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THE EFFECT OF *N,N'*-DICYCLOHEXYLCARBODIIMIDE ON ANAEROBIC AND AEROBIC PHOSPHATE UPTAKE BY BAKER'S YEAST*

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SUMMARY

N, N'-Dicyclohexylcarbodiimide (DCCD) does not inhibit anaerobic phosphate uptake by yeast when applied at concentrations sufficiently high in order to reduce the rate of aerobic phosphate uptake considerably. Moreover, inhibition of anaerobic phosphate uptake by the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, is not relieved by DCCD. These findings do not support the hypothesis that anaerobic phosphate uptake is energized by some high-energy intermediates formed under anaerobic conditions *via* glycolytically generated ATP.

INTRODUCTION

As shown by Borst-Pauwels and Jager[1] anaerobic phosphate uptake by yeast can be described as follows. Orthophosphate binds to a primary acceptor located at the cell membrane surface. The acceptor–phosphate complex then reacts with a hypothetical compound "Y" resulting in the accumulation of phosphate within the cells. Inhibitors of anaerobic phosphate uptake decrease the concentration of Y and thereby affect phosphate transport. These inhibitors not only include inhibitors of glycolysis, but also uncouplers of oxidative phosphorylation like 2,4-dinitrophenol[1], isooctyldinitrophenol and pentachlorophenol[2]. 2,4-Dinitrophenol inhibits other energy-requiring processes[3–7] as well in yeast when applied under anaerobic conditions without much affecting the level of cellular ATP[3]. In connection with these findings it has been considered that a high-energy intermediate of oxidative phosphorylation, equilibrating with ATP and produced by glycolytic activity, can provide the energy for certain cellular processes[3,5].

In the present study the possibility of Y being such a high-energy intermediate has been examined. To this end two specific inhibitors of the energy transfer from ATP to high-energy intermediates have been applied, *viz.* oligomycin and *N,N'*-dicyclohexylcarbodiimide (DCCD). The latter compound, which has an analogous activity as oligomycin[8], might be more suitable, as oligomycin does not readily penetrate into intact yeast cells[9]. It has been shown[9] that DCCD, when applied

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

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to intact yeast cells, inhibits respiration whereas glycolysis is left virtually unaffected, apart from aspecific effects exerted by DCCD at the higher concentrations on account of its strong chemical activity[10]. The specific inhibition of respiration is relieved by uncouplers[9], which supports the view that DCCD leads to an impairment of the energy transfer from the high-energy intermediate formed under aerobic conditions to ATP.

MATERIAL AND METHODS

The yeast *Saccharomyces cerevisiae* (Hansen) Dekker, strain "Delft II", was preaerated for at least 1 day in a 4% (w/v) suspension in 0.1 M sodium citrate buffer of pH 4.5 provided with 10 mM KCl in order to exhaust the internal substrates. The yeast was pretreated in a 1% suspension in fresh buffer medium during 1 h either anaerobically (in the presence of 3% (w/v) glucose, and nitrogen bubbling through) or aerobically (in the presence of 1% ethanol and air bubbling through) at pH 4.5 or pH 6.5, and at 25 °C. The pH 6.5 buffer consisted of 0.1 M Tris–citrate with 10 mM KCl. During this preincubation in the absence of added orthophosphate the phosphate transport system is induced[1,11]. After an additional incubation period of 6 min with a given concentration of DCCD or oligomycin added as alcoholic solution and a final concentration of 1% or ethanol alone as a control, the uptake of ^{32}P-labelled phosphate (Philips–Duphar, Petten, The Netherlands) was determined using the methods described previously[1]. The yeast concentration during phosphate uptake was 0.9% (w/v). Cellular ATP content was determined using a luminiscence method with firefly extract (Sigma).

RESULTS

It was examined whether relatively high concentrations of oligomycin might have an effect upon phosphate uptake by yeast cells as yet. It appeared that amounts of oligomycin as high as 0.1 mg per ml did not affect significantly phosphate uptake neither aerobically nor anaerobically after 6 min preincubation at pH 4.5 and 6.5. A longer exposure of the cells to oligomycin was not used for the following reason. The rate of phosphate uptake depends not only upon the direct supply of energy but also upon the number of acceptor sites, which number increases on extending the preincubation period[1]. This increase in itself might be sensitive to inhibitors of energy transduction.

The effect of DCCD upon phosphate uptake is shown in Table I. The sensitivity of the yeast under aerobic conditions appeared to be much higher at pH 6.5 than at pH 4.5. This might be due to the fact that the concentration of uncharged DCCD molecules will be higher at the higher pH, allowing a more rapid penetration of DCCD into the yeast cells.

It was examined whether the inhibitory action of DCCD upon aerobic phosphate uptake is accompanied by a decrease in the level of Y, the unknown compound, the level of which determines the rate of anaerobic phosphate uptake[1]. In that case not only the maximum rate of phosphate uptake (V) will be increased, but also the K_m[1]. The effect of DCCD upon the ATP content of the cells has been determined, too (see Table II). When the DCCD concentration was increased, the ATP content decreased, as did V and K_m. There was a clear correlation between

TABLE I

THE EFFECT OF VARIOUS CONCENTRATIONS OF DCCD ON ANAEROBIC AND AEROBIC PHOSPHATE UPTAKE RATE

The values indicated represent the rate of uptake of 0.1 mM phosphate, expressed in percents of the control values obtained in the absence of DCCD.

pH	*DCCD concn* (*mM*)	*Uptake* (%)	
		Anaerobic	*Aerobic*
4.5	0.1	96	80
	0.3	95	38
6.5	0.02	96	63
	0.06	104	13
	0.15	95	5
	0.30	88	4

TABLE II

THE EFFECT OF VARIOUS CONCENTRATIONS OF DCCD ON KINETIC PARAMETERS OF PHOSPHATE UPTAKE AND CELLULAR ATP CONTENT UNDER AEROBIC CONDITIONS AT pH 4.5

V and ATP content refer to the dry weights of the yeast.

DCCD concn (*mM*)	K_m (*μM*)	*V* (*mmoles·min*$^{-1}$*·kg*$^{-1}$)	*ATP* (*mmoles·kg*$^{-1}$)
0	10.7	10.2	5.4
0.1	8.4	8.0	4.3
0.3	4.9	4.1	2.1

TABLE III

THE COMBINED EFFECT OF 0.3 mM DCCD AND 0.03 mM DINITROPHENOL ON ANAEROBIC AND AEROBIC PHOSPHATE UPTAKE

The yeast is preincubated for 6 min with either 2,4-dinitrophenol (DNP), DCCD, or DCCD *plus* dinitrophenol whereafter the rate of uptake of 0.1 mM phosphate has been determined. This rate is expressed in percents of the control value obtained without added inhibitor.

	Uptake (%)	
	Anaerobic	*Aerobic*
Control	100	100
DNP	57	2.5
DCCD	102	41
DNP + DCCD	55	0.8

the inhibition of phosphate uptake and the decrease in ATP content. DCCD appeared to have but a small and only slightly significant effect upon the anaerobic ATP content of the cells at pH 4.5. 0.3 mM DCCD decreased this content by about 10%.

Finally, it was examined whether DCCD affects the inhibition of phosphate

uptake by 2,4-dinitrophenol. When dinitrophenol exerts its inhibitory effect upon phosphate uptake *via* the breakdown of a high-energy intermediate, then DCCD should abolish this effect[12]. It is seen in Table III that the inhibition of anaerobic phosphate uptake by dinitrophenol was not appreciably influenced by the presence of DCCD, whereas DCCD increased the inhibition observed aerobically. Aerobic phosphate uptake appeared to be much more sensitive to dinitrophenol than anaerobic phosphate absorption.

DISCUSSION

The results of the present study do not support the view that anaerobic phosphate uptake is energized by a high-energy intermediate of oxidative phosphorylation. In that case DCCD should have inhibited anaerobic phosphate uptake, whereas this process was virtually unaffected by those concentrations of DCCD which inhibit respiration[9] and which lead to an impairment of aerobic phosphate uptake as shown in this paper. This lack of inhibition of anaerobic phosphate uptake by DCCD was not encountered in the anaerobic bacterium *Streptococcus faecalis*. With these cells it is shown on the contrary that phosphate uptake is not only inhibited by uncouplers of oxidative phosphorylation, but also by DCCD[13,14].

An apparent correlation between the rate of aerobic phosphate uptake and the cellular ATP content was found when applying increasing amounts of DCCD. Such a correlation has also been found under anaerobic conditions in the presence of iodoacetate[1]. It is doubtful, however, whether a direct link exists between ATP content and the rate of phosphate uptake. Inhibition of anaerobic phosphate uptake by 2,4-dinitrophenol is accompanied by only a slight decrease in ATP level of the cells[1]. In addition DCCD does not affect the extent of inhibition of anaerobic phosphate uptake caused by dinitrophenol. This excludes the possibility that this uncoupler inhibits phosphate transport by inducing mitochondrial ATPase activity and indicates that dinitrophenol interacts with anaerobic phosphate transport by some mechanism other than the breakdown of a high-energy intermediate with the dissipation of energy. This is supported by the finding that much smaller amounts of dinitrophenol are needed for the inhibition of aerobic phosphate uptake than for the impairment of anaerobic uptake indicating that an inhibitory process other than the normal uncoupling process is involved under anaerobic conditions.

The present findings are similar to those of Galeotti *et al.*[15] on the effect of oligomycin and uncouplers of oxidative phosphorylation upon the respiratory adaptation of anaerobically grown yeast. These authors suggested that the uncouplers might affect this energy requiring process by the disturbance of membrane functions essential to transport processes.

Another possibility is that the main cause for the inhibition of phosphate uptake is a decrease of the cellular pH. As a matter of fact, the inhibition of anaerobic phosphate uptake by fatty acids can be attributed to cell acidification[16]. It is now examined whether inhibition of phosphate uptake by dinitrophenol is accompanied by a decrease in cell pH, and whether such a decrease can account for the inhibition of phosphate uptake quantitatively.

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REFERENCES

1 G. W. F. H. Borst-Pauwels and S. Jager, *Biochim. Biophys. Acta*, 172 (1969) 399.
2 G. W. F. H. Borst-Pauwels and P. L. M. Huygen, *Biochim. Biophys. Acta*, 288 (1972) 166.
3 L. Jarett and R. W. Hendler, *Biochemistry*, 6 (1967) 1693.
4 J. C. Riemersma, *Biochim. Biophys. Acta*, 153 (1968) 80.
5 L. Kováč and I. Istenesová, *Biochim. Biophys. Acta*, 82 (1964) 162.
6 C. E. Cliffon, *Adv. Enzymol.*, 6 (1946) 269.
7 J. M. Reiner and S. Spiegelmann, *J. Cell. Comp. Physiol.*, 30 (1947) 347.
8 R. B. Beechey, C. T. Holloway, I. G. Knight and A. M. Robertson, *Biochem. Biophys. Res. Commun.*, 23 (1966) 75.
9 L. Kováč, T. Galeotti and B. Hess, *Biochim. Biophys. Acta*, 153 (1968) 715.
10 H. G. Khorana, *Chem. Rev.*, 53 (1953) 145.
11 J. E. Leggett, *Plant Physiol.*, 36 (1961) 277.
12 E. C. Slater and H. F. ter Welle, *20. Colloq. Ges. Biol. Chem.*, Vol. 20, Springer Verlag, Berlin, 1969, p. 258.
13 F. M. Harold and J. R. Baarda, *J. Bacteriol.*, 96 (1968) 2025.
14 F. M. Harold, E. Pavlasová and J. R. Baarda, *Biochim. Biophys. Acta*, 196 (1970) 235.
15 T. Galeotti, L. Kováč and B. Hess, *Nature*, 218 (1968) 194.
16 G. W. F. H. Borst-Pauwels and J. Dobbelmann, *Biochim. Biophys. Acta*, 290 (1972) 348.