THE EFFECT OF UNCOUPLING AGENTS ON THE pH GRADIENT ACROSS THE PLASMA MEMBRANE OF THE EHRLICH ASCITES TUMOR CELL

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Received June 18, 1968

The chemiosmotic hypothesis of photosynthetic and oxidative phosphorylation (Mitchell, 1961) postulates that a pH gradient produced across a coupling membrane is the immediate driving force for phosphorylation and that uncoupling agents act by rendering the membrane permeable to H^+, thereby destroying the pH gradient. Some support for this hypothesis has come from studies of artificial phospholipid bilayer membranes (Hopfer, Lehninger, and Thompson, 1968). Uncoupling agents increase the conductance of these membranes in a manner interpreted as facilitation of transport of H^+ or OH^- or both.

These reports suggested a study of the effect of uncoupling agents on the pH gradient across the plasma membrane of cells. An appropriate object for a study of this kind is the Ehrlich ascites tumor cell, the intracellular pH of which can be readily measured from the distribution between intracellular and extracellular water of the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), when the cells are suspended in buffers (Poole, 1967). The present studies with 2,4-dinitrophenol (DNP) and bishydroxycoumarin have shown that both of these uncoupling agents abolish or greatly reduce the pH gradient across the cellular membrane in glycolyzing cells but not in non-glycolyzing cells. In both glycolyzing and non-glycolyzing cells, intracellular pH is lower in cells incubated with uncoupling agents than in control cells.

METHODS. The propagation, harvesting, and washing of the tumor have been described (Poole, Butler, and Waddell, 1964) except that the suspending buffers in the present experiments were 25 mM phosphate buffers of pH 7.4, 6.2, or 7.7. The cells incubated in buffers of pH 6.2 and 7.7 were washed with cold isotonic saline rather than with the buffer. The incubation, sampling, measurement of extracellular pH (pH_e), calculation of intracellular pH (pH_i), and determination of lactate concentration in the suspending medium have been described in detail elsewhere (Poole, 1967). Any deviation from these basic procedures is noted in the figure legends. In each experiment, the same suspension of cells was divided into two parts. The uncoupling agent was added to one part while the other served as control. Each experiment thus contains its own control, and any difference between the preparation containing the uncoupling agent and its control is significant if that difference exceeds the experimental error of the method.

RESULTS. In experiments with glycolyzing cells, as shown in Fig. 1 A and B, pH_i is significantly lower in cells incubated with either DNP or bishydroxycoumarin than in control cells, and lactate concentration in the suspending medium is greater. With both uncoupling agents the pH gradient across the membrane is either greatly reduced as compared with control values or disappears entirely. Of 25 sets of samples studied in 8 experiments, 20 showed a pH gradient of less than 0.1 pH unit. In the corresponding controls only 12 sets had a pH gradient of less than 0.1 pH unit.

Fig. 2 A and B show the results of an experiment in which the pooled cells were first washed in cold isotonic saline and then resuspended in either pH 6.2 or pH 7.7 phosphate buffer. Samples were removed at the time intervals shown after the addition of DNP. In all 5 experiments in this series there was immediate buffering of the pH of the medium. The

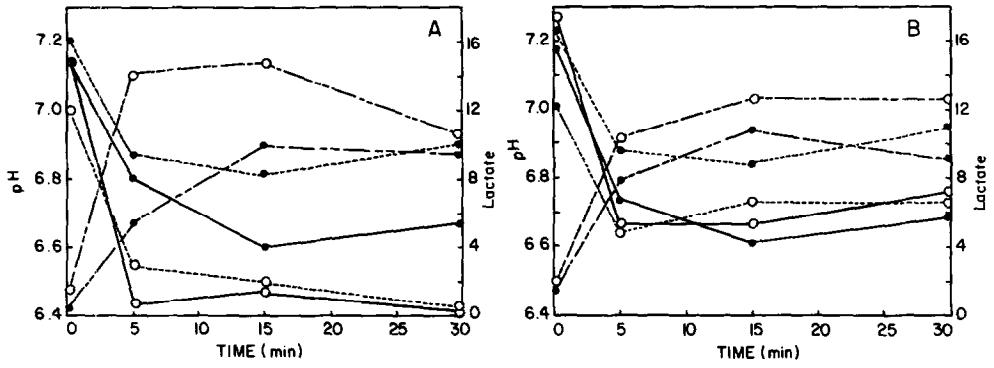

FIG. 1. Intracellular pH (pH_i) and extracellular pH (pH_e) and extracellular lactate concentration in suspensions of Ehrlich ascites tumor cells in 25 mM Krebs-Ringer phosphate buffer of initial pH 7.4 (approximately 30% cells by volume). The uncoupling agent is 0.3 mM DNP in Fig. 1A and 0.1 mM bishydroxycoumarin in Fig. 1B. Glucose was added immediately after the zero time sample to a final concentration of 5.5 mM to both uncoupled and control cell suspensions. Open circles represent pH_e (O——O), pH_i (O·····O), and lactate concentration (O— - —O) of the uncoupled cell suspension. Closed circles represent pH_e (●——●), pH_i (●·····●), and lactate concentration (●— - —●) of the control cell suspension.

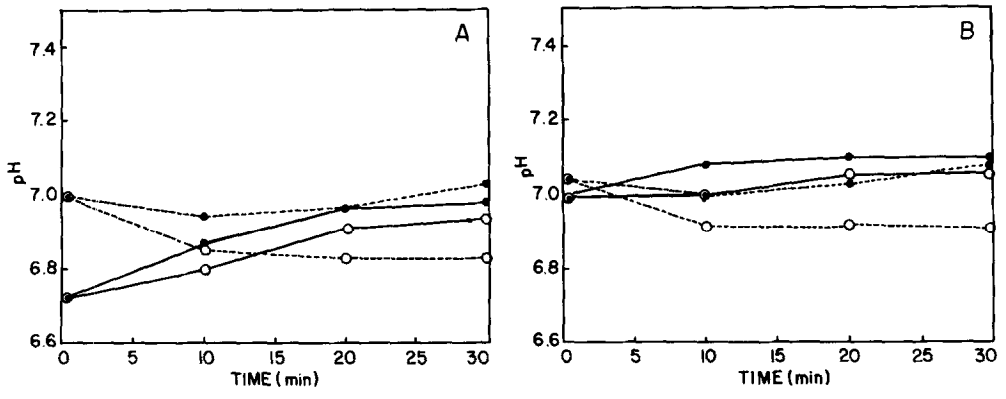

FIG. 2. pH_i and pH_e in suspensions of non-glycolyzing Ehrlich ascites tumor cells washed in cold saline and resuspended in 25 mM Krebs-Ringer phosphate buffer of initial pH 6.2 (A) or 7.7 (B). Suspension contains approximately 30% cells by volume. The uncoupling agent is 0.3 mM DNP, added at time zero. Open circles represent pH_e (O——O) and pH_i (O·····O) of the uncoupled cell suspension and closed circles represent pH_e (●——●) and pH_i (●·····●) of control cell suspension.

pH of the acid buffer was raised 0.4-0.6 pH unit and that of the alkaline buffer lowered 0.6-0.8 pH unit. There was no significant or consistent difference between the pH values of the control and experimental media in either the high or low pH buffer. Consistently, however, the pH_i of cells incubated in the presence of DNP in either buffer was significantly lower than that of the control cells. This same pattern was shown when bishydroxycoumarin was used as the uncoupling agent. In no case was there a significant decrease in the pH gradient across the membrane in the presence of either of the uncouplers.

DISCUSSION. As was found in an earlier study (Poole, 1967), in the absence of uncouplers the relationships between pH_e and pH_i in glycolyzing cells are quite similar to those found for non-glycolyzing cells. For the low values of pH_e encountered in glycolysis, this represents a considerably higher value of pH_i than of pH_e. However, in the presence of the uncouplers, there is very little difference between pH_i and pH_e.

As is shown in Fig. 2, addition of cells to buffers of high or low pH brings about large changes in the pH of the buffers without significant change in the internal pH of the cell. This is presumably due to hydrogen ion exchanges with materials on the surface of the cells. The fall of pH_i produced by uncouplers in non-glycolyzing cells could be caused by accumulation of hydrolysis products of high energy phosphate compounds. The failure of the uncouplers to abolish the pH gradient in non-glycolyzing cells is indicative that the abolition of that gradient in glycolyzing cells cannot be due to a generalized and nonspecific increase in the permeability of the membrane to H^+.

As has been discussed by Butler, Waddell, and Poole (1967), if a membrane is permeable to both the proton-donor and proton-acceptor species of an acid or base, an equilibrium state in which there is a pH gradient across the membrane is not possible. Continuous addition of or

removal of H^+ from one side would be required for maintenance of a steady state in which there is a pH gradient. Although it has been generally assumed that the concentration of undissociated phosphoric acid is too low to be of significance in passage across membranes, and the phosphate ion has often been referred to as a "permeant" ion, the ionic state of the permeant species of orthophosphoric acid has actually not been identified. There is much evidence that organic acids in general penetrate cellular membranes only in their undissociated forms. This is true even for an acid as strong as salicylic acid, with a pK of 3, which comes to rapid equilibrium across cellular membranes even when the concentration of the undissociated form is as low as 10^{-8} M, approximately the concentration of undissociated phosphoric acid in mammalian plasma. The maintenance of a pH gradient across cellular membranes is most readily comprehensible if it is assumed that the membrane is permeable only to undissociated phosphoric acid and impermeable both to the univalent and the bivalent phosphate ions. The abolition of the pH gradient in glycolyzing cells but not in non-glycolyzing cells might be explicable as an action of the uncoupling agents in rendering the cellular membranes permeable to the lactate ion but not to the phosphate ion.

This investigation was supported by Public Health Service Research Grant GM 13606 from the National Institutes of General Medical Sciences.

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