Effects of Some Inhibitors on the Temperature-Dependent Component of Resting Potential in Lobster Axon

JOSEPH P. SENFT

From the Department of Biology, The State University of New York at Buffalo, Buffalo, New York 14214, and the Department of Physiology, School of Medicine, University of Maryland, Baltimore, Maryland 21201. Dr. Senft's present address is the Department of Physiology and Biochemistry, Rutgers-The State University, New Brunswick, New Jersey 08903

ABSTRACT The resting membrane potential of the lobster axon becomes 5–8 mv more negative when the temperature of the perfusion solution is increased 10°C. This potential change is about twice that predicted if the axon membrane potential followed that expected for a potassium ion electrode potential. When the inhibitors, 2,4-dinitrophenol, sodium cyanide, and sodium azide, were added separately to the perfusion medium the potential change was reduced to about 1.4 times that predicted for a potassium ion electrode potential. Assays of axons exposed to these inhibitors showed that ATP levels were reduced to about one-fourth that obtained for control axons. Ouabain added to the perfusion medium reduced the potential change to that expected for a potassium ion electrode potential. These results suggest that the resting potential changes with temperature as a result of the activity of an electrogenic ion pump.

INTRODUCTION

The resting membrane potential of the lobster giant axon becomes hyperpolarized by about 10 mv when the temperature of the perfusion medium is increased from 2° to 16°C (Dalton and Hendrix, 1962). In contrast, the squid giant axon shows almost no change in resting membrane potential over this same temperature range (Hodgkin and Katz, 1949; Guttman, 1966). The mathematical formulation developed to describe membrane potentials in squid axons assumes that the potential is developed by the electrochemical gradients of ions across the membrane when appropriate adjustments are made for the relative ion permeabilities of the membrane (Goldman, 1943; Hodgkin and Huxley, 1952). For these equations changes in temperature should produce a change in potential proportional to the absolute temperature change. For squid axons Hodgkin and Katz (1949) suggested that the

lack of change in potential with temperature change could be accounted for by assuming that the relative ion permeabilities of the membrane are altered. This assumption which is included in the Goldman, and Hodgkin and Katz equations has proven to be satisfactory to explain the temperature effect on resting potential in squid axons. Since much of the present theory about transmembrane potentials in axons rests on squid axon data (Hodgkin and Huxley, 1952), it is of interest and importance to determine whether differences found in other excitable tissues can be explained in terms of the theory developed from squid axon data.

There are at least two possible explanations for the presence of the temperature-dependent component of the resting potential in lobster axon: (*a*) the relative ion permeabilities of the membrane are directly altered by temperature as suggested by Hodgkin and Katz (1949) and (*b*) some metabolic process contributes a hyperpolarization to the resting potential as the temperature of the perfusion medium is increased; i.e., an electrogenic ion pump is present. The second hypothesis was tested by observing the effects of some inhibitors upon membrane electrical properties and ATP levels in the lobster giant axon. The results strongly suggest (*a*) that an electrogenic ion pump is indeed present and (*b*) that the first hypotheis is not tenable.

MATERIALS AND METHODS

Lobsters 1¼—1½ lb. lobsters (*Homarus americanus*) were obtained from a local fish market where they arrived daily from Nova Scotia packed in seaweed and ice. Transport time from Nova Scotia was usually 3 or 4 days. Upon arrival in the laboratory the lobsters were placed in aquaria containing aerated artificial seawater prepared from artificial seasalt. The aquaria were kept in a cooler at 4°–5°C, a temperature which is within the range of temperatures at which lobsters are found under natural conditions (Herrick, 1909). Lobsters were usually not kept longer than a week before being used; however, they have been kept for more than 3 wk under these conditions. No differences in experimental results were observed among lobsters used immediately and those used at later times.

Nerve Preparation The nerve preparation was made as described by Dalton (1958). Two connectives were obtained from one lobster. One connective was used immediately; the second one, still sheathed, was placed in a Petri dish of control perfusion solution and was kept in a refrigerator at 4°–5°C until the experiment with the first connective was completed (usually 4–8 hr). In the present experiments dissected connectives have been kept at 4°–5°C for 24 hr without any apparant loss of the normal electrical properties which were measured or significant changes in ATP levels.

Nerve Chamber and Stimulation and Recording Apparatus The nerve chamber and the stimulation and recording apparatus have been described by Dalton (1958) and Dalton and Hendrix (1962). Microelectrodes were used in recording membrane resting and action potentials.

Potentials were recorded with the microtip in contact with the perfusion solution to determine whether the tip potential changed with temperature. No change in this junction potential was observed over the temperature range used in these experiments. This result confirms similar observations made previously by Dalton and Hendrix (1962).

Perfusion Solutions The perfusion solution (Dalton, 1958) used as a standard for these experiments contained the following millimolar concentrations of ions per liter:

Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	$SO_4^=$
465	10	25	8	533	4

The pH of the solutions was maintained at 7.4–7.8 by adding 1 ml of 0.5 Tris (tris (hydroxymethyl) aminomethane) buffer per liter of perfusion solution. The concentration of inhibitors employed was 0.2 mM 2,4-dinitrophenol, 2 and 4 mM NaCN, 3 and 10 mM NaN_3, and 10^{-5} M ouabain. No differences in results were observed between 2 and 4 mM cyanide. However, 3 mM azide was not effective; 10 mM was used in the experiments reported here. The perfusion rate was 3–5 cc/min. The temperature of the circulating solution in the nerve chamber could be controlled to give any temperature level between 4.5° and 20°C, and was monitored continuously by a thermistor-thermometer bridge (Cole, 1957).

Procedure for Recording Membrane Potentials The desheathed connective was placed in the chamber with the perfusion temperature at 4.5°–6.5°C. Resting and action potentials were recorded at low temperature, between 4.5° and 6°C, and at high temperature, between 12° and 20°C. The nerve was then perfused with solution which contained the inhibitor and potentials were again recorded at both high and low temperatures. Perfusion with control solution was restored and potentials were recorded until recovery (2 or 3 hr) was complete, or until they reached a constant level. The axons were then dissected from the nerve and were prepared for chemical analysis. The same procedure was followed for the second connective with the exception that the experiment was terminated while the connective was being perfused with the solution which contained inhibitor. Changes in resting potential were estimated to the nearest 0.1 mv.

In some nerve bundles resting and action potentials were recorded from the four largest axons. No significant differences in electrical responses were observed among these axons. However, a complete set of membrane potential measurements was not made for each axon in every nerve bundle; therefore, such measurements were not made for every axon used in the biochemical analyses.

Dalton and Hendrix (1962) have shown that the resting potential increases linearly with temperature from 0° to 20°C. This finding was confirmed by the present study. It was not necessary, therefore, to measure membrane potentials at exactly the same temperature for every axon. The ratio of the observed potential change to that expected from the change in absolute temperature was obtained by dividing the observed potential change by the potential change expected for the change in absolute temperature.

Dissection and Extraction of Axons Four giant axons lie on the surface of each connective. The largest axon (diameter 80–110 μ) is separated from the other three axons (diameters 50–90 μ) by smaller fibers and connective tissue. Nerves were kept in control perfusion solution or perfusion solution which contained inhibitor (this was dependent upon the final solution used in the nerve chamber) during dissection of axons. Axons were dissected from the nerve bundle in a water bath which could be maintained at any given temperature (± 1°C) between 4° and 20°C. The diameters of the axons were measured with an ocular micrometer in a compound microscope by taking the average of 10 separate measurements at approximately equal intervals along the axon. The length of the axon was determined and its volume was calculated on the assumption that it was a right circular cylinder. The specific gravity of axons was obtained by observing whether they floated or sank in artificial seawater solutions to which sucrose had been added to increase the specific gravity. By this method the specific gravity was found to be between 1.03 and 1.05 for the six axons tested. A value of 1.04 was used for the present calculations. (This is somewhat less than the value of 1.08 obtained by Brinley (1965).) Measurements of volume and specific gravity were then used to determine the weight of the axon (calculated wet weight).

Perchloric acid extraction of axons was used (Abood and Goldman, 1956; Cheng, 1961). The axons were homogenized in 10 μl of 1.2 M perchloric acid in an ice bath. The extract was centrifuged in the cold, the supernatant was transferred to another tube, and 10 μl of 1.2 M KOH were added to it. The sample was mixed and after standing a few minutes it was centrifuged again. This supernatant was transferred to a preweighed glass vial and was placed in a freezer until analyzed. Samples frozen for several weeks gave results similar to those obtained from samples analyzed immediately after extraction. The precipitate from the initial perchloric acid extraction was used for protein determinations.

The dissection, determination of diameters, and extraction of axons usually required 1–1½ hr. No variability in results could be correlated with the amount of handling during dissection, with the length of time required for dissection, or with the temperature at which the axons were dissected (4°–15°C).

Protein Determinations The method of Lowry et al. (1951) with slight modification was followed for the determination of protein.

Analysis of Axon Extracts for Compounds Which Contained Phosphorus The axon extract was adjusted to about pH 7.0 with 0.05 M KOH and the volume of the extract was made up to 1 ml with distilled, demineralized water. One-fourth of the extract was used for inorganic plus labile phosphate determination, one-fourth was used for total acid-soluble phosphate determination, and the remaining one-half ml was used for ATP determination.

The method for preparation of the firefly luciferin-luciferase system was that described by McElroy and Coulombre (1952). The method used to determine ATP concentrations in the extracts was similar to that described by Cheng (1961). The reaction mixture for determination of ATP consisted of 0.8 ml of 0.025 M glycyl glycine buffer at pH 7.7 which contained 0.01 M $MgSO_4$, 0.1 ml. firefly extract, and

0.1 ml axon extract. The buffer solution and the firefly extract were placed in a test tube which was contained in a light-tight box into which a phototube projected. The axon extract was squirted into the tube with a ¼ cc syringe to initiate the light-producing reaction. The light flash from this reaction was detected by a photomultiplier-photometer and the signal from the photometer was fed to a chart recorder. Usually two determinations were made for each sample and in most cases the two responses could be superimposed. The light flash was a linear function of the ATP concentration within this range and as little as 10^{-12} moles of ATP could be detected with this system. Standard ATP solutions were prepared from the disodium salt of ATP.

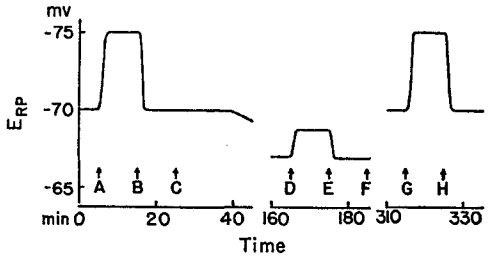

FIGURE 1. A resting potential record which shows changes for a 6°C temperature change in control perfusion solution and in perfusion solution which contained inhibitor. At *A*, *D*, and *G* the temperature was increased from 6° to 12°C and at *B*, *E*, and *H* the temperature was decreased from 12° to 6°C. At *C* 0.2 mM 2,4-dinitrophenol perfusion solution was introduced and at *F* control perfusion was restored. The change in resting potential fell and rose gradually during the discontinuities in the curve.

The method used for the determination of phosphate was that described by Lowry et al. (1954) and further investigated by Chen et al. (1956) and by Larrabee et al. (1963). Inorganic plus labile phosphate, total acid-soluble phosphate, and total phosphate content of axons were determined.

No biochemical analyses were made for ouabain-inhibited axons.

RESULTS

Control Values for Membrane Potentials The control values for the resting membrane potential, the "overshoot," maximum rates of rise and fall, negative after-potential, and duration of the action potential over the temperature range used in these experiments were in complete agreement with the results previously reported by Dalton and Hendrix (1962). These authors point out that the changes in the action potential, with the exception of the negative after-potential, can be attributed to changes in the resting potential. In the present experiments the data obtained for action potentials suggest this to be the case for control as well as inhibited axons. Therefore, only the data for resting potential changes will be considered here. The changes in the negative after-potential observed in these experiments require further

investigation before any conclusions can be drawn about their significance; these data will not be presented here.

The Decrease in the Temperature-Dependent Component of Resting Potential upon Inhibition An example of the typical effect of inhibitors on the temperature-dependent component of resting potential is shown in Fig. 1. In the control perfusion medium the resting potential increased 5mv when the temperature

TABLE I

THE CHANGE IN THE TEMPERATURE-DEPENDENT COMPONENT OF RESTING POTENTIAL WHEN PERFUSED WITH 0.2 mM 2,4-DINITROPHENOL

Axon	$\Delta E_o/\Delta E_N$*	$\Delta E_s/\Delta E_N$*	$\Delta E_{cae}/\Delta E_N$*
1	2.2	1.2	—
2	2.6	1.5	—
3	2.2	1.6	—
4	1.7	1.4	—
5	1.8	1.3	—
6	2.5	1.9	—
7	2.1	1.4	—
8	2.1	1.2	—
9	2.5	0.7	—
10	2.0	1.5	2.5
11	2.5	1.2	—
12	2.2	1.6	—
13‡	2.5	1.3	2.1
14‡	2.0	1.1	—
15	—	—	2.9
16	2.0	1.7	—
17	2.6	1.7	1.9
18	2.5	1.3	—
Average	2.2±0.07 (SEM)	1.4±0.07 (SEM)	2.3±0.23 (SEM)

* ΔE_o, ΔE_s, and ΔE_{cae} are the resting potential change observed for a change in temperature in the control perfusion solution, the experimental perfusion, and in the control perfusion after the experimental perfusion. ΔE_N is the resting potential change expected from the change in absolute temperature.

‡ Resting potentials recorded from different axons in the same nerve bundle.

of the perfusion medium was increased from 6° to 12°C. This potential change occurred in 2–3 min, which was also the length of time required to change the temperature in the nerve chamber. In experiments in which ouabain was used as the inhibitor the temperature change could be made in about 30 sec and again the resting potential change was coincident with the temperature change. Upon introduction of perfusion medium which contained 0.2 mM 2,4-dinitrophenol (or any of the other three inhibitors) the resting potential remained constant for 10–15 min and then declined gradually for 30–60 min. When the temperature was increased from 6° to 12°C the change in

potential was now 2 mv. Upon return to the control perfusion medium the resting potential gradually increased and in this axon it returned to its initial value. Fig. 1. also shows that the potential changes for control and inhibition conditions were symmetrical for increases and decreases in temperature.

The data which show the effects of 0.2 mM 2,4-dinitrophenol (DNP), 2 mM cyanide (CN), 10 mM azide (Az), and 0.01 mM ouabain on the temperature-dependent component of resting potential are summarized in Tables I to III. The values presented are the ratios obtained by dividing the observed poten-

TABLE II

THE CHANGE IN THE TEMPERATURE-DEPENDENT COMPONENT OF RESTING POTENTIAL WHEN PERFUSED WITH AZIDE OR CYANIDE

Axon	$\Delta E_c/\Delta E_N$	$\Delta E_e/\Delta E_N$	$\Delta E_{cae}/\Delta E_N$
		10 mM azide	
1	2.9	1.5	2.3
2	2.4	1.5	—
3*	2.1	1.2	—
4*	—	1.6	—
Average	2.5±0.22 (SEM)	1.4±0.08 (SEM)	2.3
		2 mM cyanide	
1	2.8	1.7	3.1
2‡	1.9	1.3	—
3‡	—	1.2	—
4	2.0	1.2	1.4
5	2.0	1.4	—
Average	2.2±0.22 (SEM)	1.4±0.09 (SEM)	2.2±0.86 (SEM)

*, ‡ Resting potentials recorded from different axons in the same nerve bundle. In Tables II and III symbols have the meaning indicated in Table I.

tial change (ΔE_c, ΔE_e, ΔE_{cae}) by the potential change expected from the change in absolute temperature (ΔE_N). These data (column 2) show that the resting potential of axons exposed to control perfusion medium increased between 2.0 and 2.5 times the increase expected from the change in absolute temperature. The data for the axons inhibited with DNP, CN, and Az (column 3) show that the resting potential change for a corresponding change in temperature was decreased significantly from the control values ($p < 0.001$ for DNP) and that similar results were obtained for all three inhibitors. The resting potential change for DNP-inhibited axons, although decreased significantly from the control condition was still significantly greater than that expected for the absolute temperature change as judged by Student's t test ($p < 0.01$). Although a sufficient number of experiments was not done to

establish a similar conclusion by statistical analysis for Az and CN, a similar trend is apparent from the data for these inhibitors. In Table III it is seen that in the experiments in which ouabain was used as the inhibitor, the resting potential change was reduced to that expected for the absolute temperature change (p for ΔE_e vs. $\Delta E_N > 0.1$). The greater reduction observed when ouabain was used as the inhibitor may suggest that some energy for ion pumping is obtained from glycolysis which was probably not blocked by the other three inhibitors. When the control perfusion solution was returned, complete recovery was usually obtained following DNP, CN, and Az perfusions. No recovery, even after 4 hr, was obtained following inhibition by ouabain.

TABLE III

THE CHANGE IN THE TEMPERATURE-DEPENDENT COMPONENT OF RESTING POTENTIAL WHEN PERFUSED WITH 0.01 mM OUABAIN

Axon	$\Delta E_c/\Delta E_N$	$\Delta E_e/\Delta E_N$	$\Delta E_{cae}/\Delta E_N$
1	2.2	1.5	—
2	2.1	1.0	1.2
3	2.1	1.3	1.0
4	1.5	1.0	—
5*	2.1	—	—
6*	—	0.8	0.9
7‡	2.1	1.1	—
8‡	—	1.0	—
9‡	—	—	1.0
10	2.1	1.3	1.3
Average	2.0±0.09 (SEM)	1.1±0.10 (SEM)	1.1±0.08 (SEM)

*, ‡ Resting potentials recorded from different axons in the same nerve bundle.

The relationships between these data and the potential change expected from the absolute temperature change will be considered in the Discussion.

Biochemical Analyses of Axons Analysis of axons was usually carried out on groups of four axons obtained from the same nerve bundle. With the present techniques it was possible to measure the protein and ATP content of single axons, but it was necessary to pool several axons for determination of total acid-soluble phosphate content. The data from these analyses are summarized in Table IV. Protein content is expressed as grams of protein per 100 g of tissue (g%) based on the calculated wet weight of the tissue. The protein content of axons was 2.1 g%. Inhibitors had no significant effect on the protein content and no significant differences were observed among the results for the three inhibitors used. Protein content of axons was used as a measure of the amount of tissue that was being used for determinations of other tissue

constituents. In the present study this was done to determine whether a linear relationship existed for the calculated wet weight of the axon vs. the protein content of the axon. Such a relationship was found. Protein contents of axons were similar to those reported for squid axons (Deffner and Hafter, 1959; Koechlin, 1955).

The amounts of compounds containing phosphate are expressed in micromoles per gram of tissue determined on the basis of calculated wet weight. Data from control axons were obtained from unperfused and perfused axons,

TABLE IV

PROTEIN, ATP, ACID-SOLUBLE, AND TOTAL PHOSPHATE CONTENT OF AXONS

	Amount* ± SEM	No. of determinations	No. of axons
Protein			
Control	2.1±0.06	29	114
Inhibited	1.9±0.06	22	80
ATP			
Control	0.66±0.05	41	87
Inhibited	0.16±0.04	18	68
Acid-soluble phosphate			
Control	27.5±2.5	15	57
Inhibited	28.3±2.1	14	55
Total phosphate			
Control	30.4±1.7	11	29

* Protein is expressed as grams of protein per 100 g of tissue based on the calculated wet weight. ATP, acid-soluble phosphate, and total phosphate are expressed in micromoles/gram of tissue based on the calculated wet weight.

and perfused axons exposed to inhibitors and returned to normal perfusion. The variation in phosphate compound content in any one of these three preparations was great and no significant differences could be determined among them. Therefore, the average of these has been included under control axons. The data for inhibited axons include 12 determinations for DNP-inhibited axons, 4 determinations for cyanide-inhibited axons, and 2 determinations for azide-inhibited axons. No significant differences were found among the results for the three inhibitors.

The data show that ATP levels were decreased in inhibited axons by 76%. This result shows that the inhibitors did penetrate the axon membrane and block ATP synthesis. Because this decrease in ATP content occurs in conjunction with the decrease in resting potential change with temperature, this result

lends additional support to the contention that the potential change in control axons is associated with energy-producing processes in the axon.

The acid-soluble phosphate fraction, which was the total phosphate content of the supernatant obtained from the perchloric acid extraction, shows no significant difference between values obtained for control and inhibited axons. The total phosphate content of axons was slightly greater than the acid-soluble phosphate fraction. Total phosphate contents of axons were similar to those reported for squid axons (Caldwell, 1960; and Deffner, 1961). However, the acid-soluble fraction accounted for almost all the phosphate in the axon in the present experiments, whereas for the squid only 60% of the phosphate was found in the acid-soluble fraction. This difference may result from the different techniques used in the determination or because in the present experiments acid-soluble phosphate was not determined for the same axons in which total phosphate determinations were made. In any event the data do not indicate any striking differences between squid and lobster axons in phosphate compounds which were measured.

DISCUSSION

Hodgkin and Katz (1949) have shown that the resting potential of squid axons is little affected by changes in temperature from 0° to 20°C. In contrast Dalton and Hendrix (1962) have reported that the lobster axon resting potential is increased by 8 to 10 mv when the temperature is increased from 2° to 16°C. These results for the lobster axon have been confirmed in the present investigation. As Hodgkin and Katz (1949) point out in their discussion of temperature effects in squid axons, the resting potential should change in direct proportion to the absolute temperature change if the resting potential is an ion diffusion potential such as that described by the Nernst relation. Hodgkin and Katz, citing earlier arguments by Bernstein and Krough, explain the divergences observed in the squid axon data by assuming that the relative ion permeabilities of the membrane varied with temperature. Similar arguments have been proposed to explain such divergences in resting potentials of other excitable tissues (e.g. Apter and Koketsu, 1960).

In the present study resting potentials of axons perfused with solutions which contained the three metabolic inhibitors were changed by slightly greater amounts than predicted by the absolute temperature change, and for ouabain were changed by the predicted amount. Therefore, for these axons it is not necessary to suggest relative membrane ion permeability changes with changing temperature. In addition this result suggests that some mechanism other than a direct effect of temperature on ion permeabilities operates to produce the hyperpolarization observed in control axons. As was suggested initially, one possible explanation is that the metabolic processes of the cell give rise to this hyperpolarization.

The importance of metabolism for maintaining nerve function is apparent from experiments with squid axons. Hodgkin and Keynes (1955) found that sodium efflux and potassium influx were reduced to 10% or less of their resting values when 0.2 mM 2,4-dinitrophenol, 2 mM cyanide, or 3 mM azide was contained in the perfusion medium. Later work by Caldwell and coworkers (Caldwell, 1960; Caldwell et al., 1960 *a*, *b*) has shown that arginine phosphate and ATP are necessary for the active transport of sodium and potassium ions. Injection of these compounds into axons which were blocked by inhibitors restored the active ion fluxes. Measurements of active ion fluxes were not made in the present experiments; however, the decrease in ATP levels in axons when inhibitors were applied suggests that these fluxes were reduced in a manner similar to that observed in squid axons. The coincidence of a decrease in axonal ATP levels with the loss of the membrane hyperpolarization for increased temperature suggests that this hyperpolarization is related to metabolic processes. Further, if lobster and squid axons are similar in requirements for active ion transport it may be suggested that active ion transport processes in the lobster axon are related to the membrane hyperpolarization.

A simple assumption then would be that the hyperpolarization results from the redistribution of ions across the membrane as a result of the ion-pumping mechanism. However, resting potentials followed temperature changes very rapidly (Fig. 1). If Brinley's value of 14.5 pmoles/cm^2 per sec (Brinley, 1965) for active potassium ion uptake is used, the ionic gradient across the membrane cannot be changed in this short time to give the observed change in resting potential for a coincident temperature change even when potassium ion outflux is disregarded. This explanation is therefore not consistent with the present data.

An alternative explanation might be that an electrogenic ion pump is present; that is, an ion pump which selectively removes positive charge from the axon. Evidence for a pump of this sort in muscle fibers has been reported by Mullins and Awad (1965), and such a system is consistent with the results obtained for lobster axons. It should be pointed out that there are a number of differences between muscle fibers and lobster axons in the potential changes resulting from temperature changes; therefore the proposed electrogenic pumps may have quite different properties in the two experimental preparations. It will be necessary to study these systems further before conclusions can be drawn about the similarity of the specific mechanisms involved. In any event it seems clear that the hyperpolarization with increasing temperature is probably related to active ion transport, especially with reference to the ouabain experiments because ouabain is known to be a specific inhibitor of the membrane sodium–potassium ATPase (e.g. Skou, 1960; Baker, 1965).

As pointed out previously, the squid axon resting potential is not altered when the temperature is varied (Hodgkin and Katz, 1949). In addition,

exposure of squid axons to 0.2 mM 2,4-dinitrophenol has little effect on the resting potential (Hodgkin and Keynes, 1955). One possible explanation for the differences observed between these two preparations may be that the relative conditions of the two experimental preparations are not the same. The lobster axon resting potential more nearly approaches the potassium ion equilibrium potential (Brinley, 1965) than does the squid resting potential (Moore and Cole, 1960). Also, the ion permeability of the lobster axon is lower and the membrane resistance is higher than those of the squid axon, and the squid axon shows a constant gain in sodium ion content during an experiment while the lobster axon maintains an almost constant sodium ion level (Keynes, 1951; Brinley, 1965).

It might be assumed that the temperature-dependent component of the resting potential in the lobster axon is "turned on" when the resting influx of sodium ions and efflux of potassium ions increase. A similar component could be present in squid axons as well. However, because squid axons apparently are in poorer condition than lobster axons, the passive ion fluxes may be so large that they mask any effect the active ion transport system may have on the resting potential with increased temperature. In other words, the active ion transport system may not be functioning to the same extent in the isolated squid axon preparation as it is in the intact animal. It is also possible, of course, that the squid axon does not possess a temperature-dependent component of resting potential similar to that observed for the lobster axon.

The possibility that the inhibitors used in the present experiments have some direct effect on the membrane is not excluded. Interactions which these inhibitors may have with the membrane are uncertain because no complete description of membrane structure is available. However, because little or no initial change is observed in the electrical responses of squid axons when inhibitors are applied, and because the length of time required for the inhibitors to reduce the electrical responses in lobster axons is fairly long as well as gradual (Fig. 1 and Methods), it seems unlikely that the results are explained more plausibly by implicating some direct surface effect rather than inhibition of metabolic function.

The data from the present experiments strongly support the hypothesis that the temperature-dependent component of the lobster axon resting potential results from a metabolically linked process. This was shown when the metabolic inhibitors, 2,4-dinitrophenol, cyanide, and azide nearly abolished this component when they were added to the axon perfusion solution. Assays of axons exposed to metabolic inhibitors showed that ATP levels were greatly reduced from levels in control axons. Ouabain abolished the temperature-dependent component, which gives further support to this argument. In addition these experiments suggest that there is no change in the relative ion permeabilities of the lobster axon membrane when the temperature of the

perfusion medium is altered, because the potential change in ouabain-inhibited axons was that expected for the change in absolute temperature when the membrane potential is considered to be an ion diffusion potential. It is suggested that an electrogenic ion pump is the most likely mechanism for the production of the temperature-dependent component of resting potential.

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