RAPID RELEASE OF FREE FATTY ACIDS DURING CELL BREAKAGE AND THEIR EFFECTS ON A SUGAR–PROTON COTRANSPORT SYSTEM IN *CHLORELLA VULGARIS*

Michael DECKER and Widmar TANNER

Botanik I, Fachbereich Biologie und Vorklinische Medizin der Universität Regensburg, West Germany

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1. Introduction

Chlorella vulgaris is able to accumulate hexose analogues more than 1000-fold [1] by an inducible proton cotransport system [2]. Recently it has been attempted to isolate vesicles from these cells which take up 6-deoxyglucose in vitro. The experiments have failed so far. However, it was observed during the progress of this work that surprisingly also those cells which remained intact during the breakage procedure were not able to take up 6-deoxyglucose anymore. It is shown in this communication that the inhibitory effect is due to a rapid release of free fatty acids during cell breakage.

2. Material and methods

The uptake experiments with *C. vulgaris* were carried out as described previously [2]. The cells (in Na-PO_4 buffer 0.025 M, pH 6.0) were disrupted with a French Press or with glass beads (0.45 mm diameter) in a Zellhomogenisator MSK. For the experiment in table 1 *S. cerevisiae* cells in their log phase (for growth conditions see [3]) were disrupted in the same way as *Chlorella*. *E. coli* KL 16 lac I, grown in 1% casamino acids and 0.2% glycerol were kindly supplied by Dr J. Lengeler; the cells were washed in 1% NaCl, resuspended in PO_4 buffer and broken with a sonifier. As crude membrane fraction of Chlorella the pellet obtained between 2000 to 48 000 *g* was used. The content of FFA* was determined in the supernatant (after 2000 *g*); it was acidified and extracted with chloroform/methanol (2:1; v/v). The FFA fraction was purified on t.l.c.-silica gel plates; qualitatively the spots were visualized with iodine. Quantitative determinations of FFA were carried out according to the method of Heinen and De Vries [4].

Table 1
FFA content in broken and whole cells

Organism	FFA content (mg/g wet weight) in Broken cells	Whole cells
C. vulgaris	4.2	0.2
S. cerevisiae	1.4	0.1
E. coli	0.7	0.1

3. Results

When extract of broken cells was added to a normal incubation mixture with intact cells, a strong inhibition of sugar uptake was observed (fig.1). With fractionated cell extract, it could be shown that only the resuspended membrane pellet possessed this inhibitory activity (fig.1). It was found that the protein of the membrane fraction was inactive and that the inhibitor could be extracted from the membranes with organic solvents.

However, chloroform/methanol (2:1) extracts of intact *Chlorella* did not contain the inhibitory substance. T.l.c. of the lipophilic fraction of isolated membranes revealed large quantities of free fatty acids which were present at extremely low amounts

* *Abbreviations:* FFA, free fatty acids.

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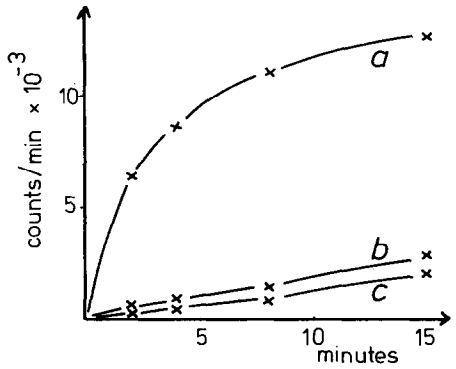

Fig.1. Effect of broken cells and washed membranes on active hexose transport of intact *C. vulgaris* cells. Uptake of [^{3}H]6-deoxyglucose was measured in a total vol of 5 ml 0.02 M Na-PO_4-buffer (pH 6.0) which contained 90 µl packed cells of *Chlorella* and 0.8 µCi [^{3}H]6-deoxyglucose (10^{-4} M). Aliquots of 0.25 ml were taken and the radioactivity in the cells determined [5]. (a) control; (b) contains 1.5 ml cell extract (4.6 mg protein); (c) contains the washed membranes from 1.5 ml cell extract (3.75 mg protein).

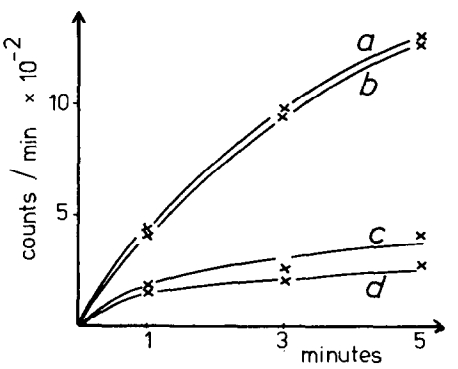

Fig.2. Increase of inhibitory activity (= amount of FFA released) after disruption of cells. Uptake of [^{3}H]6-deoxyglucose was measured in a total volume of 5 ml 0.02 M Na-PO_4-buffer (pH 6.0) which contained 32 µl of packed *Chlorella* cells and 0.33 µCi [^{3}H]6-deoxyglucose (4×10^{-4} M). Aliquots of 0.25 ml were taken and the radioactivity in the cells determined [5]. (a) control; (b) + lipid extract of whole cells; (c) + lipid extract of broken cells 1.5 min after disruption; (d) + lipid extract of broken cells 30 min after disruption.

in extract of intact *Chlorella*. Only the area on the t.l.c. plate corresponding to FFA showed inhibitory activity. Similar observations were made with *Saccharomyces cerevisiae* and with *Escherichia coli* (table 1). Obviously, therefore, free fatty acids are rapidly released by phospholipases during the process of cell breakage. Thus even when broken cells were extracted with organic solvents 1.5 min after cell breakage at a temperature of close to 0°C, this extract contained a large amount of 'inhibitor' (fig.2).

Table 2
Inhibition of active hexose transport by linolenic acid

Linolenic acid [M]	Relative rate of uptake [%]
Control	100
5×10^{-4}	68
1×10^{-3}	30

Further evidence that free fatty acids are responsible for the inhibition of the active hexose uptake of *Chlorella* is presented in table 2. Linolenic acid, one of the main fatty acids present in *Chlorella* lipids [5], inhibits the uptake of 6-deoxyglucose by 70% at a concentration of 10^{-3} M.

From these results it is obvious that in order to obtain actively transporting membrane vesicles one has to be able to prevent the action of phospholipases or at least to remove the fatty acids set free during cell breakage. Whereas neither the addition of EDTA nor that of various esterase inhibitors (like DFP, Nupercaine) showed any inhibitory effect on lipase activity, the addition of rather high concentrations of BSA completely removed the inhibitory free fatty acids (table 3). When cells were broken at a pH > 9 no FFA were released.

Table 3
Release of transport inhibition by BSA

Conditions	Relative rate of uptake [%]
Whole cells (control)	100
Whole cells + broken cells	5
Whole cells + (broken cells + 10 mg BSA/0.5 ml)	15
Whole cells + (broken cells + 50 mg BSA/0.5 ml)	80

4. Discussion

It has been known for a long time that free fatty acids at high concentrations can act as uncouplers in mitochondria and chloroplasts [6–10]. The results of this paper show that within minutes fatty acids are set free in eucaryotic cells which largely inhibit a proton dependent sugar cotransport system. Obviously this is detrimental for any attempt to obtain actively proton cotransporting vesicle preparations. Since fatty acids will not only destroy a proton gradient but may also affect any membrane potential, the observations reported here are of importance in relation to in vitro studies of ion transport and ion cotransport systems in general.

Since intact cells contain less than 5% of the amount of free fatty acids present after cell breakage, one may assume that the phospholipases responsible for the release have only limited access to their substrate. One possibility certainly would be a location within lysosomes [11]. In the case of *E. coli* it has been shown that a phospholipase A is located within the outer membrane [12]; again the enzyme obviously requires cell breakage to get in contact with sufficient substrate (table 1). It seems likely, therefore, that during the pretreatment of *E. coli* in the process of preparing 'Kaback vesicles' [13] parts of the outer membrane and with it the phospholipase A is removed before the vesicles are actually produced. An analogous situation has to be achieved for eucaryotic cells before proton driven or proton dependent transport processes can be measured in vitro.

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