POLARIZATION VARIATIONS INDUCED BY HIGH HYDROSTATIC PRESSURES IN THE ISOLATED FROG SKIN AS RELATED TO THE EFFECTS ON PASSIVE IONIC PERMEABILITY AND ACTIVE Na+ TRANSPORT

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SUMMARY

The effects of a wide range of hydrostatic pressures (from 50 to 1000 kg/cm²) have been investigated on the spontaneous potential difference (PD), the short-circuit current (SCC) and the activity of the membrane ATPases of the isolated abdominal skin from the frog *Rana temporaria* L. Two types of variations in PD are induced by pressure changes: short and transient potential variations which appear to be related to the pressure change (compression and decompression) and lasting variations which persist as long as pressure is applied and whose nature appears to be related to the pressure magnitude.

Long-lasting potential changes have particularly been investigated. At pressures lower than 500 kg/cm², the skin potential increases while a pressure over 500–600 kg/cm² induces a depolarization. Both variations consecutively occur at 500 ± 100 kg/cm². These effects of pressure have been shown to be reversible up to about 800 kg/cm².

The question of the origin of the potential changes is discussed and it is proposed that the lasting hyperpolarization results from an effect on the passive permeabilities to Na⁺, K⁺ and Cl⁻ ions inducing in turn a secondary readjustment (stimulation) of the Na⁺ active transport while the depolarization at high pressures reflects a direct inhibition of the Na⁺ pump.

These interpretations are supported by experimental data on the effects of pressure on the short-circuit current and on the activity of the skin (Na⁺ + K⁺)ATPase.

INTRODUCTION

Hydrostatic pressure is known to modify the electrical properties of the isolated skin of the frog *Rana temporaria* L. (Schoffeniels, 1967; Brouha, 1965; Brouha *et al.* 1970; Pequeux, 1971, 1972). A pressure step of 100 kg/cm² induces a small and transient depolarization phase followed by a much larger and stable hyperpolarization, while decompression is accompanied by a short hyperpolarization before the skin potential slowly decreases to reach its initial value.

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Fig. 1(a). For legend see opposite

- Plexiglass
- Steel
- Brass
- Agar agar
Fig. 1. Diagram of the apparatus used for measuring the skin potential difference and the short-circuit current at the atmospheric pressure and under hydrostatic pressure. (a) Details of the cell containing the skin (explanation in the text): A, plexiglass cell; B, electrodes holder; C, bomb head; D, plexiglass cylinder (protection); E, Ag-AgCl electrodes; F, skin; \( \mu \text{A}, \text{microammeter}; \text{mV}, \text{electrometer}; \text{e.m.f.}, \text{battery generating the outside e.m.f.} \) (b) Pressure vessel. Pressure is applied on piston G and transmitted by silicone oil to the high pressure chamber H upon where the plexiglass cell and its holder (described on the diagram (a)) are fastened.

By modifying the ionic composition of the salines (Na\(^+\) substituted by K\(^+\), Mg\(^{2+}\), Li\(^+\) or choline) or by adding oxytocin, it has been demonstrated that a pressure step of 100 kg/cm\(^2\) increases the Na\(^+\) permeability of the outer-facing cell membranes of the skin (Brouha et al. 1970; Pequeux, 1974). Similar experiments where the K\(^+\) concentration is changed and Cl\(^-\) ions partially or entirely replaced by the non-penetrating SO\(_4^{2-}\) ions further showed that a small part of the pressure-induced hyperpolarization is also related to an increase in the K\(^+\) permeability of the inner membranes of the skin and to a decrease in Cl\(^-\) permeability (Pequeux, 1974). A few experiments at pressures higher than 100 kg/cm\(^2\) have also been reported but remain fragmentary (Brouha et al. 1970).

This paper presents the results of a study of the skin potential response over a wide
range of high pressures. An attempt is also made to explain the pressure-induced potential changes by studying the effect of pressure on both the skin short-circuit current and the activity of the membrane ATPases.

MATERIALS AND METHODS

Animal: preparation of the skins. Experiments were performed on isolated abdominal skins of the frog *Rana temporaria* L. The skins were mounted between plexiglass chambers, containing 5 ml of saline at 22 °C (NaCl Ringer’s solution containing 115 mM-NaCl, 2 mM-KCl, 0.45 mM-CaCl₂, buffered at pH 7.8 with phosphate buffer 0.14 mM-KH₂PO₄-1.53 mM-Na₂HPO₄). 30 minutes before the beginning of the experiment, air was bubbled through the saline on both sides of the skin. The exposed skin area was 3 cm².

Pressure vessel: potential difference and short-circuit current measurements. PD and SCC measurements were performed with an experimental set-up similar to that described by Ussing & Zerahn (1951), but adapted for the pressure vessel. It is shown diagrammatically in Fig. 1. The skin F separated two plexiglass chambers filled with saline. The skin potential was measured using two silver-silver chloride electrodes dipped in the solutions, in the immediate vicinity of the skin. The electrodes were connected in opposition to a precision potentiometer (Cambridge) and the output of this circuit fed to an electrometer (603- Keithley Electrometer) (mV) linked to a pen recorder (Varian G14).

In the plexiglass walls farthest from the skin, a thin channel connected the large chamber to a smaller one on top of the cell wall. The thin channel and the bottom of the little reservoir were filled with agar-ringer, the rest contained saturated KCl.
Polarization variations

Saturated with AgCl. Silver wires immersed in that solution were used as electrodes through which an outer e.m.f. could be applied to cancel the skin potential difference. The current passing through the skin under these conditions (SCC) was recorded on the microammeter (μA).

The plexiglass cell and the electrode holder were fastened to the bomb head as shown in Fig. 1. The whole device containing the skin was placed in a plexiglass cylinder which was then filled with silicone oil (Dow Corning Silicone 200 Fluid, viscosity 1 CS), before being submitted to pressure. Care was taken that no gas phase was present in the chamber when under pressure. Experiments performed at atmospheric pressure with this experimental set-up showed that the potential requires about 4 min after the short-circuit release to recover its initial stable value (Fig. 2). Therefore, when both PD and SCC had to be determined successively, readings were taken every 5 min.

Pressure was applied on piston G (Fig. 1) by means of a hydraulic press for periods of up to 5 min. Compression and decompression required between 3 and 30 s depending on the pressure magnitude.

ATPase activity measurements. Frog epidermis was isolated according to the method of Kawada, Taylor & Barker (1969) and homogenized in a homogenizer with a Teflon pestle in ice-cold 0.3 M sucrose solution containing 5 x 10⁻³ M ethylenediamine-tetraacetic acid (EDTA) adjusted at pH 7.4 with Tris buffer. The homogenate was first centrifuged at 1000 g for 10 min. The pellet resulting from a second centrifugation at 10000 g for 20 min was used as enzyme preparation after washing and dilution with cold sucrose to a protein content of 1-5 mg/ml. The reaction medium used to measure enzyme activity contained, in a final volume of 2 ml, 4 mM-ATP, 25 mM-Tris buffer (pH 7.4), 0.25 mM-EGTA, 100 mM-NaCl, 25 mM-KCl, 5 mM-MgCl₂ and 0.2 ml of enzymic extract. Ouabain (final concentration 0.2 mM) was added for estimation of the 'ouabain sensitive ATPase' expressed as the difference between enzymic activities with and without the drug.

Incubation was performed at 22 °C for 30 min at atmospheric pressure (control) and under high hydrostatic pressure. The reaction was stopped by adding 0.2 ml trichloroacetic acid 50%. After centrifugation at 10000 g for 20 min, inorganic phosphate was determined in the supernatant by the method of Fiske & Subbarow (1925). Protein content of the enzymic extract was determined by the method of Lowry et al. (1951). Results are expressed as micromoles of inorganic phosphate per mg of proteins per hour (μmole Pi/mg prot/h). The activity of the (Na⁺ + K⁺)ATPase was also estimated by calculating the difference between the enzymic activities in the presence ((Mg²⁺, Na⁺ + K⁺)ATPase) and absence ((Mg²⁺ATPase)) of Na⁺ and K⁺ in the reaction medium. Mg²⁺ATPase and (Na⁺ + K⁺)ATPase activities were found to be nearly the same as the 'ouabain insensitive' and 'ouabain sensitive' ATPase activities respectively. For simplicity we have used the terminology Mg²⁺ATPase and (Na⁺ + K⁺)ATPase even though we have used ouabain.
Fig. 3. Typical records of the effects of high hydrostatic pressures (in kg/cm²) on the potential difference (PD) of the isolated frog skin bathed, on both sides, by NaCl Ringer’s solution. Pressure is applied at Cp, removed at D. The magnitude of the two types of potential changes is measured as shown by the arrows: A and C respectively fast depolarization and hyperpolarization (see the text). $B_1$ and $B_2$ respectively sustained hyperpolarization and depolarization (see the text).
RESULTS

A. Effects of hydrostatic pressures on the potential difference

Fig. 3 shows a series of typical records of pressure-induced potential variations. Whatever the pressure applied, two main kinds of PD modifications are revealed: small and fast ones which seem to be correlated to the pressure changes, and large slower ones which persist as long as pressure is applied.

The fast depolarization and hyperpolarization are maximum between 200 and 500 kg/cm² and at 500 kg/cm² respectively (Fig. 4). Their magnitude, however, appears to vary greatly from one compression to the other when several identical pressure steps are consecutively applied.

With respect to the large PD changes, pressure steps up to 500 kg/cm² induce a hyperpolarization of the skin (Fig. 3). This hyperpolarization phase is maximum for pressures of 200–300 kg/cm² and remains stable during the whole compression except when pressure exceeds 400 kg/cm². At this pressure, a potential drop follows hyperpolarization (Fig. 4). At pressures higher than 500 kg/cm² the hyperpolarization is no longer seen; a large depolarization immediately prevails.

When a series of increasing pressure steps lower than 500 kg/cm² is applied instead of a single one, each kind of electrical response is still observed (Fig. 5). Moreover, in such experimental conditions and for each value of pressure considered, the skin potential seems to reach the level at which it would have been stabilized if the skin had directly been compressed at the same pressure.

The effects of pressure steps up to 600 kg/cm² on the PD appear to be quite reversible. Control PD values are resumed upon return of compressed skins to atmospheric pressure (Table 1 and typical records of Fig. 3). However, after compressing the skins between 600 and 800 kg/cm², the PD does not completely resume its initial control value. There is only a partial repolarization. The effects of pressure steps up to 500 kg/cm² are reversible, but above 800 kg/cm², the skin potential cannot be restored (Table 2 and lower curve of Fig. 5). In these experiments, 100 kg/cm² was chosen as 'control compression' because its effects have previously been proved to be stable, entirely reversible and reproducible.

B. Effects of hydrostatic pressure on the skin short-circuit current and on the activity of the skin ATPase system

(1) Short-circuit current measurements

Fig. 6 shows that a pressure step of 100 kg/cm² produces a large increase in the skin short-circuit current. Table 3 summarizes the maximum hyperpolarizations observed at 100 kg/cm² and the corresponding changes in the short-circuit current. Similar results have been obtained at 250 kg/cm².

(2) Activity of the membrane ATPase system

The short-circuit current measurements indicate that the Na⁺ active transport seems to be enhanced when pressure steps of 100 and 250 kg/cm² are applied to the frog skin. We have therefore studied the possible effects of pressure on the ATPases bound to the membranes of this tissue.

The total activity of the membrane ATPase (i.e. \((\text{Mg}^{2+}, \text{Na}^+ + \text{K}^+)\) ATPase 38
activity) of the isolated frog epidermis at the atmospheric pressure has been found to be $0.59 \pm 0.04 \mu$ moles Pi/mg proteins per hour (mean ± standard error, $n = 18$). 39.2 ± 2.2% of that activity can be inhibited by ouabain and corresponds to the $(\text{Na}^+ + \text{K}^+)$ATPase (cf. Materials and methods).

It appears that the $(\text{Na}^+ + \text{K}^+)$ATPase activity is not significantly affected at 100 or 250 kg/cm² (Fig. 7). On the other hand, pressure steps higher than 500 kg/cm² induce a progressive and reversible decrease in enzyme activity. The activity of an extract assayed at atmospheric pressure after compression up to 1000 kg/cm² for 15 min is not significantly different from that recorded without applying any pressure step.
Fig. 4. Variations of the frog skin potential difference as a function of the hydrostatic pressure when both faces of the skin are bathed with NaCl Ringer's solution. (a) Fast and transient PD changes. (b) Sustained hyperpolarization. Vertical lines indicate standard errors at each point and the number of animals is given in brackets.

**DISCUSSION**

Application of a pressure step to the frog skin induces PD modifications of two types. First, there are short and apparently transient variations which accompany application and release of pressure. Application of a pressure step induces a depolarization while a hyperpolarization is recorded upon release of the pressure. These PD changes are variable in magnitude, are not reproducible and show variable responses to changes in the ionic composition of the salines (Brouha, 1965; Brouha et al. 1970; Pequeux, unpublished data). This suggests that they do not reflect a specific effect of pressure on ionic permeabilities.

These potential variations are apparently short-lived. However, they could be related to a constant depolarization, superimposed on the larger more slowly-developing maintained hyperpolarization, as shown in Fig. 8. So far, the existence of such a square wave remains hypothetical since it has not yet been demonstrated in our experimental conditions even when using sulphate Ringer which is known to practically abolish the lasting hyperpolarization.

Whatever the real nature of the short-depolarization phase, its great variability
could be related to changes in amplitude and time constants of the mechanisms responsible for both short and lasting types of polarization changes.

It remains, however, very likely that a pressure step immediately affects the electromotive force (e.m.f.) of the skin considered as a series of electromotive concentration cells related by ionic transport phenomena. Little effect is to be expected on transport numbers (Hamann, 1957) but effects on activity coefficients and on the dissociation of complexes into ions might explain the overall e.m.f. change being linked to a volume restriction (Dobson & Firman 1972).

Application of a pressure step also induces sustained and important potential variations. These PD modifications are reproducible whatever the way pressure is applied provided it remains lower than 500 kg/cm².
Table 1. Reversibility of the effects of high hydrostatic pressures (kg/cm²) on the frog skin potential difference (mV)

(Pressure steps of 3 min. Student test: results are not significantly different if $P > 0.05$.)

<table>
<thead>
<tr>
<th>Applied pressure (kg/cm²)</th>
<th>Skin potential difference (mV)</th>
<th>Comparison (Student test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before compression</td>
<td>After compression and stabilization at the atmospheric pressure</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>67.1 ± 2.2 (n = 5)</td>
<td>64.1 ± 3.1 (n = 5)</td>
</tr>
<tr>
<td>100</td>
<td>54.6 ± 3.3 (n = 31)</td>
<td>53.3 ± 3.4 (n = 31)</td>
</tr>
<tr>
<td>200</td>
<td>58.4 ± 10.6 (n = 7)</td>
<td>57.2 ± 10.9 (n = 7)</td>
</tr>
<tr>
<td>300</td>
<td>38.5 ± 5.8 (n = 5)</td>
<td>34.1 ± 5.1 (n = 5)</td>
</tr>
<tr>
<td>400</td>
<td>34.1 ± 5.2 (n = 5)</td>
<td>30.7 ± 4.7 (n = 5)</td>
</tr>
<tr>
<td>500</td>
<td>42.1 ± 6.8 (n = 7)</td>
<td>37.6 ± 6.0 (n = 7)</td>
</tr>
<tr>
<td>600</td>
<td>25.7 ± 3.4 (n = 5)</td>
<td>17.2 ± 0.9 (n = 5)</td>
</tr>
<tr>
<td>700</td>
<td>17.2 ± 1.0 (n = 4)</td>
<td>8.6 ± 1.6 (n = 4)</td>
</tr>
<tr>
<td>800</td>
<td>34.4 ± 5.5 (n = 5)</td>
<td>11.6 ± 2.3 (n = 5)</td>
</tr>
<tr>
<td>900</td>
<td>10.8 ± 3.7 (n = 5)</td>
<td>4.3 ± 1.9 (n = 5)</td>
</tr>
<tr>
<td>1000</td>
<td>20.8 ± 10.7 (n = 5)</td>
<td>3.2 ± 1.8 (n = 5)</td>
</tr>
</tbody>
</table>

Table 2. Reversibility of the effects of high hydrostatic pressures (kg/cm²) P on the frog skin potential difference

(Comparison of typical 'control' sustained hyperpolarization (obtained by compressing the skin at 100 kg/cm² for 3 min) recorded before and after a compression at a higher pressure P. Successive compressions (at 100 kg/cm² and at pressure P) are each preceded by a resting period (5 min for stabilization of the skin potential) at the atmospheric pressure.)

<table>
<thead>
<tr>
<th>Pressure P (kg/cm²)</th>
<th>Before the compression at pressure P</th>
<th>After the compression at pressure P</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>+23.1</td>
<td>+24.7</td>
</tr>
<tr>
<td>300</td>
<td>+42.6</td>
<td>+42.9</td>
</tr>
<tr>
<td>400</td>
<td>+39.9</td>
<td>+46.5</td>
</tr>
<tr>
<td>500</td>
<td>+8.0</td>
<td>+7.3</td>
</tr>
<tr>
<td>1000</td>
<td>+21.3</td>
<td></td>
</tr>
</tbody>
</table>
Several attempts have already been made to explain the origin of the sustained hyperpolarization induced by a pressure of 100 kg/cm² and particularly by considering a modification of the components which generate the skin potential according to the Koefoed-Johnsen and Ussing model (1958) (Brouha et al. 1970; Pequeux, 1971, 1972, 1974). Accordingly, any pressure effects on the skin potential would have to be related to an action on concentration gradients or on relative permeability coefficients. It does not seem likely that the bulk concentration gradients are affected quickly enough to account for the large potential variation although one cannot exclude that
Polarization variations

Table 3. Effect of a pressure of 100 kg/cm² on the skin potential difference and short-circuit current

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Initial PD</th>
<th>Max. hyperpolarization under pressure</th>
<th>Initial SCC</th>
<th>Max. variation* under pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.9</td>
<td>17.8</td>
<td>23.7</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>23.0</td>
<td>17.0</td>
<td>12.5</td>
<td>10.8</td>
</tr>
<tr>
<td>3</td>
<td>26.9</td>
<td>34.0</td>
<td>52.5</td>
<td>9.2</td>
</tr>
<tr>
<td>4</td>
<td>39.2</td>
<td>14.0</td>
<td>36.7</td>
<td>8.3</td>
</tr>
<tr>
<td>5</td>
<td>42.2</td>
<td>14.0</td>
<td>24.7</td>
<td>7.0</td>
</tr>
<tr>
<td>n = 5</td>
<td>35.8±4.7†</td>
<td>19.4±3.7</td>
<td>30.0±6.8</td>
<td>8.3±0.8</td>
</tr>
</tbody>
</table>

* Positive variation corresponding to an increase of the short-circuit current.
† Mean ± standard error.

Fig. 7. Effect of high hydrostatic pressures on the frog skin ATPase system. Reaction medium: 4 mM-ATP, 5 mM-MgCl₂, 100 mM-NaCl, 25 mM-KCl (+EGTA and Tris-buffer pH 7.4) + ouabaine 0.2 mM (see the Methods). Ordinate: average percentage of variation calculated on n (number of experiments is given between brackets) absolute data obtained at atmospheric pressure and under pressure. Vertical lines indicate the standard error.

local concentration gradients could be created by pressure, thus modifying the skin potential.

The simplest interpretation of the observed potential variations is that they reflect changes in the relative values of the passive permeability coefficients. The sustained hyperpolarization recorded at 100 kg/cm² has indeed previously been shown to reflect
increases in the passive Na\(^+\) permeability of the outer-facing cell membranes of the skin and of the passive K\(^+\) permeability of the inner cell membranes and also to reflect a decrease in Cl\(^-\) permeability. However, our results support the idea that the skin also adjusts the activity of its Na\(^+\) active transport to these permeability changes in order to maintain the intracellular Na\(^+\) concentration at a normal level.

Indeed, when a pressure step of 100 kg/cm\(^2\) is applied to the skin, the short-circuit current greatly increases. According to Ussing's theory, this corresponds to an increase in the net active Na\(^+\) flux. It thus appears that, within this pressure range, the integrity of the active transport mechanism is maintained. Another argument in favour of this view is that the lasting hyperpolarization observed at 100 kg/cm\(^2\) decreases and generally disappears when the active transport has been inhibited by ouabain (Brouha et al. 1970) or 2:4 (α)-dinitrophenol (unpublished data).

The Na\(^+\) pump appears thus to be directly stimulated in response to an increase in passive permeability but would remain unaffected by pressures of 100 and 250 kg/cm\(^2\). This last point is substantiated by the fact that the activity of the (Na\(^+\) + K\(^+)\)ATPase remains unaffected by pressure steps of 100 and 250 kg/cm\(^2\). Another argument favouring this view is the fact that the hyperpolarization at 100 kg/cm\(^2\) does not appear when Cl\(^-\) is replaced by SO\(_4\)\(^2-\) in the incubating saline (Brouha et al. 1970). In this situation, the Goldman equation adapted by Hodgkin & Katz then becomes more nearly a Nernst equation since permeability coefficients disappear and the total skin potential can then be related almost entirely to the ionic concentration gradients.

It could be suggested that the potential variations result from the action of hydrostatic pressure on a Na\(^+\) pump which would operate electrogenically. This view remains however much contested, and we believe that there is a lack of evidence to

Fig. 8. Hypothetical decomposition of the pressure induced potential variations (lower curve) into a fast small depolarization (upper curve) and a slower large hyperpolarization (middle curve). Explanations in the text.
Polarization variations

extend such a model to any experimental condition, and that it is unprofitable to consider a hypothetical effect on a mechanism which is also hypothetical.

The above interpretations account satisfactorily for the effects of pressures up to 400 kg/cm². At higher pressures, however, our results suggest that another kind of perturbation has to be considered.

At 500 ± 100 kg/cm², a depolarization follows a transient hyperpolarization. This situation can be explained by considering that pressure immediately acts on passive permeabilities so as to induce the first hyperpolarization phase; the depolarization would reflect an action at another level which is closely bound to the maintenance of the skin potential difference. The mechanism most likely affected is the Na⁺ pump. The pressure induced potential fall (± 8 mV per minute) is of the order of magnitude of the change which would result from an increase in the intracellular Na⁺ content when the active output is inhibited. Similar results are obtained at atmospheric pressure using specific chemical inhibitors like ouabain (Brouha et al. 1970) and 2,4-(2)-dinitrophenol (Pequeux, 1974). According to this view at 500 ± 100 kg/cm² a transient hyperpolarization occurs but subsequently the Na⁺ pump activity is directly inhibited by pressure, causing the skin potential to decrease (depolarization). From 600 kg/cm² onwards, the potential immediately drops and the hyperpolarization does not appear. In this situation, the pump is inhibited immediately.

The above explanation of the pressure-induced depolarization agrees with the observed effects of pressure on the (Na⁺ + K⁺)ATPase activity (i.e. significant and strong decrease of the (Na⁺ + K⁺)ATPase activity from 500 kg/cm² on). It would thus appear that pressure, in this high range, directly affects the Na⁺ active transport by acting on the pump bound enzyme. The decrease in enzymic activity, however, does not reflect a pressure-induced denaturation of the enzyme since we have demonstrated that the membrane ATPase activity at the atmospheric pressure is not affected even after submitting the enzyme for some time to pressure steps up to 1000 kg/cm².

The decrease in activity at 500 and 1000 kg/cm² thus most likely reflects a pressure-induced decrease in the reaction speed. Little is known of the level at which hydrostatic pressure acts on enzyme activity: it is very likely that pressure could affect the native configuration or the allosteric sites by disturbing the amount of ionization.

In the light of this work, hydrostatic pressure seems to be a valuable tool in the study of mechanisms involved in ion transfer across biological membranes. The selectivity of its effects, for example, suggests different and spatially separated sites for the passage of anions and cations in the cell membrane.

Little can be said at the present time as to the molecular aspect of the pressure-induced changes in electrical potential. However, as hydrostatic pressure is known to favour phenomena involving a volume decrease such as the ionization of weak electrolytes including proteins, it seems logical to conclude that pressure enhances the passive permeability by modifying the structure of the channels involved in ion transport. In a next paper, an attempt will be made to approach this question by studying the effects of pH changes on the potential variations induced by pressure.

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