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THE HYPERPOLARIZING AND DEPOLARIZING EFFECTS OF 2,4-DINITROPHENOL ON EHRLICH CELLS

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Summary

The ability of glucose to reverse the effects of dinitrophenol on amino acid uptake in Ehrlich cells is a function of pH. At pH 6.0, the presence of glucose does not reverse the inhibitory action of the uncoupler. Nearly complete restoration occurs with glucose at pH 7.4. At pH 8, the presence of glucose may cause a modest increase in amino acid uptake in presence of dinitrophenol. At all pH values, glucose restores ATP and cellular K^+ to the control levels at the same pH. Although the cytoplasmic pH changes with changes in the external pH, the cell interior is more alkaline than the medium near pH 6.0 and more acid than the medium at pH 7.8 even after 45 min incubation at 37°C. With dinitrophenol and in presence of glucose the difference in pH between the medium and the cell is minimal at both pH 6.0 and 7.8.

It has been known for some years that 2,4-dinitrophenol does not affect amino acid transport in Ehrlich cells when glucose is also present [1—3]. These data have been interpreted as showing that the high glycolytic rates characteristic of the Ehrlich cells restored ATP levels to normal, thereby overcoming the inhibitory effects of dinitrophenol. It was shown that with glucose and dinitrophenol, ATP levels can be maintained which are similar to those in control cells [3].

Recently several groups [4—8] have concluded that a membrane potential may be an important component of the driving force for amino acid uptake in Ehrlich cells. That Na^+-dependent amino acid uptake is electrogenic in Ehrlich cells and plasma membrane vesicles as well as other systems has been widely accepted [4—17].

Since dinitrophenol is considered to increase proton permeability [18,19], the question may be raised whether dinitrophenol can cause changes in the

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; TPMP, triphenylmethylphosphonium bromide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

membrane potential, and therefore, whether glucose addition would overcome dinitrophenol inhibition of amino acid transport when dinitrophenol is likely to enhance depolarization (medium pH < cell pH). Conversely, the question is whether dinitrophenol, in the presence of glucose, would enhance amino acid transport via a hyperpolarization when medium pH > cell pH.

Studies by Poole [20] have shown that a pH difference may exist across the Ehrlich cell membrane during glucose metabolism when the medium acidifies more rapidly than the cell interior. Navon et al. [21] have shown with NMR spectroscopy and 'pH jump' experiments that the pH of the cell interior does not adjust to that of the medium for 50—60 min. We have therefore examined the effects of dinitrophenol on amino acid uptake with and without glucose and dinitrophenol between pH 6.0 and pH 8.0, knowing that freshly isolated cells have an internal pH near 7.0 [20].

Materials and Methods

Cells were prepared and used as described earlier [3]. The buffer in these experiments was a mixture of 1,3 bis [tris (hydroxymethyl)-methylamino] propane and *N*-2 hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (Bis-Tris/HEPES) over the entire pH range. ATP was assayed by a modification of the method of Stanley and Williams using a commercial preparation of firefly extract [22].

Ions were measured by flame photometry using Li^+ as internal standard. Potential changes were measured using the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide as described earlier [8]. Uptake of ^3H-labeled triphenylmethylphosphonium bromide (TPMP) was also measured using routine procedures for uptake except that the cells were separated from the medium without washing. Duplicate samples were incubated with [^{14}C]dextran to correct for extracellular space.

Internal pH was measured using ^{14}C-labelled dimethyl oxazolidine 2,3 dione. The pH was calculated from the distribution of this compound using a p*K* of 6.13 as reported [23]. Corrections for extracellular space were made with [^{14}C]dextran.

[^3H]TPMP was a generous gift from Dr. R.H. Kaback, Roche Institute of Molecular Biology, Nutley, N.J. All other radioisotopes were purchased from New England Nuclear, Boston, Mass. Reagents were purchased from Fisher Scientific, Montreal, Canada. Firefly extract was obtained from Sigma Biochemicals, St. Louis, Mo. and the fluorescent carbocyanine dye was obtained from P. Laris, Santa Barbara, Calif.

Results and Discussion

The results in Fig. 1 show that (a) at pH 7.4, 7.0 or 6.5, 2,4-dinitrophenol reduces glycine uptake by 50% (or more) at all pH values. In line with earlier observations [24] transport activity decreases as the pH decreases below pH 7.4. (b) Addition of glucose at pH 7.4 or 7.0 restores uptake to within 80% of the control level (no glucose or dinitrophenol). The presence of glucose generally has no effect on glycine uptake in freshly prepared Ehrlich cells [25]. (c)

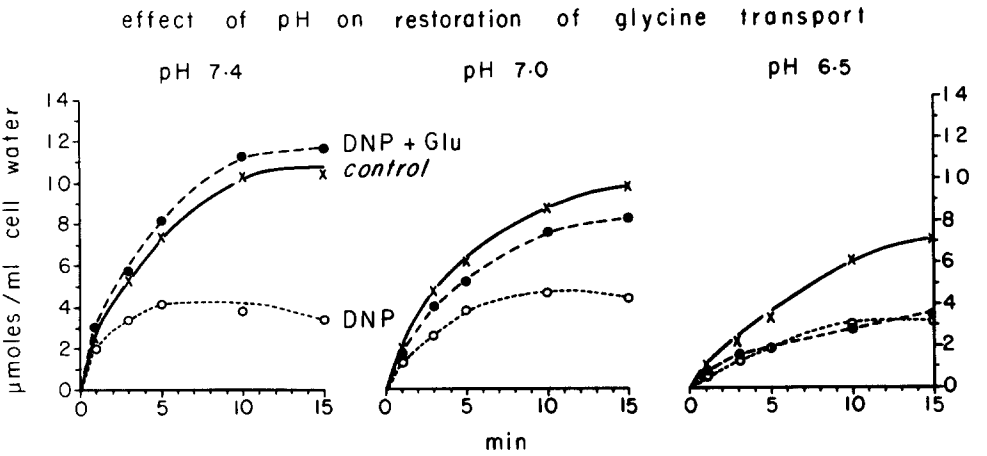

Fig. 1. Incubation was carried out with shaking at 37°C in a Ca^{2+}-free Krebs Ringer medium. The buffer was 10 mM Bis-Tris/HEPES at the pH given. [1-^{14}C]glycine was used at a final concentration of 1 mM, specific activity 300 cpm/nmol. The cell cytocrit was approximately 2%. With this cytocrit, little pH change occurs in the medium during the incubation period. After an equilibration period of 5 min at 37°C dinitrophenol (DNP) was injected. 2 min after dinitrophenol, glucose (Glu) was injected followed by [1-^{14}C]glycine 4 min after dinitrophenol. Uptake of glycine was terminated by adding 1 ml of the suspension to 10 ml cold, isotonic NaCl and rapidly centrifuging [3]. The glucose concentration was 10^{-2} M and dinitrophenol was 10^{-4} M. ●- - - - -●, dinitrophenol + glucose; X———X, control; ○- - - - -○, dinitrophenol.

Below pH 7.0 glucose addition does not restore transport to levels approaching those in the control. At pH 6.5, the effect of glucose is variable. In some experiments, there is little restoration by glucose (as seen in Fig. 1). In others, glucose addition may double the uptake seen with dinitrophenol (as seen in Table I). At pH 6, uptake with glucose and dinitrophenol is within 10% of the uptake seen with dinitrophenol alone (10 experiments).

Above pH 7.4, the addition of glucose in presence of dinitrophenol results in modestly higher (10—25%) glycine uptake than that seen in the corresponding control. Of ten experiments done at pH values above 7.4, glycine uptake with glucose and dinitrophenol was never less than the corresponding control and greater than control in 80% of cases. In two experiments at pH 7.4, the difference was experimentally small (≤10% difference) but larger with dinitrophenol.

At all pH values examined, the presence of glucose restored the cellular K^+ and ATP levels in the dinitrophenol-treated cells to those observed in the controls without dinitrophenol. Tables I and II show representative values for the restoration by glucose of cellular K^+ levels and ATP levels at different pH values. In Table I it may be seen that at pH 6.5 with dinitrophenol, glucose restores the cellular K^+ level to 87% of the control at 15 min whereas glycine uptake is only 57% of the control level. Although most studies were conducted with dinitrophenol, similar results were obtained with carbonylcyanide *m*-chlorophenylhydrazone (CCCP). For example, after 5 min incubation, the glycine uptakes at pH 6.0 were: control, 1.12 μmol/ml cell water; with glucose, 1.17; with $3 \cdot 10^{-5}$ M CCCP, 0.45; and with CCCP and glucose, 0.37.

Since ATP and K^+ levels were restored by glucose at pH values below 7.0, the reason for the persistance of the reduced glycine uptake was examined. The

TABLE I

ACTION OF DINITROPHENOL AND GLUCOSE ON CELLULAR K^+ AND GLYCINE UPTAKE IN EHRLICH CELLS

At pH 8.0, 7.4 and 6.5 the cellular concentrations of [^{14}]glycine at 15 min were 7.0; 8.4 and 5.5 times greater than the concentration in the medium. Cells were incubated as described in Fig. 1. Uptake of glycine and cell K^+ levels were computed as a percentage of the uptake in the corresponding control (no glucose, no dinitrophenol) at the same pH and the corresponding time of incubation. Measurements of the pH of the incubation medium showed that the medium pH did not change significantly during the experiment except at pH 8.0 in presence of glucose and dinitrophenol where the pH at the end of the incubation was 7.7. K^+ was measured by flame photometry.

pH	Time (min)	Glycine uptake (% control)		Cellular K^+ (% control)	
		Dinitrophenol	Dinitrophenol + glucose	Dinitrophenol	Dinitrophenol + glucose
8.0	5	67	125	68	92
	10	50	136	—	—
	15	41	125	41	98
7.4	5	42	109	54	91
	10	32	115	—	—
	15	23	117	57	100
6.5	5	27	54	69	81
	10	32	62	—	—
	15	23	57	50	87

possibility that dinitrophenol was collapsing the membrane potential below pH 7.0 and increasing it (albeit slightly) above 7.4 was considered. The membrane potential was examined in two ways: (1) uptake of [^{3}H]TPMP, (2) with a fluorescent probe, 3,3'-dipropylthiadicarbocyanine iodide. The results in Table III show that uptake of [^{3}H]TPMP is reduced by dinitrophenol at both pH 6.0 and 7.8, but that at pH 6.0 glucose does not enhance [^{3}H]TPMP uptake in presence of dinitrophenol. In experiments with [^{3}H]TPMP, the cells must be incubated for at least 30 min to achieve a steady-state distribution of the cation (un-

TABLE II

RESTORATION OF CELLULAR ATP LEVELS BY GLUCOSE AT DIFFERENT pH VALUES

Cells were incubated at 37°C in a Ca^{2+}-free Krebs Ringer medium with Bis-Tris/HEPES buffer at the three different pH values given. Cells were incubated for 2 min with dinitrophenol (10^{-4} M) and then glucose (10^{-2} M final concentration) was added. After 5—15 min exposure to glucose, samples were taken for ATP analyses. 1-ml samples were injected into cold trichloroacetic acid to give a final concentration of 5% trichloroacetic acid. Although not shown, complete restoration of ATP was also seen with glucose at pH 7.0.

Conditions	Cellular ATP concentration (mM)					
	6.0		6.5		7.4	
	5 min	15 min	5 min	15 min	5 min	15 min
Control	1.81	1.51	4.40	4.40	4.60	4.40
+ 10^{-4} M dinitrophenol	0.26	0.15	0.32	0.21	0.46	0.26
+ 10^{-4} M dinitrophenol + glucose	1.70	2.30	4.60	4.60	4.00	3.70

TABLE III

UPTAKE OF TPMP BY EHRLICH CELLS

Cells were incubated in a Ringer medium containing Bis-Tris/HEPES buffer at pH 6.0 and 7.8 with [^{3}H]-TPMP ($350 \cdot 10^3$ cpm/ml) at 37°C for 30 min. The glucose concentration was 10 mM and dinitrophenol was 10^{-4} M. Parallel samples were incubated with [^{14}C]dextran to correct for the extracellular space which ranged from 36 to 38% of the wet weight in these experiments. After incubation the cells were centrifuged, superficial fluid removed and the tubes weighed. The cell pellets were extracted with 5% trichloroacetic acid and counted. Samples of the supernatant adjusted to contain 5% trichloroacetic acid were also counted.

Additions	pH	[^{3}H]TPMP accumulation $\frac{\text{(cpm/ml cell water}}{\text{cpm/ml medium)}}$	Estimated potential difference (mV)
1			
None	6.0	5.1	—42.5
Glucose	6.0	3.6	—33.4
Dinotrophenol	6.0	1.6	—12.2
Dinitrophenol + glucose	6.0	1.6	—12.2
None	7.8	10.2	—60.5
Glucose	7.8	10.9	—62.2
Dinitrophenol	7.8	1.3	—7.0
Dinitrophenol + glucose	7.8	6.3	—48.0
2			
None	7.8	4.9	—41.4 *
Glucose	7.8	4.8	—41.0
Dinitrophenol	7.8	2.0	—18.0
Dinitrophenol + glucose	7.8	5.0	—42.0

* Cells, preincubated for 60 min at 37°C in Krebs Ringer at 1 : 300 dilution, were used for this experiment.

published observations). The lack of complete restoration by glucose at pH 7.8 may be a consequence of the additional time of incubation. However, if cells are preincubated at high dilution (a procedure which results in depletion of cellular amino acids and depolarization of the cells (ref. 8 and Laris et al., submitted to Biochim. Biophys. Acta)) a restored uptake of TPMP may be seen with glucose and dinitrophenol (Expt. 2). Initially, glycine uptake was 25% greater with dinitrophenol and glucose than the control uptake in these experiments.

In studies with the fluorescent cyanine dye, it was shown that with glucose and the uncoupler CCCP (at high dilution) cells tend to depolarize on the acid side (increased fluorescence) and hyperpolarize on the alkaline side (decreased fluorescence) (Table IV). For fluorescence studies CCCP was used because at high dilution, unlike dinitrophenol, it does not interfere with the fluorescence of the dye itself. It should be noted that these measurements were carried out less than 5 min after CCCP addition so that a direct comparison with [^{3}H]-TPMP distribution after 30 min with dinitrophenol is not valid.

The data with the fluorescence measurements and TPMP uptake suggest that at alkaline pH, but not at acid pH, the addition of glucose will repolarize cells treated with uncoupler.

The data on glycine transport and on membrane potentials suggest that the proton distribution is not at equilibrium under control experimental conditions. They suggest that when cells are introduced into a medium at pH 6 the cell interior is more alkaline for the duration of these experiments and that in a

TABLE IV

ACTION OF CCCP ON FLUORESCENCE CHANGES AT DIFFERENT pH VALUES

Cells were preincubated at 37°C for 30 min prior to measuring fluorescence changes. For measurement of fluorescence the cell cytocrit was approximately 0.3%. The cells were suspended in Ca^{2+}-free Krebs Ringer solution. Bis-Tris/HEPES buffer at 10 mM was used over the entire pH range. Measurements of dye fluorescence were described earlier [8] using 3,3'-dipropylthiadicarbocyanine iodide. After obtaining a steady baseline of fluorescence, uncoupler was injected and the percent change in fluorescence noted. A positive change indicates depolarization relative to the initial level and a negative change indicates hyperpolarization. The uncoupler used was CCCP at 10^{-8} M. Glucose was present in all conditions. In absence of glucose, only a transient hyperpolarization was obtained above pH 7.0 on addition of uncoupler, followed by depolarization (increased dye fluorescence). At acid pH values, depolarization was observed with and without glucose. The changes reported above were maintained for 5 min. Results of a typical experiment are shown.

pH of medium	Percent change in fluorescence after addition of uncoupler
6.1	+8.0
6.5	+5.0
6.9	+4.0
7.3	+1.0
7.5	—0.5
7.7	—2.0
8.0	—4.0

medium near pH 8.0 the interior is more acid. Experiments with radioactive dimethyloxazolidine 2,4 dione are consistent with this conclusion (Table V).

From these data it cannot be concluded that Ehrlich cells maintain a pH difference for the long term, but rather that for the duration (12 min) of these 'pH jump' experiments a pH difference is maintained. However, under the present experimental conditions after 45 min incubation, a pH difference in control cells (no glucose, no dinitrophenol) of 0.4 to 0.5 pH units (inside alkaline) was still maintained at pH 6.0, whereas at pH 7.8, the pH difference was only 0.1—0.2 pH units (inside more acid) (not shown).

The possibility exists that the low internal pH in presence of glucose and

TABLE V

CELLULAR pH CHANGES WITH AND WITHOUT DINITROPHENOL

Cells were incubated as in Table III. After a warm-up period of 10 min, dinitrophenol was added ($3 \cdot 10^{-4}$ M) followed by glucose (10 mM final) 2 min later. ^{14}C-labeled dimethyl oxazolidene 2,4 dione was added 2 min after the glucose and samples were taken at 2 and 12 min. The cells were centrifuged and aliquots of the medium and the pellet were taken for counting. The adherence of medium water was corrected using [^{14}C]dextran as a marker of the extracellular space in parallel experiments.

Additions	Medium pH	Cell pH at	
		2 min	12 min
None	6.00	6.4	6.3
Glucose	6.00	6.4	6.3
Dinitrophenol	6.00	6.0	5.8
Dinitrophenol + glucose	6.00	6.1	6.1
None	7.80	7.4	7.5
Glucose	7.80	7.4	7.5
Dinitrophenol	7.80	7.4	7.3
Dinitrophenol + glucose	7.80	7.6	7.6

dinitrophenol inhibits glycine uptake. Although this possibility cannot be eliminated it appears unlikely because with glucose present the difference in internal pH with and without dinitrophenol is quite small (0.2 pH units, in Table V, compare line 2 with line 4). In fact, although with dinitrophenol the presence of glucose may increase cellular pH from 5.8 to 6.1, there is no increase in glycine uptake at pH 6.0 (see above).

These experiments are consistent with the conclusion that the ability of glucose to restore glycine transport in presence of dinitrophenol is a function of the pH of the medium. At acid pH, even in presence of glucose (below 7), dinitrophenol depolarizes the cell and a membrane potential (inside negative) cannot be maintained or is reduced compared to the control level. In contrast, at alkaline pH (above 7.4), the presence of dinitrophenol may enhance transport of glycine in presence of glucose since dinitrophenol may increase the membrane potential (at least transiently).

The effects with dinitrophenol are reminiscent of the actions of K^+ conductors on the membrane potential and amino acid uptake in Ehrlich cells. Valinomycin [7,8] and propranolol [26] have hyperpolarizing and depolarizing actions on the cell membrane at low and high medium K^+, respectively. Amino acid uptake is increased with hyperpolarization and decreased with depolarization with valinomycin [4] and propranolol [26,27].

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