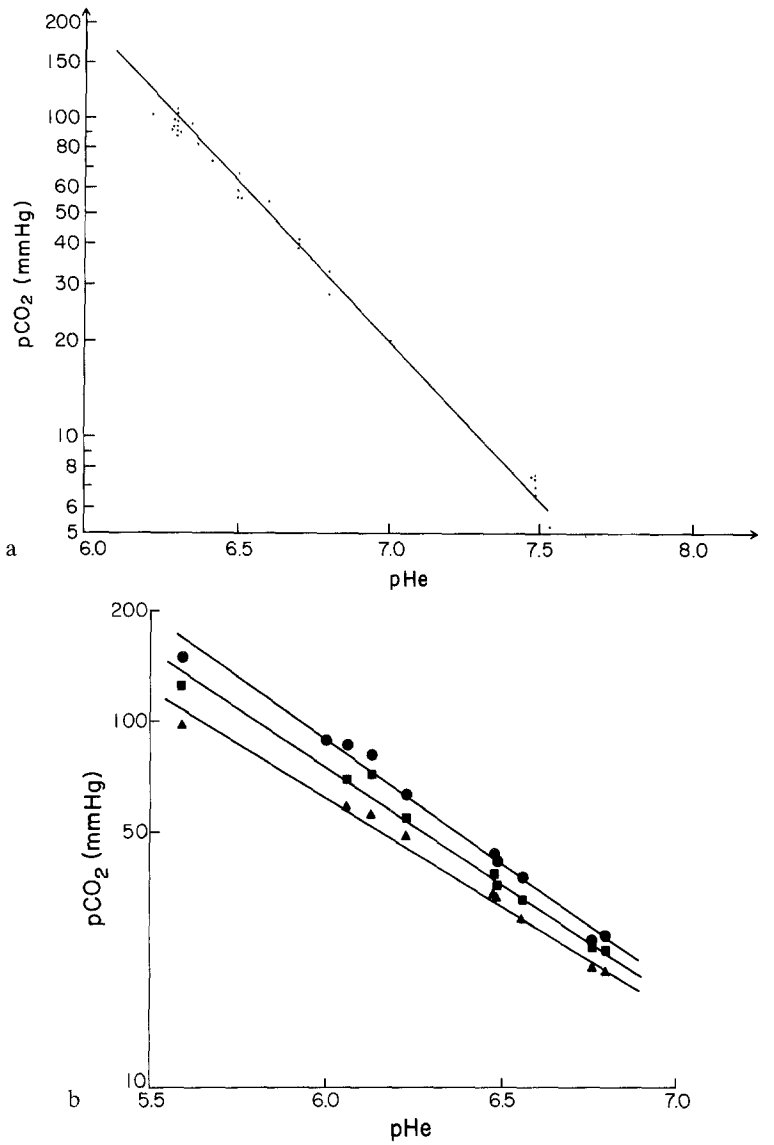

Fig. 5. (*a*) The relation between pCO_2 and pH of ASW at 24 °C. Measurement of pCO_2 was carried out immediately following the adjustment of pH. The straight line was fitted by eye. (*b*) The relation between pCO_2 and pH of ASW (at 24 °C) almost at 0, 60, and 120 min following external acidification. The lines were drawn by the method of least squares. ■ 0 min; ▲ 60 min; ● 120 min

when pH_e is less than 7 is the buffering capacity of these fibers appreciably compromised. An indication of their buffer capacity, e.g. to CO_2, is provided by an estimate of Δ log pCO_2/ΔpH. The values obtained are 3–4, which is thrice that reported for rat brain (Siesjö & Messeter, 1971).

Table 2. Internal pH of barnacle fibers bathed in HCO_3^-- and TRIS-containing ASW at 24°C[a]

HCO_3^--ASW						
pH_e	7.8	7.4	7.0	6.6	6.2	5.8
Mean pH_i	7.09	7.07	6.84	6.80	6.63	6.46
Range	6.85–7.32	6.93–7.34	6.62–6.98	6.63–6.89	6.29–6.77	6.41–6.54
SD	±0.106	±0.097	±0.100	±0.073	±0.117	±0.040
n	62	17	40	20	36	12
TRIS-ASW						
	7.8	7.4	7.0	6.6	6.2	5.8
	7.28	7.37	7.17	7.25	6.97	6.98
	7.05–7.47	7.21–7.59	6.88–7.46	7.09–7.39	6.46–7.25	6.65–7.11
	±0.130	±0.112	±0.131	±0.117	±0.206	±0.158
	46	12	40	12	38	11

The concentration of HCO_3^- in ASW at pH 7.8 was 10 mM; the concentration of TRIS in ASW was 25 mM.

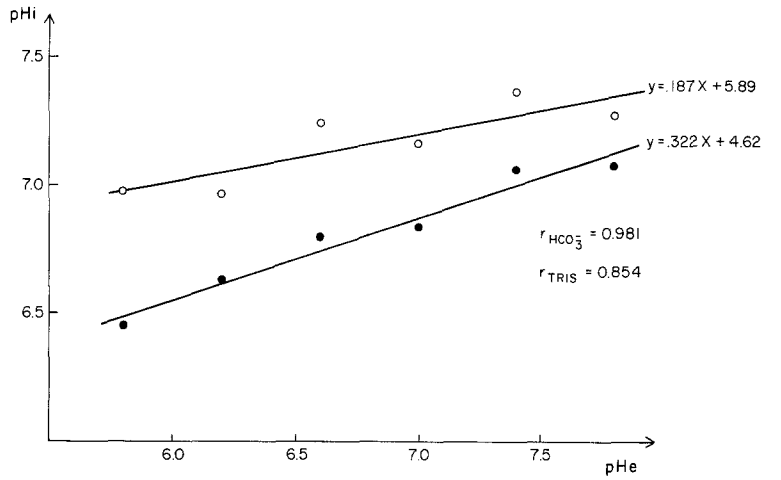

Fig. 6. The relation between pH_e and pH_i. The lines were drawn by the method of least squares. ○ Fibers bathed in TRIS-ASW; ● Fibers bathed in HCO_3^--ASW

The experiments with ^{14}C-DMO raised the question whether this indicator is bound by muscle protein or trapped in an alkaline compartment inside the fiber. Hence it seemed important to microinject DMO into cannulated fibers and check by washout experiments whether the indicator can leave the fiber readily or not. In six washout experiments,

it was found that the efflux curve was a composite of three exponential phases, and that only approximately 0.3% of the fiber activity was left after $2^1/_2$ hr. This ruled out the possibility of irreversible binding of injected DMO. ^{14}C-DMO was therefore injected into cannulated fibers bathed in 10 mM HCO_3^--ASW at pH 7.8, and after $2^1/_2$ hr the fibers were digested and counted. The activity of DMO in the ASW was also measured. Taking the extrafiber fluid volume as 6%, an average value for pH_i of 7.25 ± 0.1 (SD) ($n=10$) was obtained. This figure may represent a slight over-estimate on the alkaline side as the result of some damage to an alkaline compartment, e.g. the actomyosin compartment, caused by the procedure of microinjection. Nonetheless, it compares fairly well with that obtained by means of the soaking method using *uncannulated* fibers and with the results reported by Graves and Moore (1976) where the pH of sartorius muscle of the frog was estimated on the basis of the washout kinetics of DMO.

Depolarization by External Acidification

The effect of external acidification on the resting membrane potential was also examined. Reduction in the pH of 10 mM HCO_3-ASW from 7.8 to 6.3 caused a gradual fall in the E_m of the cannulated fiber. The onset of depolarization was rapid. Restoration of the pH back to 7.8 resulted in repolarization. Both depolarization and repolarization processes were complete within 60 min ($n=6$). To gather information about the threshold pH for depolarization and to establish the relationship between pH_e and E_m over a wide pH range, measurements were next made of E_m at pH_e 7, 6.5, 6.0, 5.5 and 5.0. Inspection of the time course shows that as the external medium was rendered more acid, the longer it took for the E_m to stabilize. Fig. 7 shows that depolarization is a linear function of pH_e and that the threshold for depolarization is a pH_e value of 7.3. Estimates of $\Delta E_m/\Delta$pH indicated an average value of 8.2 ± 1.1 mV/pH unit (SEM). To check whether this effect of pH_e on E_m is HCO_3^--dependent, measurements were made using 3 mM TRIS ($n=4$) and 3 mM HEPES ($n=5$) as well as a medium containing no buffer ($n=3$). Depolarization took place upon lowering pH_e from 7.8 to 6.3 only when HCO_3^- was present (in this instance, 10 mM HCO_3^-, $n=6$). Since these experiments were performed in an open system, it is possible to translate the observed threshold pH (*see* Fig. 7) into a threshold pCO_2 using the results in Fig. 5*a* from which it can be seen

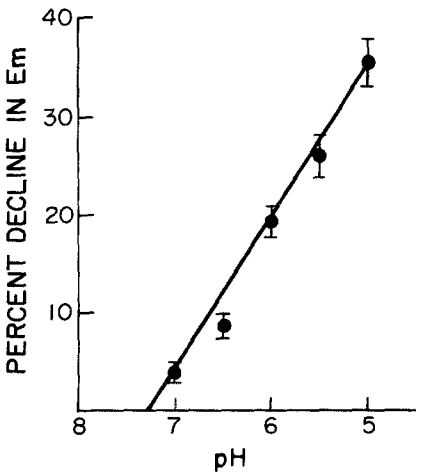

Fig. 7. Influence of external pH on E_m. Each point represents the mean of six measurements carried out 60 min after external acidification, as recorded in the preceding series of experiments. Vertical bars span ±SE

that pH_e 7.3 corresponds to a pCO_2 of approximately 10 mm Hg. The calculated external HCO_3^- concentration for pH_e 7.3 and $pCO_2 \sim 10$ mm Hg is approximately 7 mM.

To ascertain the threshold pCO_2 more accurately, it seemed essential to employ a closed measuring system, as described in Materials and Methods, in order to eliminate the problem of diffusion of CO_2 into the atmosphere during membrane depolarization. The first eight experiments carried out confirmed the observation that relatively large increment in pCO_2 resulted in depolarization but reversibility of the depolarization was not always complete when pH_e was restored to its original value. In a second group of 12 experiments, measurements were made of relative membrane resistance in the presence of an external pH of 6.3 and 5.8. The results showed no change in resistance. This indicated that changes in P_k were not involved in the mechanism of depolarization. In the third group of nine experiments, the effect on E_m of small changes in pCO_2 was tested. The experiments were designed to see if the theoretical threshold pH of depolarization was in fact 7.3 as found with an open CO_2 system. Fig. 8 shows that when pH_e was changed from 7.8 to 7.3 (i.e. when a pCO_2 of 7 mm Hg was achieved) E_m was reduced by approximately 0.3 mV. This result is to be compared with that shown in Fig. 9 where at pH_e 7.1 (and a pCO_2 of approximately 11 mm Hg) the fall in E_m was twice as great. As in the preceding experiment, restoration of pH_e to 7.8 resulted in reversal of the depolarization. The muscle

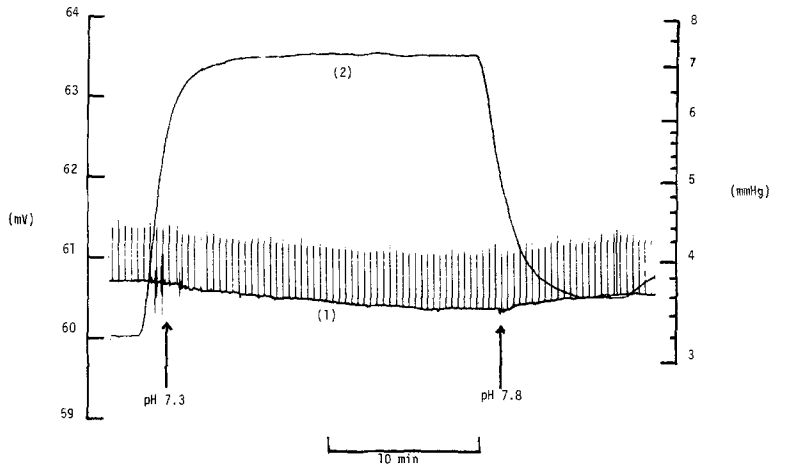

Fig. 8. Effects of reducing pH_e from 7.8 to 7.3 on E_m in a closed CO_2 system, and then restoring pH_e back to 7.8. Trace (1) is E_m (mV) and trace (2) the measured pCO_2 (mm Hg) in the solution flowing through the sealed chamber containing the fiber (*see* Fig. 1)

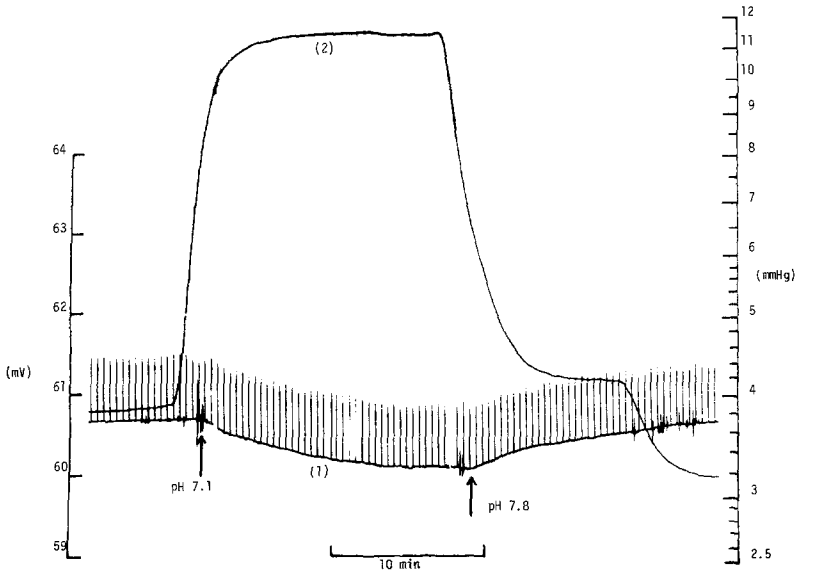

Fig. 9. Effects of reducing pH_e from 7.8 to 7.1 on E_m in a closed CO_2 system and then restoring pH_e back to 7.8

fiber membrane and the pCO_2 electrode respond to the change in pH_e at the same time, as nearly as could be observed. The apparent time lag shown in Figs. 8 and 9 is a result of the fact that the pens on the multirecorder (Watanabe) all use the full width of the chart paper and are thus offset along the time axis. It could be objected that the recorded E_m changes are of doubtful significance, since they are in the

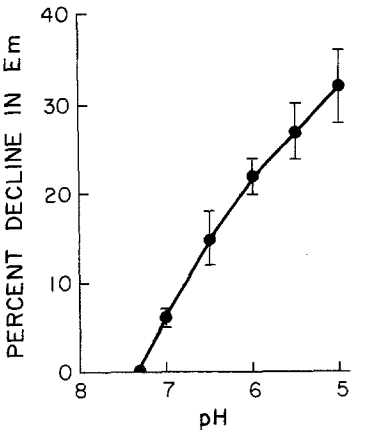

Fig. 10. Dependence of E_m on pH_e at 0 °C. Each point represents the mean of data obtained from six fibers. Vertical bars span ±SE

noise range or they could be due to an effect on the tip potential of the electrode. These objections are dismissed for two reasons: one is the experiments done at pH 7.4 have failed to show similar changes. The other is that a pH of 7.3 is a value which corresponds with the theoretical threshold value obtained on the basis of the results given in Fig. 7.

To investigate the possibility that depolarization is the consequence of phosphorylation of the membrane following activation of the cAMP-dependent protein kinase by cAMP (*see* Bittar *et al.*, 1976, for evidence that CO_2 activates the adenyl cyclase system), three experiments involving the microinjection of the protein inhibitor of Walsh before external acidification were done. The results showed no alteration in the pattern or magnitude of the depolarization caused by raising pCO_2.

The effect of external acidification on E_m can be interpreted as indicating activation of an outwardly directed HCO_3^-- or Cl^--pump. The possible existence of a HCO_3^--pump in muscle was first suggested by Mainwood (1966) who noted that CO_2 must generate a current in frog sartorius which counters E_K – a conclusion originally drawn by Boyle and Conway (1941). More recently, Woodbury (1971), working with frog muscle, produced evidence supporting Mainwood's theory. To distinguish between the possibility of an electrogenic HCO_3^-- or Cl^--pump, and a process involving outward diffusion of HCO_3^-, E_m measurements were carried out at 0 °C over a wide pH range. Cooling caused an immediate fall in E_m and subsequent acidification caused the membrane to depolarize further ($n=6$). The relationship between E_m and pH_e, as shown in Fig. 10,

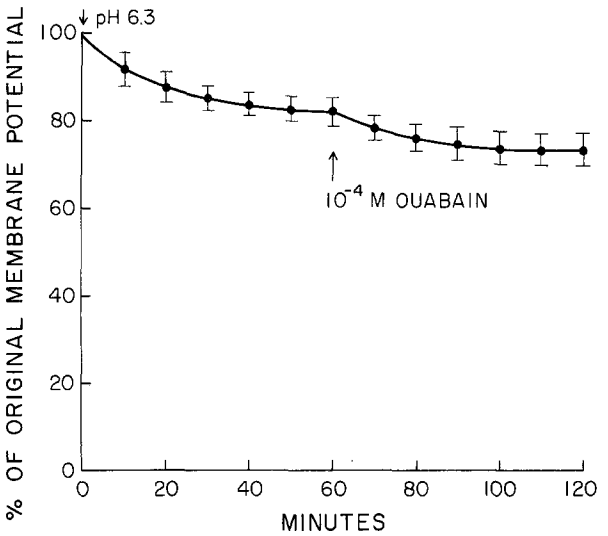

Fig. 11. Effects on E_m of reducing pH_e from 7.8 to 6.3, followed by 10^{-4} M ouabain at pH 6.3. Each point represents the mean of data obtained from six fibers. Vertical bars span ± SE

is not exactly linear. There is a transition phase in the pH_e region of 6.0–6.5 but the measured threshold pH is 7.3, as found at 24 °C. Significantly, the fall in E_m per pH unit turned out to be 7.1 ± 1.1 mV, which is not very different from the sensitivity observed at room temperature.

The effect of ouabain on E_m before and after external acidification was studied to see whether the Na-pump includes an electrogenic component and whether the CO_2-sensitive component of the E_m is in any way linked to a ouabain-sensitive component. Ouabain (10^{-4} M) was found to cause a prompt but gradual depolarization over a period of 40 min, the magnitude of which averaged 6.3 ± 1.5% ($n=6$) at pH 7.8 and 6.3 ± 0.8% ($n=6$) at pH 6.3. The time-course of the experiments done at pH 6.3 is shown in Fig. 11.

Ethacrynic acid, a known inhibitor of the CO_2-sensitive Na efflux in barnacle fibers (Danielson, Bittar, Chen & Tong, 1972) was applied internally and externally following external acidification. Since external treatment with a 10^{-3} M solution usually leads to contraction of the fiber, the experiments done included a series involving a Ca-free medium. Fig. 12*a, b* and *c* show that ethacrynic acid typically caused a fall in E_m but only when the agent was applied externally. This implies that the site of action of ethacrynic acid is the external side of the fiber membrane. Depolarization following external treatment is also known to occur in *Maia* fibers (Bittar, 1966) and the cortical ascending limb of

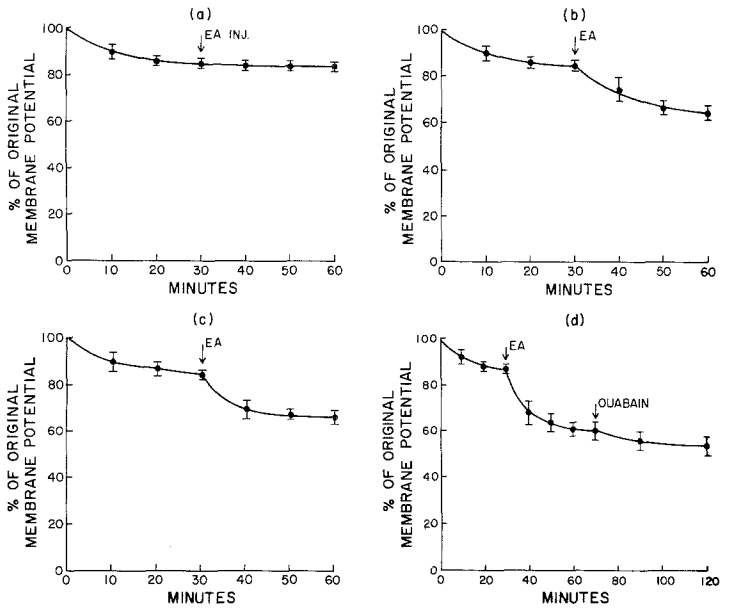

Fig. 12. (*a*) Effect on E_m of reducing pH_e from 7.8 to 6.3, followed by a lack of effect of microinjection of 8×10^{-2} M ethacrynic acid (pH 7.0). Each plotted point represents the mean of data obtained from four fibers. Vertical bars span ± SE. (*b*) Effect on E_m of reducing pH_e from 7.8 to 6.3, followed by effect of external application of 10^{-3} M ethacrynic acid in 10 mM Ca^{2+}-ASW at pH 6.3. Each point represents the mean of data obtained from four fibers. Vertical bars span ± SE. (*c*) Effect on E_m of reducing pH_e from 7.8 to 6.3, followed by effect of external application of 10^{-3} M ethacrynic acid in Ca^{2+}-free ASW at pH 6.3. Each point represents the mean of data obtained from four fibers. Vertical bars span ± SE. (*d*) Effect on E_m of reducing the pH of Ca-free ASW from 7.8 to 6.3, followed by effect of external application of 10^{-3} M ethacrynic acid and then 10^{-4} M ouabain. Each point represents the mean of data obtained from four fibers. Vertical bars span ± SE

Henle's loop from rabbit kidney (Burg & Green, 1973). In view of these results the possibility was explored that ethacrynic acid and ouabain might act on the same site, viz. the site mediating an uncoupled Na efflux. Measurements were first made with fibers suspended in Ca-free ASW at pH 7.8. These showed that (*i*) addition of 10^{-3} M ethacrynic acid, followed by 10^{-4} M ouabain caused in each instance a fall in E_m (EA: 27.06% and ouabain 6.55%, $n=2$), and (*ii*) addition of 10^{-4} M ouabain, followed by 10^{-3} M ethacrynic acid in Ca-free ASW at pH 6.3 caused a fall in E_m of 6.01% and 31.26%, respectively ($n=2$). Acidification itself caused a 14.26% fall. Summarized in Fig. 12*d* are the results of the E_m measurements made using Ca-free ASW at pH 6.3, to which 10^{-3} M ethacrynic acid, followed by 10^{-4} M ouabain, had been added.

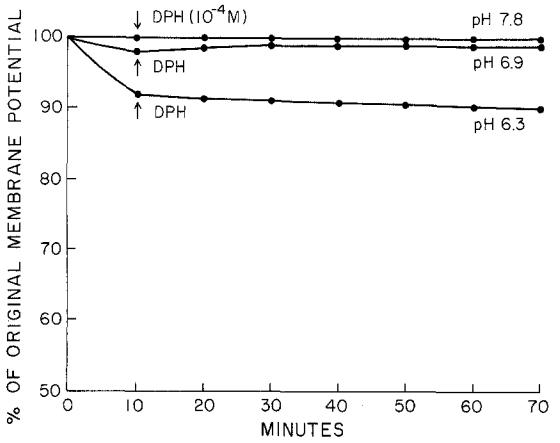

Fig. 13. Effect on E_m of reducing pH_e from 7.8 to 6.9 and 6.3, followed by effect of external application of 10^{-4} M DPH at pH 6.9 and 6.3. Also shown is the lack of effect of DPH at pH 7.8 on E_m. In this type of experiment the three fibers used were isolated from the same muscle bundle

From such results the conclusion must be drawn that ethacrynic acid and ouabain act on two different sites.

Another agent known to inhibit the CO_2-sensitive Na efflux is diphenylhydantoin (DPH) (Bittar, Chen, Danielson & Tong, 1973). Experiments carried out by applying externally 10^{-4} M DPH failed to show any effect on E_m when pH_e was 7.8 ($n=5$). However, the same was not true when DPH was applied following depolarization by CO_2 at pH 6.9 ($n=3$) or 6.3 ($n=3$). These results are illustrated by Fig. 13 where it can be seen that at pH_e 6.9, DPH promptly caused partial reversal of the depolarization, while at pH_e 6.3 DPH stopped the depolarization process. The ability of DPH to stop depolarization was more closely examined by methods using fibers from the same muscle bundle and also acidifying the medium to the same extent. Clearly, though DPH does not completely prevent depolarization from occurring, it stops the depolarization process. One possible explanation for the effect of DPH is the ability of the drug to stimulate Ca^{2+} efflux in barnacle fibers as the result of mobilizing internal 'bound' Ca^{2+}. (S. Chen, *unpublished data*) or its ability to increase g_K by raising the internal free Ca^{2+} concentration. In either case a fall in myoplasmic pCa in barnacle fibers is known to lead to hyperpolarization of the membrane (Bittar *et al.*, 1974). To gain more insight into the mode of action of DPH, fibers were cooled down to 0 °C and then exposed to DPH at pH 7.8 and 5.8. Fig. 14*a* summarizes the six experiments done with fibers bathed

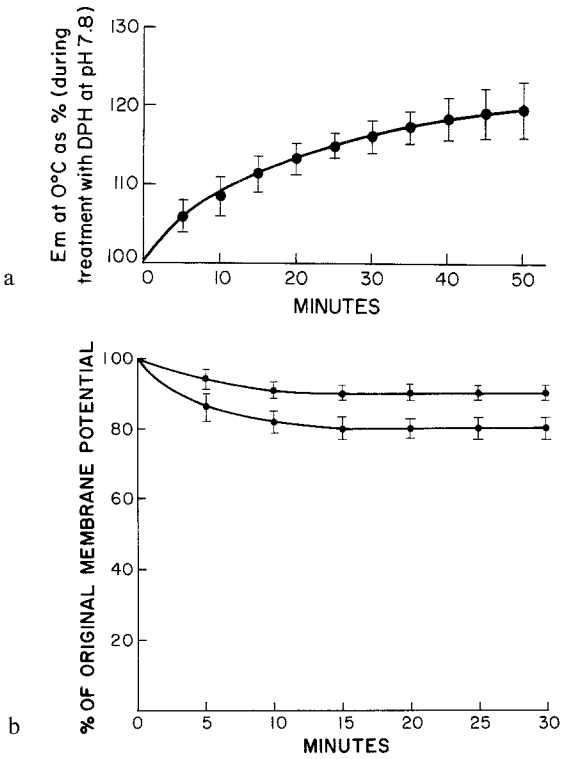

Fig. 14. (*a*) Time-course of hyperpolarization of the fiber membrane at 0 °C by external DPH (10^{-4} M) at pH 7.8. Each point represents the mean of data obtained from six fibers. Vertical bars span ± SE. (*b*) Time-course of depolarization recorded in fibers bathed in ASW at 0 °C and pH 5.8 (lower curve) and fibers bathed in ASW at 0 °C and pH 5.8 containing 10^{-4} M DPH (upper curve). Each plotted point represents the mean of data obtained from six fibers. Vertical bars span ± SE

in ASW at 0 °C and pH 7.8. These results show that 10^{-4} M DPH at pH 7.8 caused a slow, gradual rise in E_m. Similar striking results were obtained with DPH following its application in ASW at pH 5.8. These are summarized in Fig. 14*b* where the upper curve described the response of fibers to 10^{-4} M DPH at pH 5.8, and the lower curve describes the behavior of control fibers at pH_e 5.8. Though the time-course for stabilization of the E_m is exactly the same in both situations, the magnitude of depolarization in the presence of DPH is half that in its absence. Thus, the only possible conclusion to be drawn is that DPH repolarizes the fiber membrane at 0 °C by means of a mechanism which is probably similar to that observed in unchilled fibers. Whether the action of DPH involves abolition of the inward Na current is not yet known, but DPH is without effect on Na efflux at 0 °C (E.E. Bittar and D. Brown, *unpublished data*).

Discussion

The work as a whole reported in this paper shows that the barnacle muscle fiber is a useful preparation for studying the problem of how a raised pCO_2 causes stimulation of the ouabain-insensitive Na efflux, as well as depolarization of the fiber membrane. The observation that whenever the bathing medium pH is reduced below 6.8, Na efflux rises and that unless HCO_3^- is present beforehand, such an effect fails to occur, is of considerable physiological interest. For example, little is yet known about the way in which external pH stimulates the ouabain-insensitive Na efflux in the renal cortex (Proverbio, Condrescu-Guidi, Perez-Gonzalez & Whittembury, 1976). In an open CO_2 system, stimulation of the Na efflux by raising pCO_2 is a completely reversible process. The mechanism by which a raised pCO_2 brings about stimulation of the Na efflux appears to involve activation by CO_2 of the membrane adenyl cyclase system, since not only is the effect abolished by microinjecting ethacrynic acid (Bittar *et al.*, 1976) but also by microinjecting the protein inhibitor of Walsh shortly before external acidification (Bittar, Schultz & Pennington, 1977). A notable feature of CO_2 action is that activation of a plasmalemma-sensitive site would precede any action secondary to a fall in pH_i caused by the raised pCO_2. Present information concerning the response of pH_i in skeletal muscle to an increased CO_2 tension reveals that within 15 min pH_i begins to slowly return to normal (Aickin & Thomas, 1975). If this were the case in barnacle fibers, one would then expect a transitory slowing of the adenyl cyclase system, the pH optimum for the system being on the alkaline side. (Sutherland, Rall & Menon, 1962). Thus, the only conclusion possible would seem to be that a reduced pH_i is not likely to be the cause of stimulation of the ouabain-insensitive Na efflux, but as explained earlier (p. 229) internal acidosis appears to be a factor maintaining the stimulatory response.

The experiments involving E_m measurements led to unambiguous evidence that depolarization of the fiber membrane following external acidification is due to CO_2 and that the process of depolarization in an open system is reversible. A striking finding is that the threshold pH for depolarization both at room temperature and 0 °C is 7.3, a value quite close to that required for stimulating the Na efflux in fibers pretreated with ouabain. More important perhaps is the further finding that CO_2 causes as much depolarization of the membrane at 0 °C as at room temperature. For if cooling abolishes electrogenic mechanisms, it then follows that the depolarization observed following external acidifi-

cation could not be due to activation of a Cl^--pump. However, there is one valid reason why this line of argument is fallacious. This is that evidence is now available that an energy-dependent mechanism is indeed activated at 0 °C since (*i*) the Na efflux at 0 °C is stimulated by microinjecting cAMP and that this mechanism is readily abolished by prior microinjection of the inhibitor of Walsh (Bittar *et al.*, 1976), and (*ii*) that the Na efflux at 0 °C is stimulated by external acidification of a HCO_3^--containing medium and that this response can be abolished by prior microinjection of the inhibitor of Walsh (E.E. Bittar & G. Chambers, *unpublished*). Thus, the question which remains is whether the two effects caused by CO_2, namely, stimulation of the Na efflux and depolarization of the membrane, can be accounted for in terms of a theory that assumes the existence in the fiber membrane of an adenyl cyclase system which is activated by CO_2, and an outwardly directed electrogenic Cl^--pump, the activity of which is closely related to that of the adenyl cyclase system. Support of the latter idea comes from the work of DiPolo (1972) and Russell and Brodwick (1976) showing that the mechanism of Cl^- extrusion in dialyzed barnacle fibers has a Q_{10} of 3–4. It should be remembered, however, that these workers as well as Hagiwara, Gruener, Hayashi, Sakata and Grinnell (1968) measured Cl^- movements in the absence of HCO_3^- as buffer. This point needs to be emphasized in view of the possibility that a second pump consisting of a carbonic anhydrase system, if activated by a fall in myoplasmic free Cl^- concentration, could lead to depolarization as the result of HCO_3^- efflux. At the moment, all that can be said is that barnacle fibers possess some carbonic anhydrase activity (H. Deutsch, *private communication*).

The results of the experiments carried out with DMO are in accord with the conclusion reached by Caldwell (1958) that the internal pH of skeletal muscle lies in the region of 7.0 and not 6.0. A similar value for pH_i in barnacle fibers has been obtained by Boron and Roos (1976) with both the DMO and microelectrode methods. Such values however are at variance with those reported by Carter (1972) who found pH_i to be 6.68 with the DMO method, 6.71 with the nicotine method and 6.09 with a pH-sensitive microelectrode. As pointed out by Caldwell (1956), a weak acid indicator, e.g. DMO, provides an overall *p*OH value that corresponds to the mean *p*OH activity of the cell. Failure by Carter to obtain a higher pH_i value with DMO is clearly difficult to understand. This is all the more true since the DMO microinjection method as used by us gave a pH_i value of 7.2.

The experiments done with ouabain, ethacrynic acid and DPH led to new information about the sensitivity of E_m to drugs that are known to reduce the Na efflux as the result of inhibition of membrane enzyme systems. First, it is clear that ouabain and ethacrynic acid can reduce E_m before or after external acidification and that they act at two different sites. This is an observation which strengthens the view that both agents have the ability to abolish the uncoupled ouabain-sensitive and insensitive Na effluxes. Second, it is clear that DPH has the ability to hyperpolarize the fiber membrane but only when the membrane is already depolarized. This is easy to understand if DPH slows down the Cl^-- and/or HCO_3^--pump. Hyperpolarization by DPH of other membranes is known to take place, e.g. in frog skin (Watson & Woodbury, 1972; Sousa & Grosso, 1973). Why DPH hyperpolarizes the fiber membrane at pH_e 7.8 following cooling to 0 °C is a result which is not easy to explain. One possibility is that cooling results in a rise in pH_i. Another is that DPH acts more readily at 0 °C by increasing the hydrophobic component of the membrane (Carnay & Grundfest, 1974). It remains to point out that the experiments with DPH may have some bearing on the problem of how the drug acts as an anticonvulsant, for it is quite conceivable that depolarized neurones lying in an epileptic focus of the brain may undergo repolarization when exposed to DPH.

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