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Uncouplers of oxidative phosphorylation promote derepression of the hexose transport system in cultures of hamster cells

(2,4-dinitrophenol/glucosamine/"alactacid" repression/cycloheximide)

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ABSTRACT **Long-term (18–24 hr) preincubation of NIL hamster cell cultures with D-glucose or D-glucosamine (both of which repress the hexose transport system) gave rise to a striking loss of the hexose transport system ("super-repression") when cycloheximide was also present in the culture medium. However, if 0.2 mM 2,4-dinitrophenol (DNP) was also present, the cycloheximide-mediated super-repression was prevented. Moreover, the presence of DNP at this low concentration contributed to an increase in hexose uptake such that it was substantially higher than that permitted by either of the two repressive sugars alone. When the cultures were maintained in medium containing D-fructose in place of glucose, a marked increase in uptake occurred, and this increase (derepression) was not affected by DNP. The derepression due to glucose deprivation and the increases caused by DNP treatment were also observed when 3-*O*-methylglucose was used to measure hexose transport. Although cultures maintained in the presence of glucosamine exhibited a repressed hexose transport rate, they did not generate significant amounts of lactic acid. DNP, and other uncouplers of oxidative phosphorylation, promoted a derepressed state of hexose transport but did not stimulate the generation of lactate from glucosamine. These data suggest that the metabolic repression phenomena of hexose transport do not depend on glycolysis but rather on the "energized" state of the cell. The energized state of the cell may also be required for the super-repression of hexose transport that is especially apparent when protein synthesis is blocked by cycloheximide.**

It is well known that depriving fibroblast cultures of glucose tends to enhance the hexose transport system (1, 2). This gradual deregulation has been ascribed to transcriptional changes, derepression (3–5). On this basis the glucose effects may be considered a type of catabolic repression, because fibroblast cultures generate large amounts of pyruvate and lactate from glucose. Moreover, replacement of glucose by fructose, which generates only small amounts of lactic acid, especially in NIL hamster fibroblast cultures (6), brings about a pronounced derepression of the hexose transport system as well as of the amino acid transport systems (6–10).

In the present article it will become evident that the rate of lactic acid production does not correlate with the regulatory state of the transport systems, especially not with the state of repression of the hexose transport system.

We have found the so-called glucose repression effect on the hexose transport system to be elicited just as well by glucosamine (6), yet this amino sugar, when replacing glucose in the culture medium of NIL monolayer cultures, generates only small amounts of lactic acid (11).

This observation prompted us to examine the effect of the respiratory pathways and the effect of oxidative energy couplings on the regulation of the hexose transport system.

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MATERIALS AND METHODS

Hamster fibroblasts (NIL strain) were obtained originally from the Imperial Cancer Research Fund, London. Cells were grown in Dulbecco's modified Eagle's medium with glucose at 4 mg/ml and 10% fetal calf serum in 30-mm plastic culture dishes (Lux) in a humidified CO_2 incubator. The cells were routinely grown to near confluency (about 4 days). Usually 16–24 hr before the uptake test the cells were rinsed twice with sugar-free medium and given fresh modified Eagle's medium without pyruvate and containing 10% dialyzed fetal calf serum. Glucose was omitted or replaced by other sugars and inhibitors were added as indicated for the individual experiments. Just prior to the uptake test, cells were rinsed three times with phosphate-buffered saline (P_i/NaCl), pH 7.4, then given [*U*-^{14}C]-galactose in P_i/NaCl for 10 min. at 37°C. Usually L-[^{3}H]glucose was added to the radioactive mixture to test for leakiness of the cells and completeness of washing. After the uptake test the cells were washed three times with ice-cold P_i/NaCl, then extracted with 500 μl of 70% (vol/vol) ethanol for at least 15 min. A portion of the ethanol extract was assayed for radioactivity in a Beckman scintillation counter. For 3-*O*-methylglucose transport the dishes were rinsed at least three times with P_i/NaCl, given 3-*O*-[*U*-^{14}C]methyl-D-glucose containing L-[^{3}H]glucose for 15 sec at 23°C, and washed rapidly (less than 15 sec) five times with 2 ml of ice-cold P_i/NaCl. Lactate was determined by the lactate dehydrogenase method (Sigma). Media and serum were from GIBCO and Microbiological Associates (Bethesda, MD). Radioactive sugars were from New England Nuclear. Cycloheximide (CHx), sodium malonate, 2,4-dinitrophenol (DNP), and oligomycin were obtained from Sigma. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a generous gift from P. G. Heytler, Du Pont.

RESULTS

NIL cultures maintained in DNP/modified Eagle's medium show derepressed levels of the hexose transport system (12). Table 1 shows this feature as applied to glucose as well as glucosamine, which also shows repressive ability on the transport system (6, 11). Cycloheximide, if present in glucose-containing media, exerts an additional repression (4, 7). This repression we shall call "super-repression." The CHx super-repression can be prevented by the additional presence of ammonium salts (13). In Table 1 it can be seen that DNP is able to counteract the super-repression as well as the repression. This constellation

Abbreviations: P_i/NaCl, phosphate-buffered saline; DNP, 2,4-dinitrophenol; CHx, cycloheximide.

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Table 1. DNP counteracts glucose and glucosamine repression and CHx-imposed "super-repression" of galactose uptake

Culture medium	[^{14}C]Galactose uptake, nmol/mg protein		
	Glucose	Glucosamine	Fructose
No additions	1.58	1.18	3.63
DNP	3.02	3.12	3.55
CHx	0.23	0.71	1.44
DNP + CHx	2.03	1.05	1.97

Cells were cultured for 20 hr in modified Eagle's medium containing 22 mM glucose, 22 mM fructose, 5.5 mM glucosamine, with or without 0.2 mM DNP and CHx at 2 μg/ml as indicated. Uptake tests used 0.1 mM [^{14}C]galactose for 10 min.

provides an enhancement of the transport system that could well be described as a "promotion of derepression" (12).

The simultaneous addition of DNP at the time of the shift to fructose medium did not affect the final development of the derepression; i.e., fructose and fructose + DNP cultures in Table 1 were derepressed to about the same degree. The further addition of CHx (i.e., DNP + CHx) partly interfered with the development of derepression due to glucose depletion, but the interference was less pronounced than with CHx alone (cf. figure 2 in ref. 7). The most dramatic effect by DNP was , as mentioned, the bypass of the CHx super-repression seen in standard glucose media. The DNP effect was evident in concentrations as low as 20 μM, and these low concentrations also affected the regulation of the transport (as assayed by initial rate determinations of 3-*O*-methylglucose transport). This is illustrated in Table 2.

Oligomycin, another uncoupler of oxidative phosphorylation, also showed a marked ability to counteract the CHx super-repression and to some extent also the usual glucose repression (see Table 3).

A third type of uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, operated much like oligomycin (data not shown).

It should be added that the use of tritiated L-glucose was considered essential in order to monitor any trace of leakiness of the cell membrane. None was found, even if cyanide was used as an inhibitor of respiration. However, the activity of the transport system was neither stimulated nor inhibited by cyanide. In contrast, 6-hr anaerobiosis tended to counteract glucose repression as did malonate (data not shown).

From Table 4 it seems clear that the glucose repression cannot be considered a simple catabolic repression elicited by glycolysis. It appears that a combination of glucose and DNP in the culture medium generates more lactic acid than does glucose alone, yet the transport system became markedly derepressed. Conversely, glucosamine did not generate lactic acid, yet this sugar analogue brought about an efficient "alactacid" repression of the uptake system (see Table 4).

Table 2. Derepression of the hexose transport system by DNP at a low concentration (20 μM)

Culture medium	[^{14}C]Galactose uptake (relative)	3-*O*-[^{14}C]Methylglucose transport (relative)
Glucose	1.00	1.00
Glucose + DNP	1.38	2.07
Fructose	3.87	4.68

Uptake test: [^{14}C]galactose for 10 min at 37°C. Transport test: 3-*O*-[^{14}C]methylglucose for 15 sec at 23°C. Concentration of ^{14}C-labeled ligands was 100 μM.

Table 3. Oligomycin promotes derepression of the hexose transport system and prevents CHx super-repression

Culture medium	[^{14}C]Galactose uptake, nmol/mg protein	Transport (relative)
Glucose	1.25	1.00
Glucose + CHx	0.14	0.11
Glucose + oligomycin	2.06	1.65
Glucose + oligomycin + CHx	1.30	1.04
Fructose	3.75	3.01

Culture conditions were as stated for Table 1. When present, CHx was 2 μg/ml and oligomycin was 10 μg/ml. Galactose uptake was for 10 min at 37°C.

DISCUSSION

The demonstration that uncouplers of oxidative phosphorylation promote derepression of the hexose transport system even in the presence of excess glucose raises a number of new problems.

Because oxidative phosphorylation, or at least the "energized state of the cell," must now be considered one of the main vectors in the repressive effect of glucose and glucosamine, one may wonder why glucose starvation or replacement of glucose by fructose so readily elicit derepression. This is so much the more puzzling because the culture medium always contains a large excess of glutamine and even starved cells contain detectable amounts of glutamic acid (unpublished data). The latter feeds into the Krebs tricarboxylic acid cycle, which is considered the main generator of oxidative phosphorylation. Evidently this avenue alone seems to be ineffective in regard to transport regulation.

Two other aspects deserve special attention. The stimulation of sugar transport by anoxia or by inhibition of oxidative phosphorylation has been reported from various observers [ref. 14; see also a recent review by Gould (15)]. Moreover, as reported by Ozand *et al.* (16), enhancement of hexose transport by anoxia need not alter the intracellular ATP level (see also ref. 15). In our own case, replacement of glucose (or glucosamine) by fructose brings about a marked enhancement of the hexose transport system, yet the cellular ATP level remains practically unaltered (17, †).

It has been stated earlier (4, 7) that the combined effects of CHx and glucose on the regulation of the hexose transport system (in this paper called super-repression) may imply that factors responsible for protein turnover also govern the extent

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Table 4. Noncorrelation between lactate production and repression of hexose uptake

Culture medium	[^{14}C]Galactose uptake (relative)	Lactate (relative)
Glucose	1.00*	1.00†
Glucosamine	0.693	0.076
Fructose	2.51	0.102
Glucose + DNP	1.81	2.73
Glucosamine + DNP	1.47	0.121
Fructose + DNP	2.45	0.128

* Galactose uptake over 10 min (mean of seven experiments ± SD) amounted to 1.58 ± 0.68 nmol/mg of cell protein.

† Lactic acid accumulated in the medium over 20 hr (average of 10 experiments) amounted to 30.2 μmol/mg of cell protein.

of regulation of this sytem. These factors may even be considered closer to our observations described in this paper and especially with regard to the so-called super-repression. Although "transcriptional events" have been cited in previous articles (3, 5), their role in this type of regulation still remains to be defined. At present, the role of protein turnover in transport regulation is being more actively explored (13, 18, ‡).

The observations described here touch on the broader aspects regarding the mandatory requirement of oxidative phosphorylation for protein turnover, including not only biosynthesis of proteins but also their degradation (19–21).

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