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POLLUTANT-INDUCED DEPRESSION OF THE TRANSMEMBRANE SODIUM GRADIENT IN MUSCLES OF MUSSELS

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Summary

This study deals with the effects of chemical pollutants on the transmembrane potential difference for sodium ($\Delta\tilde{\mu}_{Na}$) in smooth muscle cells of *Mytilus edulis*. A method for indirect determination of extracellular space, intracellular ion concentrations and $\Delta\tilde{\mu}_{Na}$ has been developed and is applied in the investigations. The determination is based on concentration data from haemolymph and muscle tissue samples. The precision of the method used was tested by direct measurements of the apparent intracellular concentration of sodium and the membrane potential. On the basis of these tests, the method was evaluated as reasonably good.

The method was used to study the sensitivity of the transmembrane $\Delta\tilde{\mu}_{Na}$ in *Mytilus edulis* to 96 h exposures to various sublethal concentrations of formaldehyde, methanol and mercury. Both formaldehyde and mercury induced a depression of $\Delta\tilde{\mu}_{Na}$. The observed depressions could be ascribed to a change in both the electrogenic and the chemical components of $\Delta\tilde{\mu}_{Na}$. A depression of $\Delta\tilde{\mu}_{Na}$ was associated with subsequent clinical injury and death. Methanol did not cause death or any changes in $\Delta\tilde{\mu}_{Na}$. Because of the observed correlation between depression of $\Delta\tilde{\mu}_{Na}$ and clinical injury, $\Delta\tilde{\mu}_{Na}$ is suggested to have a potential as an indicator of toxicity.

Introduction

The intracellular concentration of sodium in animal cells is regulated at a level substantially lower than the concentration in the extracellular fluid. Together with the negative membrane potential, this concentration gradient for sodium is reflected in the high transmembrane electrochemical potential difference for sodium ($\Delta\tilde{\mu}_{Na}$), which is maintained by the membrane-bound sodium/potassium

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pump. The pump transports sodium and potassium across the cell and is powered by ATP: a substantial fraction of cellular energy turnover is required to maintain the sodium gradient. In resting frog muscle, the maintenance of the transmembrane electrochemical potential difference for sodium ($\Delta\tilde{\mu}_{Na}$) may consume up to 75 % of the total energy turnover (Florey, 1966). The sodium gradient is, in turn, the energy source for a number of important physiological processes, such as the generation of action potentials, cellular accumulation of free amino acids and cellular extrusion of calcium (Ganong, 1987).

The ATP-dependence of the pump implies that the sodium gradient depends on a number of physiological factors, such as an adequate supply of oxygen, an intact metabolic system for the production of ATP, an intact Na^+/K^+-ATPase and a low membrane permeability to sodium (Ganong, 1987). These requirements probably make the sodium gradient sensitive to any disturbance of metabolism, blocking of the pump or changes in membrane permeability induced by chemicals. The present study is an investigation of the sensitivity of $\Delta\tilde{\mu}_{Na}$ across the posterior adductor muscle of blue mussels (*Mytilus edulis*) to the chemicals formaldehyde, methanol and mercury.

The calculation of $\Delta\tilde{\mu}_{Na}$ requires that the intracellular as well as extracellular concentrations of sodium and the membrane potential (E_m) are known. E_m can be calculated from intracellular and extracellular concentrations of solutes such as chloride, which are in electrochemical equilibrium across the cell membrane. The intracellular concentration of a solute is normally not determined directly, but from the total tissue content of the solute and the solute content in the extracellular tissue fluid. This requires that the extracellular space is known. One method for determining this variable is to calculate inulin space, but this is a complicated procedure.

The present study describes a method for estimating extracellular space as well as E_m from the distribution of two solutes, Cl^- and K^+, both assumed to be in electrochemical equilibrium. Since K^+ is not in exact equilibrium, the method must include a correction for this deviation. The present study determines this correction factor from direct measurements of inulin space. The precision of the value is evaluated by direct measurements of apparent intracellular Na^+ concentration and E_m by intracellular microelectrodes. The method is then used to determine the effect on $\Delta\tilde{\mu}_{Na}$ of exposing the mussels to various chemicals.

Theory of the calculation method

Determination of intracellular ion concentrations

Ion concentrations are related to each other by the mass balance equation:

$$\{X\}_t = [X]_e y + [X]_i(1 - y)\,, \tag{1}$$

where the wavy brackets '{}' mean the content and the square brackets '[]' the concentration of the ion X. The indices t, e and i mean total, extracellular (haemolymph) and intracellular, respectively. y is the extracellular fraction of

tissue water. From this equation the intracellular concentration of X can be calculated as:

$$[X]_i = \frac{\{X\}_t - [X]_e y}{1 - y}. \tag{2}$$

Since the content of X is defined as:

$$\{X\}_t = [X]_t V, \tag{3}$$

where V is the total volume, which in this case is identical to a volume of unity ($V=1$), equation 2 can be written as:

$$[X]_i = \frac{[X]_t - [X]_e y}{1 - y}, \tag{4}$$

where $[X]_t$ is determined from whole tissue samples and $[X]_e$ from haemolymph.

Values of y are available in the literature, but they differ substantially (Krogh, 1939; Martin *et al.* 1958; Potts, 1958; Berger *et al.* 1980), probably because the extracellular fluid fraction is influenced by a number of natural variables, such as medium osmolality (Berger *et al.* 1980). Thus, it is necessary to know the exact value of y for each particular muscle sample which is to be analyzed. The value of y can be determined directly as inulin space for each muscle sample, but a method that allowed the extracellular fluid fraction and ionic concentrations to be determined simultaneously would offer several advantages. It would save the time it takes for inulin to equilibrate in the extracellular space. It would also reduce the handling stress associated with the inulin method, which might influence the extracellular fluid fraction. Samples can be taken directly in the field without the necessity for repeated handling, and only standard analytical methods are required.

We describe a method for calculating the extracellular fluid fraction and intracellular concentrations of chloride, potassium and sodium from extracellular and whole-tissue levels of the same ions. The values are used to calculate the membrane potential and $\Delta\tilde{\mu}_{Na}$. The precision of the method has been evaluated by direct measurements of apparent intracellular sodium concentration and membrane potential using intracellular microelectrodes.

The method is based on equilibrium considerations of the dominating inorganic ions. If both chloride and potassium are in electrochemical equilibrium across the muscle cell membrane, the two ions will have the same equilibrium potential. By expressing the equilibrium potentials of the two ions in terms of the Nernst equation, the intracellular and extracellular concentrations of the two ions are related to each other by the following equation:

$$\frac{RT}{F} \ln \frac{[Cl^-]_e}{[Cl^-]_i} = \frac{RT}{F} \ln \frac{[K^+]_i}{[K^+]_e}.$$

That is:

$$\frac{[Cl^-]_e}{[Cl^-]_i} = \frac{[K^+]_i}{[K^+]_e}, \tag{5}$$

where R is the universal gas constant, T the absolute temperature and F the Faraday constant. In the above equations it has been assumed that the activity coefficients on the two sides of the membrane are almost equal and hence only concentrations appear. By convention, no distinction is made between apparent concentration and total concentration in the development of the calculation method.

However, potassium is not in exact electrochemical equilibrium across the muscle cell membrane. Potassium is pumped into the cell and sodium is pumped in the opposite direction by the membrane-bound sodium/potassium pump. The effect of this transport on the transmembrane distribution of the ions has been dealt with by Hodgkin (1958), who, by introducing sodium and a correction factor b, modified equation 5 to give:

$$\frac{[Cl^-]_i}{[Cl^-]_e} = \frac{b[Na^+]_e + [K^+]_e}{b[Na^+]_i + [K^+]_i}. \tag{6}$$

According to Hodgkin (1958), the factor b represents the membrane permeability ratio of sodium and potassium.

By substituting the intracellular concentrations of the respective ions in equation 6 with the concentrations expressed in terms of equation 4, equation 6 becomes:

$$\frac{[Cl^-]_t - y[Cl^-]_e}{(1-y)[Cl^-]_e} = \frac{(1-y)b[Na^+]_e + [K^+]_e}{b([Na^+]_t - y[Na^+]_e) + [K^+]_i - y[K^+]_e}. \tag{7}$$

From this equation, the extracellular fraction of tissue water can be found as:

$$y = \frac{b[Cl^-]_t[Na^+]_t + [Cl^-]_t[K^+]_t - b[Cl^-]_e[Na^+]_e - [Cl^-]_e[K^+]_e}{-2b[Cl^-]_e[Na^+]_e - 2[Cl^-]_e[K^+]_e + b[Cl^-]_t[Na^+]_e + [Cl^-]_t[K^+]_e + b[Cl^-]_e[Na^+]_t + [Cl^-]_e[K^+]_t}. \tag{8}$$

When y has been calculated from equation 8, the intracellular concentrations of the respective ions can be found from equation 4.

Finding a correct value of b

Calculation of the extracellular fluid fraction y from equation 8 requires that the value of b is known. Hodgkin (1958) interpreted b as reflecting the differences in membrane permeabilities. In addition to correcting for the difference between the membrane permeabilities of sodium and potassium, b is influenced by the stoichiometric pumping ratio of the two ions. In introducing equation 6, Hodgkin (1958) assumed that the pumping ratio is 1:1, whereas most sodium/potassium pumps have a pumping ratio between sodium and potassium of about 3:2 (Hodgkin and Keynes, 1955; Post, 1960). If b is determined experimentally from the transmembrane distribution of ions, the value will take into account any pumping ratio:

$$b = f\frac{p_{Na}}{p_K}, \tag{9}$$

where f is the pumping ratio, and p_{Na} and p_K are the permeabilities of sodium and potassium, respectively.

The pumping ratio of sodium/potassium pumps is characteristic of each particular cell type. Although relative membrane permeabilities vary with muscular activity level, the average permeability ratio of the two ions is probably constant over time. This implies that, in contrast to the extracellular fluid fraction, the value of b ought to be determined experimentally: this value could then be used to determine y from equation 8 for different *Mytilus* adductor muscle samples analyzed under different conditions.

In the present study the value of b has been estimated by the use of three different methods: by direct determination of y as inulin space, by direct measurement of the apparent intracellular sodium concentration and by direct measurement of the membrane potential. The two latter methods were based on the use of intracellular microelectrodes. For each b value, corresponding sets of values of y, intracellular ionic concentrations, E_m and $\Delta\tilde{\mu}_{Na}$ have been calculated to evaluate the consequences of the uncertainty one is faced with in the determination of b.

When y was determined experimentally as inulin space, and the extracellular and total tissue ion concentrations were measured, the value of b was found by solving equation 7 with respect to b:

$$b = \frac{[Cl^-]_e[K^+]_t - [Cl^-]_e[K^+]_e + 2b[Cl^-]_e[K^+]_e - y[K^+]_e[Cl^-]_e - y[Cl^-]_e[K^+]_t}{-2y[Na^+]_e[Cl^-]_e + y[Na^+]_t[Cl^-]_t + y[Na^+]_t[Cl^-]_e - [Na^+]_t[Cl^-]_t + [Na^+]_e[Cl^-]_e}. \quad (10)$$

After b had been determined in this way, the intracellular concentrations were calculated from equation 4.

When the apparent intracellular sodium concentration was measured directly, and the extracellular and total sodium concentrations were determined, y was found by solving equation 4 with respect to y:

$$y = \frac{[Na^+]_t - [Na^+]_i}{[Na^+]_e - [Na^+]_i}. \quad (11)$$

The intracellular concentrations of potassium and chloride were then found using equation 4. Thereafter, the value of b was determined by solving equation 6 with respect to b:

$$b = \frac{[Cl^-]_e[K^+]_e - [K^+]_i[Cl^-]_i}{[Na^+]_i[Cl^-]_i - [Cl^-]_e[Na^+]_e}. \quad (12)$$

When the membrane potential was measured directly and it was assumed that chloride was in electrochemical equilibrium across the cell membrane, the intracellular concentration of chloride could be calculated from the Nernst equation using the equation:

$$[Cl^-]_i = e^{\ln[Cl^-]_e - E_m F/RT}. \quad (13)$$

$[Cl^-]_i$ was used to find y by solving equation 4, as was done for the sodium concentration in equation 8. Thereafter, the intracellular concentrations of sodium

and potassium were calculated using equation 4. The value of b was then calculated using equation 10.

Calculation of E_m *and* $\Delta\tilde{\mu}_{Na}$

The membrane potential was estimated by inserting the intracellular and extracellular concentrations of chloride, which is assumed to be in electrochemical equilibrium across the cell membranes, into the Nernst equation.

The electrochemical potential difference of sodium across the cell membrane was calculated from the standard equation:

$$\Delta\tilde{\mu}_{\mathrm{Na}} = FE_{\mathrm{m}} + RT\ln\frac{[\mathrm{Na}^+]_{\mathrm{i}}}{[\mathrm{Na}^+]_{\mathrm{e}}}. \tag{14}$$

All later reference in this paper to the 'calculation method' will include the first of the equations used to determine y, intracellular concentrations of ions, E_{m} and the $\Delta\tilde{\mu}_{\mathrm{Na}}$. This will not necessarily mean a determination of the coefficient b, which is thought of as a relative constant, variable in this context, and for which a predetermined and fixed value is to be used.

Materials and methods

The measurements were carried out on *Mytilus edulis* L. with a shell length of 40–50 mm obtained from a local shellfish farm in 1989/1990. Prior to the experiments the mussels were kept in the laboratory for 2–8 days in 35‰ continuously flowing sea water at 10±0.5 °C and under continuous light. The animals were not fed during the acclimation period or during the exposure experiments.

Inulin space

To calibrate and test the calculation method used to determine the extracellular fluid fraction y of the muscle tissue, this fraction was directly determined by measuring the dilution of [^{14}C]inulin injected into the central sinus of the muscle (100 μl of 1.2 mCi g^{-1} injected after removal of the same volume of haemolymph). After an equilibration period of 4 h, 500 μl of haemolymph was removed, and a muscle tissue sample similar to that used for ionic content measurements was dissected. The haemolymph and muscle tissue samples were treated as described on the next page. 200 μl samples of the muscle extracted in trichloroacetic acid (TCA) and TCA-diluted haemolymph were added to 10 ml of Hionic Fluor, and the radioactivity was measured on a LKB Wallace scintillation counter. The fraction of extracellular space was calculated as:

$$y = \frac{A_{\mathrm{m}}}{A_{\mathrm{h}}},$$

where A_{m} is the radioactivity per unit mass of tissue water and A_{h} is the radioactivity per unit mass of haemolymph.

Haemolymph and tissue sampling, and ionic measurements

Extracellular ionic concentrations were measured on samples of haemolymph obtained from the central sinus of the posterior adductor muscle by means of a 10 ml syringe. The haemolymph was centrifuged for 15 min at 8860 *g* to remove haemocytes and stored at −20°C until analysis.

Total tissue contents of ions were determined on muscle tissue samples dissected from the shells, dried with tissue paper, and transferred to preweighed plastic tubes to be stored in liquid nitrogen (−196°C). The dry mass was determined after 12 h of freeze drying, after which the samples were dissolved in 10 % TCA.

$[Na^+]$ and $[K^+]$ were determined on a Radiometer FLM3 flame photometer, whereas $[Cl^-]$ was measured on a Radiometer CMT10 chloride titrator. Values from measurements with these techniques on haemolymph and muscle tissue were used to calculate extracellular space, intracellular ion concentrations and $\Delta\tilde{\mu}_{Na}$ with the equation given above and with a chosen b value of 0.038.

Apparent intracellular sodium concentration and membrane potential

The apparent intracellular sodium concentration, $[Na^+]_i$, and membrane potential were recorded simultaneously on mussels from the same group used to determine inulin space. A double-barrelled electrode (Zeuthen, 1980) was introduced through an opening in the shell and individual cells of the muscle fibre were penetrated. Fluka sodium ionophore I, cocktail A (71176), was applied as a liquid membrane in the sodium-selective barrel, and 2 mol l^{-1} KCl solutions were used as a reference electrolyte. The mussel was immersed in filtered sea water at 10°C during the experiments. The results are presented as apparent concentrations, i.e. the recording from the electrode is directly compared with an external reference solution of a known concentration. This means that the apparent intracellular Na^+ concentration is equal to the true concentration if the intracellular activity coefficient is similar to that of the external solution. Before and after the intracellular recordings, the sodium microelectrodes were individually calibrated against three different standard sodium solutions. The sodium standards were made by reciprocal dilution of Na^+ and K^+, i.e. using K^+ to substitute isosmotically for Na^+. The criterion used for selecting acceptable cell penetrations and intracellular recordings was the ability to measure an instantaneous (within milliseconds) and stable membrane potential (±2 mV) of at least 1 min duration simultaneously with the recording of apparent ion concentration. The signals were recorded on a Macintosh II Apple computer *via* a MacADIOS II analog-to-digital peripheral board (GWInstruments) and the software SuperScope (GW Instruments).

Exposure to chemicals

Mussels were exposed to the chemicals in specially designed flow-through exposure equipment. The water flow through the system was passively regulated, but the chemicals were pumped from a stock solution into the water stream by

means of a four-channel peristaltic pump (Ismatec MS Reglo). The mussels were placed individually in exposure chambers and allowed to acclimate to the chambers for 2–3 days prior to exposure. The mussels used this period to make new byssus threads. They were then exposed to various chemicals for standard periods of 96 h. The mussels were held at a temperature of 10±1.0 °C from the time they were taken into the laboratory until the end of an experimental series. The approximate lethal concentrations of the different chemicals were determined by mortality screening tests, and high sublethal concentrations of the chemicals were chosen in the experiments in order to obtain physiological changes related to acute toxicity within the period of exposure (96 h).

Results

Calibration and testing of the calculation method

Results from the direct measurements of inulin space, apparent intracellular sodium concentration and membrane potential, made in order to calibrate and test the method, are given in Table 1. The measurement with inulin on 23 mussels gave a mean value of the extracellular water fraction of 0.335±0.030. The individual values of directly determined apparent intracellular sodium concentration and membrane potential in five mussels are shown in Figs 1 and 2.

The individual values of apparent sodium concentration and membrane potential are highly variable, and this cannot entirely be ascribed to methodological uncertainty. The main source of error in intracellular recordings is probably ion leakage through the penetrated membrane, but the stable values of the membrane potential during the recording periods indicate that this error did not contribute significantly. Since leakages are probably not the explanation of the great variability, the present results indicate that the muscle fibres are heterogeneous

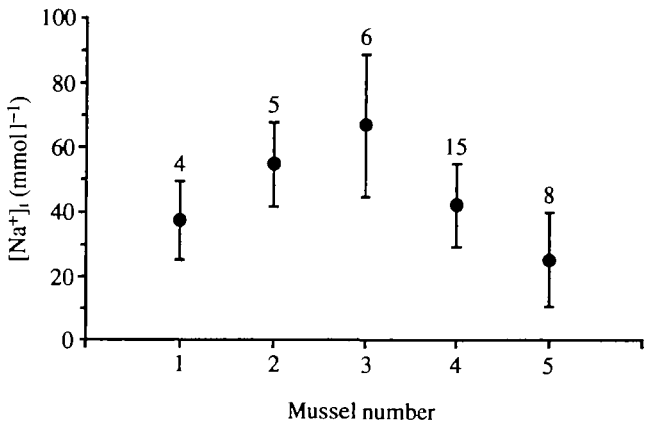

Fig. 1. Apparent intracellular Na^+ concentration in the posterior adductor muscle of *Mytilus edulis* determined by intracellular microelectrodes. Numbers of cells tested from each individual are indicated above bars. Values are mean±S.D.

Table 1. *Comparison of the three different series of estimations*

	Estimation series					
y	$[Na^+]_i$ ($mmol\,l^{-1}$)	$[K^+]_i$ ($mmol\,l^{-1}$)	$[Cl^-]_i$ ($mmol\,l^{-1}$)	E_m (mV)	b	Δ (kJ
0.335±0.030 (**23**)	33.2±11.8 (23)	201±9 (23)	77.8±17.1 (23)	−48.0±5.9 (23)	0.038±0.013 (23)	11.
0.326±0.027 (23)	**39.3** (**38**)	198±9 (23)	84.3±10.2 (23)	−45.6±3.4 (23)	0.042±0.008 (23)	10.
0.343±0.028 (23)	28.2±9.4 (23)	203±8 (23)	72.5±0.9 (23)	**−49.1** (**31**)	0.034±0.002 (23)	11.
0.335	33.6	200	78.2	−47.6	0.038	

based on a directly measured variable: (1) extracellular space; (2) apparent intracellular Na^+ concentration; (3) r

he directly measured variables are given in bold type.
f determinations is given in parentheses; mean±S.D.

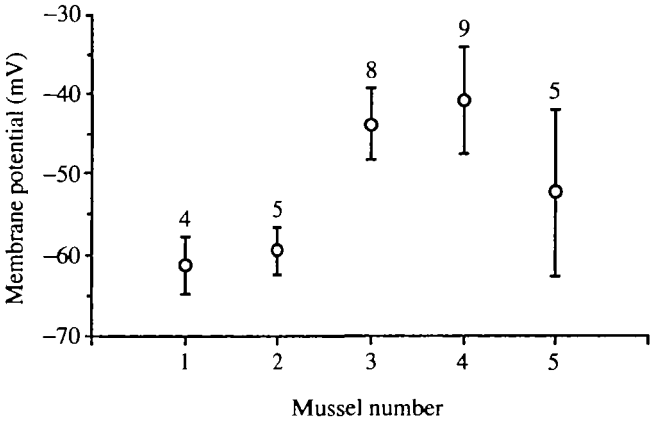

Fig. 2. Membrane potentials in the posterior adductor muscle of *Mytilus edulis* determined by intracellular microelectrodes. Numbers of cells tested from each individual are indicated above bars. Values are mean±S.D.

with respect to membrane potential and apparent intracellular sodium concentration. Therefore, values calculated from chemical measurements made on whole muscle represent a mean value for all fibres. There are also large differences between animals in these variables. This appears to be typical for *M. edulis*, which has great physiological plasticity in order to cope with the changing estuarine environment (Depledge, 1990). Thus, the immediate environmental history and physiological status are reflected as differences between animals in the ionic concentrations of their body fluids. In spite of the heterogeneity of the data from direct measurements made both within and between individuals, mean values are calculated for membrane potential as well as for apparent intracellular sodium concentration. The mean membrane potential is −49.1 mV and the mean apparent intracellular sodium concentration is 39.3 mmol l^{-1}.

The mean values of the directly measured variables y, E_m and $[Na^+]_i$ have been used to make independent estimates from equations 1–14 of the values of the constant b and the other variables: intracellular concentrations of the inorganic ions, membrane potential and the transmembrane electrochemical potential difference for sodium. The values are presented in Table 1.

The data in Table 1 show that corresponding values determined by the different methods are in good agreement. The agreement is particularly good for y, b, intracellular potassium concentration and electrochemical potential difference for sodium.

The effects of formaldehyde, methanol and mercury

The mortality of *M. edulis* during the 96 h exposures to different concentrations of the chemicals tested is shown in Table 2. Increased mortality was found at formaldehyde concentrations higher than 30 mg l^{-1} and at mercury(II) chloride

Table 2. *Mortality of mussels exposed to formaldehyde and mercury at different concentrations*

Formaldehyde		$HgCl_2$	
Concentration ($mg\,l^{-1}$)	Mortality (%)	Concentration ($mg\,l^{-1}$)	Mortality (%)
1	0	0.05	10
10	0	0.5	40
30	0	2.0	60
60	20	–	–

Table 3. *Effects of formaldehyde on the calculated values of* y, *intracellular ion concentrations and membrane potential using the method described in the text*

Formaldehyde concentration ($mg\,l^{-1}$)	Calculated values				
	y	$[Na^+]_i$ ($mmol\,l^{-1}$)	$[K^+]_i$ ($mmol\,l^{-1}$)	$[Cl^-]_i$ ($mmol\,l^{-1}$)	E_m (mV)
Control	40.4±12.2 (10)	55.5±21.3 (9)	216±21 (9)	67.5±10.8 (10)	−50.4±4.7 (10)
1	35.1±4.6 (10)	51.4±17.7 (10)	203±9 (10)	74.8±4.2 (10)	−47.6±1.2 (10)
10	36.2±4.1 (10)	51.6±26.6 (10)	207±18 (10)	74.3±6.2 (10)	−47.8±1.9 (10)
30	32.1±7.5 (10)	140.7±35.4* (10)	184±19 (5)	81.0±9.5 (5)	−40.8±5.9* (10)

Mytilus edulis were exposed for 96 h to three different concentrations of formaldehyde.
Values significantly different from the control are indicated with an asterisk (Student's *t*-test, $P<0.05$).
Number of determinations are given in parentheses; mean±S.D.

concentrations higher than $0.05\,mg\,l^{-1}$. Methanol did not cause any mortality even at $5000\,mg\,l^{-1}$. The effects of chemical exposure on y, intracellular ion concentrations and E_m of the adductor muscle are shown in Tables 3, 4 and 5. During 96 h of exposure to formaldehyde, $[Na^+]_i$ increased significantly from $55.5\,mmol\,l^{-1}$ to $140.7\,mmol\,l^{-1}$ and E_m decreased significantly from −50.4 to −40.8 mV at the highest concentration of the chemical ($30\,mg\,l^{-1}$) (Table 3). Following the exposures to mercury(II) chloride, similar changes were observed at both 0.5 and $2.0\,mg\,l^{-1}$. In addition, this chemical induced significant decreases in $[K^+]_i$ and increases in $[Cl^-]_i$ at the two highest exposure concentrations (Table 4). Methanol had no significant effect (Table 5). Examples of the reduction of $\Delta\tilde{\mu}_{Na}$ induced by different chemicals are shown in Figs 3 and 4. Significant depressions were

Table 4. *Effects of mercury on the calculated values of* y, *intracellular ion concentrations and membrane potential using the method described in the text*

$HgCl_2$ concentration ($mg\,l^{-1}$)	Calculated values				
	y	$[Na^+]_i$ ($mmol\,l^{-1}$)	$[K^+]_i$ ($mmol\,l^{-1}$)	$[Cl^-]_i$ ($mmol\,l^{-1}$)	E_m (mV)
Control	38.0±7.2 (9)	38.1±16.2 (9)	196±15 (9)	78±7 (9)	−47.3±1.9 (9)
0.05	36.2±6.0 (9)	38.2±21.0 (9)	189±12 (9)	81±5 (9)	−46.5±1.3 (9)
0.5	42.9±10.1 (6)	115.8±31.7** (6)	147±39** (6)	107±26** (6)	−40.3±6.0** (6)
2.0	30.2±9.0 (5)	151.9±67.3** (5)	121±45** (5)	135±45** (5)	−34.9±8.5** (5)

Mytilus edulis were exposed for 96 h to three concentrations of inorganic mercury.
Values significantly different from the control are indicated with asterisks (Student's *t*-test, $P<0.01$).
Number of determinations are given in parentheses; mean±S.D.

Table 5. *Effects of methanol on the calculated values of* y, *intracellular ion concentrations, membrane potential and electrochemical potential difference for sodium using the method described in the text*

Exposure type	Calculated values					
	y (%)	$[Na^+]_i$ ($mmol\,l^{-1}$)	$[K^+]_i$ ($mmol\,l^{-1}$)	$[Cl^-]_i$ ($mmol\,l^{-1}$)	E_m (mV)	$\Delta\tilde{\mu}_{Na}$ ($kJ\,mol^{-1}$)
Control	26.7±4.1 (10)	41.8±15.6 (10)	176±16 (10)	91.5±8.1 (10)	−43.1±2.1 (10)	9.98±1.02 (10)
Methanol $5000\,mg\,l^{-1}$	25.0±4.6 (10)	35.1±6.3 (9)	177±13 (10)	89.5±8.1 (10)	−43.5±2.1 (10)	10.26±0.57 (10)

Mytilus edulis were exposed for 96 h to $5000\,mg\,l^{-1}$ methanol.
Numbers of determinations are given in parentheses; mean±S.D.

observed at $30\,mg\,l^{-1}$ of formaldehyde and at 0.5 and $2.0\,mg\,l^{-1}$ of mercury(II) chloride.

Discussion

Test and calibration of method

The calculated *b* values of the posterior adductor muscle (PAM) of *Mytilus*

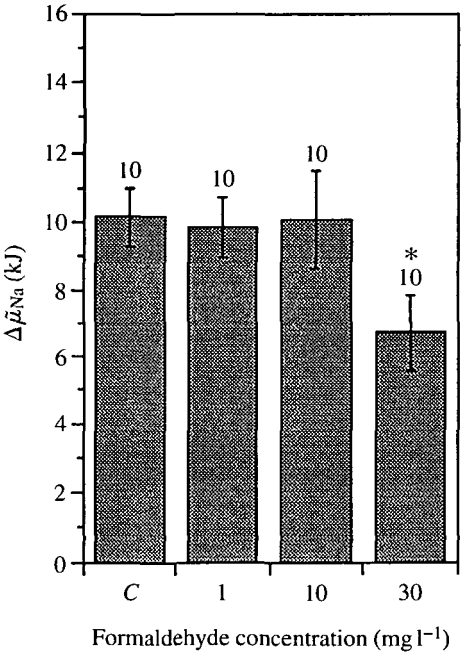

Fig. 3. $\Delta\tilde{\mu}_{Na}$ of smooth muscle cells of *Mytilus edulis* after 96 h of exposure to three concentrations of formaldehyde. Numbers of mussels tested are shown above bars and an asterisk indicates a significant decrease compared with the control value (Student's *t*-test, $P<0.05$). Values are mean±S.D. *C*, control.

edulis range from 0.034 to 0.042, with an average of 0.038. These values are of the same magnitude as those found for the PAM of *M. edulis* by Potts (1958).

Potts distinguished between fast and slow muscle fibres, and found a value of 0.026 for the fast and 0.036 for the slow fibres. Since the present study was made on whole muscle, the *b* value obtained should be considered as a mean of both categories. A correlation between the speed of the muscle and the *b* value is also indicated by other data presented by Potts. He found that the fast adductor muscle of *Pecten maximus* had a *b* value below 0.01, the slower byssus retractor muscle of *M. edulis* a value of 0.059, while the slow adductor muscle of *Anodonta cygnaea* had a *b* value of 0.071.

The relative permeability of sodium and potassium (p_{Na}/p_K), a variable in equation 9 which defines the coefficient *b*, is dependent on temperature. Gorman and Marmor (1970) reported that p_{Na}/p_K in muscle tissue of the snail *Anisodoris nobilis* is affected by temperature, increasing from 0.028 at 4°C to 0.068 at 18°C. Potts (1958) incubated his blue mussels at 10–16°C. This is only slightly above the temperature used in our study (10°C). A difference in temperature would make the discrepancy more pronounced, but both his *b* values, 0.026 for fast and 0.036 for slow fibres, lie only slightly outside the whole tissue value found in our study. Minor differences in sampling procedures and chemical analysis used by Potts and

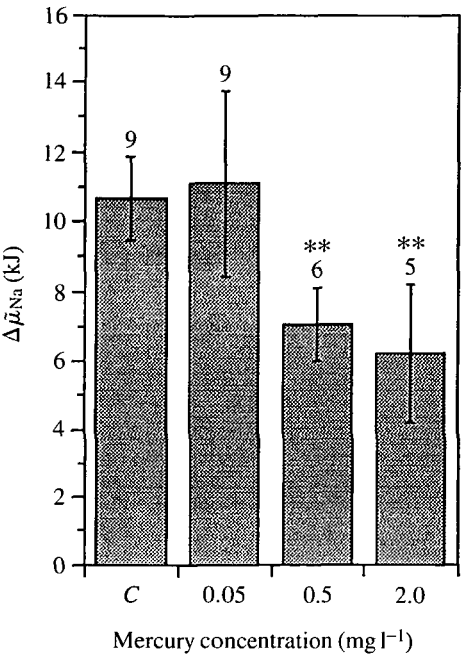

Fig. 4. $\Delta\tilde{\mu}_{Na}$ of smooth muscle cells of *Mytilus edulis* after 96 h of exposure to three concentrations of mercury(II) chloride. Numbers of mussels tested are shown above bars and asterisks indicate a significant decrease compared with the control value (Student's *t*-test, $P<0.01$). Values are mean±S.D. *C*, control.

us might also contribute to this discrepancy. Potts (1958) did not find any effect of medium salinity on the *b* value of the adductor of *Anodonta*, i.e. medium salinity seems to be of no importance.

The data listed in Table 1 reveal that there are relatively large differences between directly measured and chemically estimated values of intracellular sodium concentration. Both values of chemically estimated intracellular sodium concentration are lower than the apparent concentration determined by direct measurement with intracellular microelectrodes. Since a fraction of the intracellular sodium is bound because of mutual electrostatic interaction of ions in the solution or bound to macromolecules, one might expect that the sodium activity would be somewhat lower than the total concentrations (activity coefficient approximately 0.67). But, the high apparent Na^+ concentration indicates the opposite, i.e. an activity coefficient greater than 1.0. However, a significant fraction of the water content of the cells is probably associated with macromolecules (Dick, 1978) and may therefore be inactive as a solvent. If this fraction of bound water is large enough, it might explain the high values of potentiometrically measured apparent sodium concentration compared to the chemically measured concentration. Since direct measurements of apparent intracellular K^+ and Cl^- concentrations have not been made, it is not possible to know whether similar

differences exist between the apparent intracellular concentrations and chemical concentrations of these ions.

Postulating a proportion of bound water does not invalidate a comparison of the three methods, but the actual values (Table 1) are critically dependent on it and the discrepancy in sodium measurements indicates that there must be a lot of bound water. The proportion of bound water must be at least 16% if the measurements of sodium concentration with inulin and ion-selective microelectrodes are to be reconciled. If only 84% of the water is free, intracellular chloride concentration rises from 78 to 93 mmol l^{-1} and potassium concentration from 200 to 237 mmol l^{-1}. Calculated with equation 12, b then increases from 0.038 to 0.064. There is a very interesting corollary to all this that has perhaps not been recognised before. The higher the proportion of bound water in a cell the larger b becomes, i.e. the less selective the membrane needs to be.

The results in Table 1 imply that even if the b values should vary to some extent, the resulting estimated values for the extracellular fraction of tissue water and $\Delta\tilde{\mu}_{Na}$ will be only moderately affected. Thus, the method appears to provide a relatively precise measure of $\Delta\tilde{\mu}_{Na}$. The agreement between the estimated and directly measured values of extracellular water fraction is important because it indicates that the chemical method also gives reasonably correct values for this variable, and thus for the intracellular concentration of a solute. The present method also offers an advantage because it allows a directly measured value of y to be compared with a calculated value of y based on data from direct measurements of membrane potential and apparent intracellular sodium concentration. The good precision by which $\Delta\tilde{\mu}_{Na}$ can be estimated from chemically determined extracellular and total tissue concentrations of Na^+, K^+ and Cl^- provides an opportunity for making reliable estimates of the sodium gradient under various conditions.

It is important to notice that the measurements with inulin and microelectrodes are incorporated in this study only in order to determine the b value and to evaluate the precision of the determination of this variable from chemical measurements. These procedures will not be needed when the method is applied in routine measurements, which will be carried out using a b value of 0.038.

Exposure to chemicals

Control values of the sodium gradient obtained by the different exposure series indicate that the sodium gradient is relatively stable under normal conditions (Figs 3 and 4, Table 5). This suggests that the sodium gradient is well-regulated, which makes sense against the background of its physiological importance as an energy source for important cellular processes.

The sodium gradient is depressed both by the organic formaldehyde and by the heavy metal mercury. Both $[Na^+]_i$ and E_m increase during exposure to the highest levels of the pollutants. Therefore, the simultaneous depression of $\Delta\tilde{\mu}_{Na}$ is explained as a change in the electrogenic as well as the chemical concentration component, which both contribute to the value of $\Delta\tilde{\mu}_{Na}$.

Methanol appears to be non-toxic in blue mussels at the level of 5000 mg l^{-1} used, which is a relatively high concentration in connection with toxicity testing of this chemical. Against the background of the severe effects observed for methanol on the central nervous system of vertebrates, involving blindness and death, our results indicate that blue mussels probably lack the alcohol dehydrogenase responsible for the transformation of methanol to the toxic intermediate formate (Klaassen *et al.* 1986). Similar observations with methanol are found for rainbow trout, which has a 96 h LC_{50} value as high as 20 100 mg l^{-1} (Poirier *et al.* 1986).

There is a correlation between the observed clinical effects and depression of $\Delta\tilde{\mu}_{Na}$ (Figs 3 and 4 and Table 5). During exposure to mercury, $\Delta\tilde{\mu}_{Na}$ is depressed at the same time as an increase in mortality (Table 2). The median tolerance limit values (TL_m) for formaldehyde from comparable experiments with guppies are 50–200 mg l^{-1} (Verschueren, 1977). Marking *et al.* (1984), who studied the effects of different diets on the sensitivity of rainbow trout to formalin, found a 96 h LC_{50} of 124 μl l^{-1}. Also, our mortality studies with mussels gave an LC_{50} of 50–100 mg l^{-1}, and the formaldehyde concentration causing depression of $\Delta\tilde{\mu}_{Na}$ is very close to these concentrations. This provides further support for the view that the depression of the sodium gradient is involved in the mechanisms leading to clinical injury. A causal link between a depression of the sodium gradient and clinical injury is supported by similar results from exposure to oils and dispersants (Zachariassen *et al.* 1989) and also by quantitative depression effects measured as the degree of depression at different intervals of exposure and recovery time (Zachariassen *et al.* 1991).

Whenever $\Delta\tilde{\mu}_{Na}$ drops below a certain minimal value, during exposure to both organic and inorganic pollutants, injuries develop. Depledge (1990) interpreted this minimum as the value where the limit is reached for the mechanisms designed to maintain the variables at a normal level and at which injuries will appear. This should be of interest because it could be relevant to use the sodium gradient as a measure of toxicity, since it represents a mechanistic link between physiological disturbance and injury to an animal. It implies that $\Delta\tilde{\mu}_{Na}$ may have an application as an ecologically relevant toxicity parameter.

Normally, calcium is regulated at a very low intracellular level. The low level of calcium is obtained by extrusion through a Na^+/Ca^{2+} exchange mechanism and a Ca^{2+}/Mg^{2+}-ATPase (Bronner, 1990). A depression of the transmembrane sodium gradient should, therefore, be expected to change the calcium gradient. When the sodium gradient decreases, less energy will be available for the transport of calcium out of the cell, which leads to an increase in $[Ca^{2+}]_i$. A sustained increase in the level of cytosolic Ca^{2+} is known to cause irreversible cellular damage and malfunction (Viarengo and Nicotera, 1991). It is therefore reasonable to believe that the increased mortality observed during the depression of $\Delta\tilde{\mu}_{Na}$ is mediated by a breakdown in the regulation of $[Ca^{2+}]_i$.

Biotransformation enzymes, for example the cytochrome-P_{450}-dependent enzymes, are frequently used by biochemists to monitor environmental pollutants (Goksøyr *et al.* 1991; Guengerich, 1988; Payne, 1984). The P_{450}-related enzymes

are known to be highly sensitive to pollution by organic chemicals, but they do not respond to heavy metals. However, the ecological significance of these effects is unclear. In contrast, $\Delta\tilde{\mu}_{Na}$ appears to be directly related to lethal effects. In addition to being sensitive to organic pollutants, $\Delta\tilde{\mu}_{Na}$ is also sensitive to the heavy metal mercury. $\Delta\tilde{\mu}_{Na}$ also seems to respond to the presence of oil and oil dispersants (Aarset and Zachariassen, 1983; Zachariassen *et al.* 1989). Thus, it seems that $\Delta\tilde{\mu}_{Na}$ is a more general and nonspecifically responding, but less sensitive, parameter than those based on biotransformation enzymes. Further studies should determine whether a change in the sodium gradient is also induced at chronic exposure levels.

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