ON THE pH- AND TEMPERATURE DEPENDENCE OF THE *p*-NITROPHENYLPHOSPHATASE ACTIVITY OF INTACT EHRLICH ASCITES TUMOR CELLS

Monika LÖFFLER and Friedhelm SCHNEIDER
Physiologisch-chemisches Institut der Universität Marburg/Lahn, West Germany

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1. Introduction

In a previous communication [1] we have reported our experiments on the *p*-nitrophenylphosphatase activity of intact Ehrlich ascites (EAT) tumor cells and the effect of SH-blocking agents as well as the action of neuraminidase on this enzymatic activity at the outer surface of the intact cells. The nature and function of this phosphatase activity remains obscure although some authors have observed a phosphatase activity of purified plasma membranes [2]. They agree with other investigators [3] that the Na^+, K^+-ATPase and the K^+-stimulated *p*-nitrophenylphosphatase activities both may be manifestations of the same enzyme. Some later experiments from our laboratory and some recent publications on enzyme activities at the surface of intact EAT cells [4] suggest however that the *p*-nitrophenylphosphatase activity measured with purified plasma membranes [2] is not identical with the activity of intact cells which we are studying in our laboratory. In the present communication we describe our results on the further characterization of the phosphatase activity of the outer surface of intact EAT cells and its possible relation to the state of membrane components. We therefore have studied the pH- and temperature dependence of the activity and the effect of ouabain; furthermore we have tried to estimate the $K_{m\ app}$ for *p*-nitrophenylphosphate.

2. Materials and methods

2.1. *Substances*

p-nitrophenylphosphate (*p*-NPP), ouabain and buffer substances were purchased from Merck, Darmstadt. Pyruvate, NADH and ATP were from Boehringer, Mannheim.

2.2. *Cell suspensions*

Hyperdiploid EAT cells, grown in female NMR mice after the first passage in vitro were used for all experiments. For further details see [5]. Viability of the cells was tested by staining with nigrosin according to Kaltenbach [6].

2.3. *Chemical and enzymatic tests*

Measurement of *p*-NPPase activity was performed as described in [1]. Tris–HCl buffer (pH 6.3–8.2) containing 75 mM Na^+, 5 mM K^+, 2 mM Mg^{2+}, 20 mM cholin and 5 mM glucose was used. Before starting the reaction with *p*-NPP the cells were preincubated 10 min at the appropriate temperature. For the experiments in the presence of ouabain and ouabain + ATP the cells were preincubated with 0.1–5 mM ouabain and 0.5 mM ATP respectively 10 min before *p*-NPP was added. The reaction was stopped after 7.5 and 15 min by centrifugation of the cells at 0°C. Proteins delivered from damaged cells were precipitated with perchloric acid and determined by the

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microbiuret method [7]. Lactate dehydrogenase (LDH) in the supernatant was determined after addition of 1.2 mM pyruvate and 0.5 mM NADH by following the change in optical density at 366 nm. The pH dependence of the LDH of the intact cells was determined after ultrasonic disruption of the cells.

3. Results and discussion

3.1. *Effect of ouabain on the phosphatase activity of the intact EAT cells*

It is well known that Na^+, K^+-ATPase is strongly inhibited by ouabain. Only a slight but reproducible inhibition of a Mg^{2+} and Ca^{2+} stimulated ATPase by ouabain on the other surface of ascites cells was recently reported by Ronquist et al. [4] who pointed out that ATP hydrolysing activities other than the Na^+, K^+-ATPase might exist in the plasma membrane of EAT cells. We have therefore tested the effect of this inhibitor on the phosphatase activity of the intact cell in order to get further information on the possible relationship between this enzyme and the Na^+, K^+-ATPase. Concentrations of ouabain between 0.1 and 5 mM did not significantly affect the phosphatase activity, the $K_{m\ app}$ of which with *p*-nitrophenylphosphate as substrate was 5 mM at 25°C and pH 7.0. On addition of 1–5 mM ATP the stimulation of the *p*-NPPase activity described in our previous [1] paper was also not impaired by ouabain. This lack of inhibition by ouabain is a further argument that the activity is not part of the Na^+, K^+ stimulated ATPase of the plasma membrane.

3.2. *The pH dependence of the phosphatase activity*

p-NPPase activity of intact EAT cells can readily be measured by incubating living cells with the substrate in an appropriate isotonic buffer [1]. To further characterize the enzyme we have studied the pH dependence because this is a useful criterion for the classification of unspecific phosphatases. Concerning the *p*-NPPase activity of intact cells, it is important to differentiate it if possible from other phosphate ester hydrolyzing activities, particulary the Na^+, K^+-ATPase. Because the incubation of living cells in buffers of various pH values raises some problems, only the pH range 6.3–8.2 was investigated; the number of damaged cells and enzyme activities in the supernatant were thoroughly controlled (see below). The results of these experiments are demonstrated in fig.1. One recognizes that the phosphatase at the cell surface should be regarded as an acid phosphatase. The pH dependence makes it rather unlikely that the activity is part of the Na^+, K^+-ATPase since the pH optimum for this enzyme in the plasma membrane is about 7.5–8.0 [2,3,8].

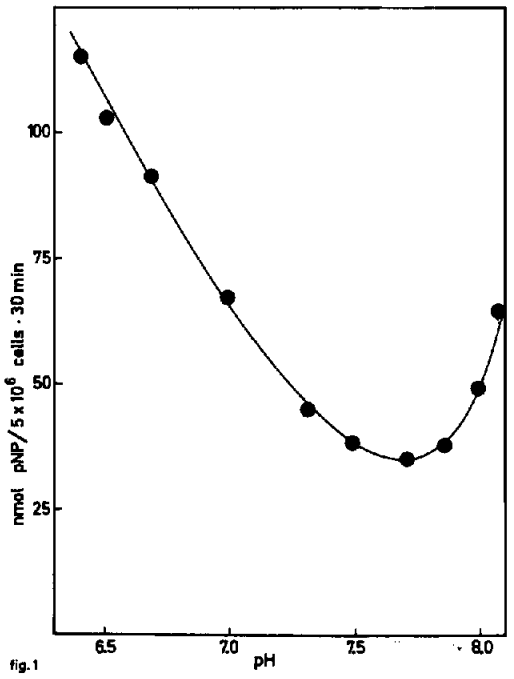
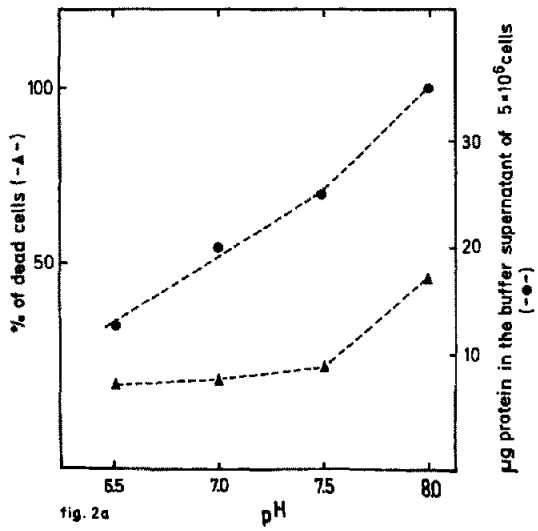


Fig.1. pH dependence of the *p*-NPPase activity of intact EAT cells.

3.3. *Control of the viability of the cells, the LDH activity and protein content of the supernatant*

The number of dead cells at different pH values, μg protein in the supernatant of the incubation medium, activity of LDH in the supernatant and in a cell homogenate are given in fig.2 a and b. These criteria were used to test how much of the phosphatase activity may originate from leaky or lysed cells. The results of these experiments reflect the well known fact that mammalian cells are generally more sensitive to alkaline than to acidic pH values. The increasing amount of protein in the supernatant with increasing pH is presumably at least partially derived



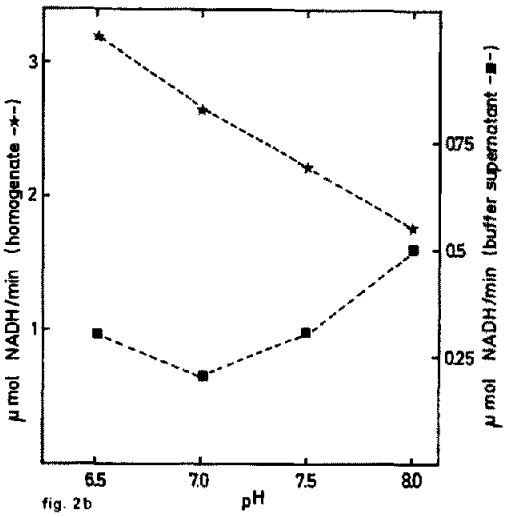
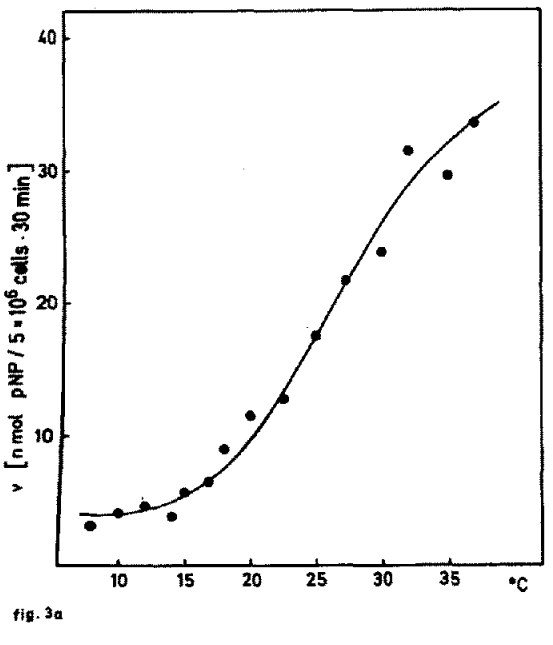


Fig.2a. Number of dead cells at various pH values (-▲-) and µg protein in the supernatant (-●-). Fig.2b. pH dependence of LDH-activity in the supernatant of 5 X 10^6 cells (-■-) and in the cell homogenate of 5 X 10^6 cells (-★-).



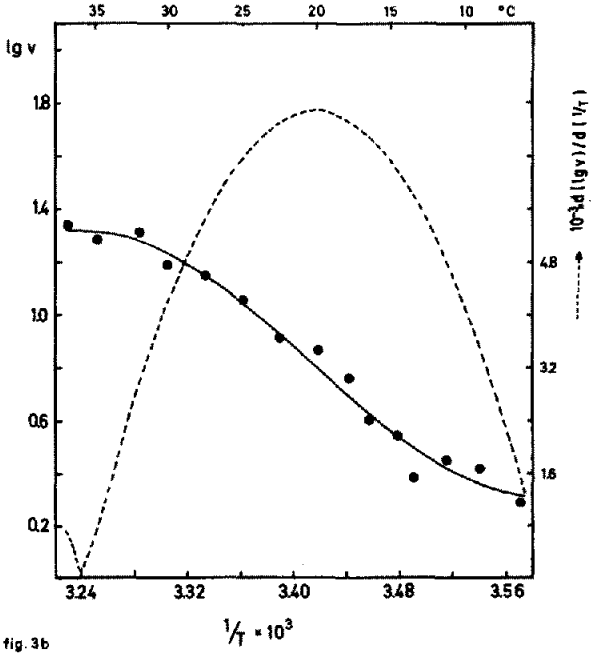


Fig.3a. Temperature dependence of the *p*-NPPase activity of intact EAT cells. Each point represents the mean of 15–25 individual measurements. Fig.3b. Arrhenius plot of the temperature dependence of the phosphatase activity of intact EAT cells.

from the surface of the cells and not only from the interior. Similar observations for the case of certain glycolytic enzymes were recently reported by Wernstedt et al. [9]. The most unequivocal test for leaky cells is considered to be the measurement of glycolytic enzymes in the supernatant. Therefore we have chosen to measure the LDH activity in the supernatant. In fig.2b the pH dependence of the LDH activity in the supernatant is compared with the total activity of a cell homogenate of the same number of cells. From these experiments it follows that a part of the *p*-NPPase activity above pH 7.5 seems to originate from the interior of the damaged cells, whereas the acidic limb actually represents only the activity of the intact cells.

3.4. *Temperature dependence of the phosphatase activity of intact EAT cells*

Since the phosphatase of the intact cells is a membrane-bound enzyme the temperature dependence of the activity is of especial interest in connection with problems of membrane structure and flexibility. There are several investigations in the literature on the temperature dependence of membrane-bound enzymes which reflect transition processes or lateral phase separations in the lipid structures of membranes [10]. The temperature dependence of the phosphatase activity of intact EAT cells between 8°C and 37°C is illustrated in fig.3a. As can be recognized the temperature dependence gives a nonlinear plot. The data shown in fig.3a were used to construct an Arrhenius plot; the solid line was calculated with the aid of a Fortran program which fits a 4th degree polynomial to the data. The inflection point of the graph could then be readily determined by calculating the first derivative (dashed line) of the polynomial. The calculated inflection point corresponds to a temperature of 20°C. Comparable results were found in several other studies on the temperature dependence of membrane-bound enzymes. It is believed that this phenomenon results from changes in the interactions involving the lipids and proteins of the membrane [10,11]. The measurement of the phosphatase activity of intact cells seems to be therefore a very convenient method for studying temperature dependent changes of the membranes.

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