Uptake of Leucine and Tyrosine and Their Intracellular Pools in *Chlorella fusca* var. *vacuolata*

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Chlorella fusca var. *vacuolata* has high affinity active transport systems for the amino acids L-leucine and L-tyrosine. Leucine at 0.2 μM was concentrated at least 222-fold and tyrosine 920-fold. Neither uptake process was inhibited by high concentrations of serine, threonine or phenylalanine. The concentration giving half-maximum rate of uptake was 2.5 μM for leucine with a smaller value for tyrosine. The maximum uptake rate for leucine was 10 amol cell⁻¹ min⁻¹. Uptake of leucine and tyrosine was not inhibited by 0.2 mM-2,4-dinitrophenol, which completely inhibited the incorporation of radioactive leucine and tyrosine into protein. Intracellular radioactive leucine did not exchange with an excess of unlabelled leucine added to the medium, except during nitrogen starvation, and only partial exchange occurred with tyrosine. The apparent overall pool size for these amino acids was dependent upon the growth state of the algal cells. The kinetics of incorporation of radioactive amino acids from intracellular pools into protein suggested that both leucine and tyrosine occupied at least two distinct pools within the cells. Tyrosine and methionine were present in smallest amounts (38 and 37 amol cell⁻¹, respectively). Leucine was present at 90 amol cell⁻¹. At least 11 amino acids had higher pool concentrations than leucine, whilst only alanine and glycine were more abundant than leucine in *C. fusca* protein.

INTRODUCTION

The active uptake of phenylalanine by cells of *Chlorella vulgaris* and *C. fusca* has been investigated by Van Sumere & Dedonder (1971) and Pedersen & Knutsen (1974), respectively. This process exhibits saturation kinetics, with a V_{max} of 10 amol cell⁻¹ min⁻¹ and K_m of 5 μM , and is substrate-specific, although high concentrations of analogues or other amino acids cause partial inhibition of uptake. Phenylalanine is accumulated 1000-fold inside the cells by an energy-dependent process which is abolished by preincubating low culture densities of cells with 2,4-dinitrophenol prior to addition of the amino acid. The system was found to be constitutive, operating at maximum rate immediately on amino acid addition. Uptake is a one-way process, in that no exchange occurs between intracellular phenylalanine and that in the medium, even during uptake.

In *C. pyrenoidosa*, the accumulation of proline from the medium requires intracellular glucose, although it is inhibited by large quantities of carbohydrates in the cells (McNamer & Stewart, 1973). The process occurs chiefly in late-exponential phase cells, when the glucose in the medium of dark-grown cells is depleted.

Little is known about the accumulation of other amino acids in this organism, although there has been some work done on the uptake of other solutes, such as guanine (Pettersen & Knutsen, 1974) and uracil (Knutsen, 1972). Maximum uptake of uracil occurs in the last 4 h of the light period in a synchronous culture. This is also the case for phenylalanine (Pedersen & Knutsen, 1974).

Active transport also occurs for sodium and potassium ions in *Chlorella*, as in other algae and higher plants (Barber, 1968). These transport processes appear to be ATP driven, since they are susceptible to inhibition by compounds such as 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone (Jeschke, 1972), and are light-dependent (Rybova *et al.*, 1972).

Komor & Tanner (1974) investigated hexose uptake by *C. vulgaris* using the glucose analogues 2-deoxyglucose and 6-deoxyglucose. Transport of these sugars was stoichiometrically linked to metabolic energy (Komor *et al.*, 1972) and took place against a concentration gradient. Their studies with the tranphosphorylation inhibitors *N,N'*-dicyclohexylcarbodiimide, azide and oligomycin led them to conclude that uptake is driven by a high-energy intermediate prior to ATP, and that the process cannot be entirely explained in terms of the electron transport hypothesis. The sugar appeared chemically unaltered inside the cell, and was later phosphorylated by an ATP-dependent mechanism. Transport of hexose in *C. vulgaris* thus differs from the phosphoenolpyruvate-energized type of sugar uptake seen in *Escherichia coli* (Kornberg & Jones-Mortimer, 1977).

The present study of the uptake of leucine and tyrosine by *C. fusca* was undertaken to investigate the suitability of these amino acids as markers for labelling proteins to measure intracellular protein turnover. Suitability for such studies depends upon rapid accumulation of such compounds from the external medium, and incorporation into protein. It is also important that an exchange equilibrium exists in both directions with the medium if an external 'trap' of unlabelled amino acid is used to avoid the problem of isotope re-incorporation.

METHODS

Culture of organism. *Chlorella fusca* var. *vacuolata* (Culture Collection of Algae and Protozoa, Cambridge, strain no. 211-8P) was grown autotrophically in a medium containing (per litre distilled water): 7.76 g KH_2PO_4 , 2.32 g K_2HPO_4 , 0.75 g NH_4NO_3 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg iron as an Fe^{3+} -EDTA complex, and 1 ml A5 trace element solution (Arnon, 1938). The pH of the medium was 6.1, and 200 ml volumes were aerated with sterile air containing 0.5% (v/v) CO_2 at 200 ml min^{-1} . Cultures were grown at 25 °C, and illuminated by four 60 cm, 40 W 'warm-white' fluorescent tubes, on two sides at a mean distance of 15 cm from the surface of each culture vessel, giving a mean light intensity at the surface of the culture vessel of 3.7×10^3 lx. Under these conditions, growth of the alga was exponential until the cell density reached 2×10^7 cells ml^{-1} , after which growth was light-limited, cell density increasing linearly to about 6×10^7 cells ml^{-1} .

For each experiment, 100 ml cultures were adjusted to 5.5×10^6 cells ml^{-1} and incubated under the conditions described above for 1.5 h prior to the start of the experiment (usually the addition of a radioactive amino acid).

Cell number was determined using a Coulter counter model Z_{BI} (Coulter Electronics, Dunstable, Beds.).

Nitrogen starvation was achieved by resuspending washed cell suspensions in medium from which NH_4NO_3 had been omitted.

Measurement of uptake of radioactive amino acids. L-[U- ^{14}C]Leucine or L-[U- ^{14}C]tyrosine (2 to 10 μCi , 74 to 370 kBq) was added aseptically to cultures, at the specific activities indicated in Results. At intervals, 5 ml samples were withdrawn; 3 ml of each sample was immediately filtered through a 0.45 μm pore-size membrane filter (2.5 cm diam., HAWP 02500, Millipore), and the filtrate (a) was retained. The remaining 2 ml was rapidly mixed with 2 ml 10% (w/v) trichloroacetic acid, centrifuged after standing for 1 h in crushed ice, and the supernatant (b) was retained. At the beginning and end of each experiment, triplicate 0.1 ml samples of unfractonated cell suspension (c) were taken for determination of the total radioactivity per unit volume of the culture. These and triplicate 0.5 ml samples of the filtrate (a) and supernatant (b) were mixed with 8 ml scintillation fluid [toluene/Triton X-100/2-(4'-*tert*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole (100:50:1, v/v/w)] and counted for 10 min periods in a scintillation counter at 8 °C. D.p.m. were calculated using the external standard channels ratio method of quench correction. The total uptake of radioactivity by cells was calculated as $(5c - a)$, the uptake into trichloroacetic acid-insoluble material (protein) as $(5c - 2b)$, and the uptake into the trichloroacetic acid-soluble fraction (pool) as $(2b - a)$. All values of sample radioactivity were then normalized to d.p.m. cell $^{-1}$. Radioactivity in representative samples (a) and (b) was shown to be unchanged leucine or tyrosine, as appropriate, using chromatographic separations described fully by Richards (1978).

Fig. 1. Uptake of radioactive leucine (a) and tyrosine (b) by *C. fusca*. Total uptake by *C. fusca* cells (○), uptake into the trichloroacetic acid-insoluble (protein) fraction (●) and uptake into the trichloroacetic acid-soluble (pool) fraction (■) are shown. (a) Cells were harvested from a culture at 1.35×10^7 cells ml⁻¹ and resuspended at 5.5×10^6 cells ml⁻¹. At zero time, L-[¹⁴C]leucine was added to give a concentration of $0.2 \mu\text{M}$ at a specific activity of $104.8 \text{ Ci mol}^{-1}$ ($3.88 \text{ TBq mol}^{-1}$). If all of the leucine had been taken up by the cells the total uptake would have been 8.3×10^{-3} d.p.m. cell⁻¹. (b) Cells were harvested from a culture at 1.17×10^7 cells ml⁻¹ and resuspended at 3.5×10^6 cells ml⁻¹. L-[¹⁴C]Tyrosine was added to give a concentration of $0.2 \mu\text{M}$ at a specific activity of 50 Ci mol^{-1} ($1.85 \text{ TBq mol}^{-1}$). If all of the tyrosine had been taken up by the cells the total uptake would have been 6.85×10^{-3} d.p.m. cell⁻¹.

Chemical analysis of total amino acid pools. A sample of 3.75×10^{10} cells was harvested by centrifugation from a *C. fusca* culture at a density of 1.25×10^7 cells ml⁻¹. The cells were washed and resuspended in ice-cold distilled water, broken by passage twice through a French press at 135 MPa and 4 °C, made 5% (w/v) in trichloroacetic acid and held in crushed ice for 1 h. The supernatant recovered by centrifugation at 30000 *g* for 1 h at 4 °C was extracted three times with an equal volume of ether (to remove trichloroacetic acid) and then freeze-dried. The amounts of the pool amino acids were determined using an automated amino acid analyser (Locarte, London) with the buffer system described by Donald (1973). Serine, glutamine and asparagine have identical retention times in this system and so were not separated. Thus, the amount of serine plus glutamine plus asparagine in Table 2 is an approximation derived using an average value of the sensitivity of the analyser detection system for the three amino acids, determined from standard samples run separately.

Chemical analysis of cell protein. The amino acid composition of *C. fusca* protein was determined by hydrolysis in 6 M-HCl followed by automated analysis for all amino acids, except glutamate, glutamine, aspartate, asparagine and tryptophan which were measured enzymically. Two fractions of cell protein, 'soluble' and 'insoluble', were analysed. Full details of the extraction of these two fractions and their analysis are described in the following paper (Richards & Thurston, 1980). The protein composition data of Table 2 are an average for the two fractions, weighted according to their relative abundances in the cell.

Radiochemicals. These were all obtained from The Radiochemical Centre, Amersham.

RESULTS

Uptake of radioactive leucine and tyrosine by C. fusca

When leucine was added to a *C. fusca* culture it was rapidly assimilated. Figure 1(a) shows the incorporation of $0.2 \mu\text{M}$ -leucine into whole cells and the division of the incorporated leucine between a trichloroacetic acid-soluble pool fraction and protein. The pool fraction contained only a small proportion of the total incorporated leucine except at the earliest sampling times, but retained a significant amount even when the rate of incorporation into protein approached zero. Uptake of $0.2 \mu\text{M}$ -tyrosine showed a similar pattern

Fig. 2

Fig. 3

Fig. 2. Effect of concentration of L-[^{14}C]leucine on uptake by *C. fusca*. Total uptake of radioactive leucine by *C. fusca* suspensions containing 5.5×10^6 cells ml^{-1} is shown. The concentrations of leucine supplied at zero time were: $4 \mu\text{M}$ (○); $1.5 \mu\text{M}$ (●); $0.2 \mu\text{M}$ (□); $0.06 \mu\text{M}$ (■).

Fig. 3. Effect of 2,4-dinitrophenol on assimilation of leucine by *C. fusca*. Suspensions of *C. fusca* containing 5.5×10^6 cells ml^{-1} were supplied with $0.2 \mu\text{M}$ -L-[^{14}C]leucine. Uptake, in the absence (○, □) and in the presence (●, ■) of 0.2 mM -2,4-dinitrophenol, into the trichloroacetic acid-insoluble fraction (○, ■) and the trichloroacetic acid-soluble (pool) fraction (●, □) is shown. Where 2,4-dinitrophenol was used, cells were preincubated for 0.5 h in the presence of the inhibitor before adding the radioactive amino acid.

(Fig. 1b), but a higher proportion of incorporated amino acid was initially found in the pool fraction and it was progressively assimilated into protein.

Total incorporation of leucine supplied at a range of concentrations is shown in Fig. 2. The initial rates of uptake calculated from these results were transformed as double reciprocal plots (not shown), together with comparable data for tyrosine. From these, the apparent K_m for uptake of leucine was $2.5 \mu\text{M}$ and the calculated maximum rate of uptake was $10 \text{ amol cell}^{-1} \text{ min}^{-1}$. The corresponding values for tyrosine were $0.4 \mu\text{M}$ and $6 \text{ amol cell}^{-1} \text{ min}^{-1}$, respectively.

Uptake of $0.2 \mu\text{M}$ -leucine and $0.2 \mu\text{M}$ -tyrosine was not inhibited by the simultaneous addition to the medium of phenylalanine, threonine or serine at $2 \mu\text{M}$ (results not shown). The effect of the uncoupling agent 2,4-dinitrophenol is shown in Fig. 3. Although incorporation of radioactive leucine into protein was completely inhibited, uptake into the pool fraction was not affected. The same result was obtained when the experiment was repeated with a *C. fusca* culture in the dark, supplied with 55 mM -glucose as carbon and energy source. This effect was also observed with tyrosine, both in the light and in the dark with glucose supplied.

Effect of growth state on apparent pool size

Table 1 shows the amount of radioactive leucine in the pool fraction 15 min after adding $0.2 \mu\text{M}$ -L-[^{14}C]leucine to suspensions of cells (adjusted to 5.5×10^6 cells ml^{-1}) taken from cultures which had grown to a range of cell densities. Suspensions of cells from the exponential phase of growth showed similar apparent pool sizes, but as the growth rate declined due to light limitation, the amount of leucine taken up into the pool fraction increased.

Table 1. Apparent leucine pool size of *C. fusca* suspensions harvested at different stages of batch culture

Samples of *C. fusca* suspensions adjusted to 5.5×10^6 cells ml^{-1} were supplied with $0.2 \mu\text{M}$ -L-[^{14}C]-leucine for 15 min, and then radioactivity in the pool fraction was determined. All values of pool size are $\pm 9\%$.

$10^{-6} \times$ Culture density* (cells ml^{-1})	Leucine pool (amol cell $^{-1}$)
4.4	0.47
19	0.53
25	0.56
40	1.7
53	2.8

* Points at which samples were taken for measurement of leucine taken up.

Fig. 4. Effect of cycloheximide and 'cold traps' on leucine (a) and tyrosine (b) pools in *C. fusca*. Suspensions of *C. fusca* containing 5.5×10^6 cells ml^{-1} were supplied with $0.2 \mu\text{M}$ -L-[^{14}C]leucine (a) or $0.2 \mu\text{M}$ -L-[^{14}C]tyrosine (b) for 15 min, and then the cells were harvested, washed and resuspended in fresh medium (○), medium containing $2.5 \mu\text{g}$ cycloheximide ml^{-1} (●), medium containing 1 mM unlabelled leucine or tyrosine as appropriate (■), or medium containing both $2.5 \mu\text{g}$ cycloheximide ml^{-1} and 1 mM unlabelled amino acid (□). Radioactive amino acid in the pool fractions was determined as described in Methods.

Exchange of leucine and tyrosine in the pool fraction with unlabelled amino acids added to the medium

The leucine pool of an exponentially growing *C. fusca* suspension was loaded with radioactive leucine, and then the cells were washed and resuspended in medium containing 1 mM unlabelled leucine (Fig. 4a). Radioactivity in the pool remained constant for 50 min with no measurable incorporation of radioactivity into protein or exchange of radioactive leucine with the unlabelled 'trap' in the medium. In the absence of the trap, pool radioactivity declined as more than 80 % of the leucine radioactivity was incorporated into protein, and this incorporation was reduced to 20 % when cycloheximide was present (Fig. 4a). This experiment was repeated with a light-limited *C. fusca* suspension where five times more leucine radioactivity was present in the pool fraction (see Table 1). Again, more than 80 % of the radioactive leucine was incorporated into protein within 50 min in the absence of unlabelled leucine or cycloheximide, but no exchange into the medium was detected in the presence or in the absence of the trap or cycloheximide.

The behaviour of the tyrosine pool fraction in similar experiments is shown in Fig. 4(b). When a cold trap was added the pool radioactivity was initially reduced by about 30 % both

Fig. 5. Efflux from the leucine pool in nitrogen-starved *C. fusca*. Cells were washed and resuspended in nitrogen-free medium at 5×10^4 cells ml^{-1} for 2 h, and then $0.2 \mu\text{M}$ -L-[^{14}C]leucine (50 Ci mol^{-1} , $1.85 \text{ TBq mol}^{-1}$) was added. After 20 min the cells were harvested, resuspended in nitrogen-free medium containing 1 mM unlabelled L-leucine and sampled for radioactivity in the pool fraction (○), in the culture medium (●) and in cell protein (□), as described in Methods.

Table 2. *Amino acid composition of C. fusca protein and intracellular pool fractions determined by automated analysis*

The amino acid content of the trichloroacetic acid-soluble (pool) and the trichloroacetic acid-insoluble (protein) fractions was determined as described in Methods. Values for protein are the mean of three experiments on separate cultures, and pool values are the mean of nine determinations made on cells from two cultures.

Amino acid	Pool fraction			Protein fraction	
	amol cell $^{-1}$	% total pool	Standard error	mol %	Standard error
Aspartate	492	6.89	0.18	3.21	0.08
Threonine	201	2.82	0.31	5.17	0.08
Serine	798	11.18	0.17	6.47	0.04
Asparagine				5.17	0.08
Glutamine				7.73	0.10
Glutamate	1794	25.15	0.07	1.91	0.01
Proline	1352	18.95	0.60	6.96	0.03
Glycine	219	3.08	0.20	8.89	0.08
Alanine	827	11.59	0.10	10.23	0.04
Cysteine	131	1.83	1.81	3.12	0.11
Valine	125	1.75	0.22	6.98	0.02
Methionine	37	0.51	0.82	1.24	0.66
Isoleucine	55	0.77	0.38	4.23	0.06
Leucine	90	1.26	0.34	8.84	0.09
Tyrosine	38	0.53	0.45	2.57	0.03
Phenylalanine	60	0.85	0.32	4.01	0.19
Histidine	42	0.59	0.26	1.91	0.11
Tryptophan	234	3.28	0.36	0.38	0.01
Lysine	117	1.65	0.22	6.21	0.13
Arginine	521	7.31	0.15	4.68	0.04

in the presence and in the absence of cycloheximide, but the residual two-thirds of the pool radioactivity did not exchange into the medium nor was it incorporated into the protein fraction.

Effect of nitrogen starvation

The uptake and incorporation of $0.2 \mu\text{M}$ -leucine and $0.2 \mu\text{M}$ -tyrosine by cells which had been nitrogen-starved for 2 h were the same as shown above for growing cells. When $0.2 \mu\text{M}$ -leucine was added for 20 min to cultures after 2 h of nitrogen starvation and the cultures were then washed and resuspended in fresh nitrogen-free medium containing an

Table 3. Uptake of 0.2 μM -leucine and 0.2 μM -tyrosine into intracellular pools of *C. fusca*

	Leucine	Tyrosine
Available internal volume*	100 $\mu\text{m}^3 \text{ cell}^{-1}$	100 $\mu\text{m}^3 \text{ cell}^{-1}$
Amount of exogenous amino acid entering pool fraction in an uptake experiment (after 10 min incorporation)	3.395 amol cell^{-1}	13.87 amol cell^{-1}
Internal amino acid concn	33.95 μM	138.7 μM
Residual amino acid concn outside cell	0.153 μM	0.151 μM
Concentration factor	222 $\times$	920 $\times$

* This value is based on Coulter counter measurements of mean cell volume, and assumes the whole volume of the cell is available to the internal pool. If this assumption is not true (for example, if the pool is located in a specific compartment such as the vacuole) the available volume will be less, and the concentration factor will be correspondingly greater than that calculated here.

external trap and cycloheximide, the pool radioactivity was progressively lost to the medium (Fig. 5).

Chemical estimation of amino acid pools in *C. fusca*

Amino acids in the trichloroacetic acid-soluble fraction of *C. fusca* cells taken from an exponentially growing culture are shown in Table 2, both as absolute amounts per cell and as percentages of the total amino acid pool, in order to allow comparison with their relative abundance in total *C. fusca* protein. The amount of proline shown in the pool fraction may be an overestimate as this amino acid was eluted next to glutamate which is present in relatively large amounts. The amounts of both leucine and tyrosine found in the pool fraction are 10 to 20 times greater than the amounts of radioactive amino acid taken up from the medium into this pool fraction by exponentially growing cells, where the initial extracellular concentration of amino acid was 0.2 μM .

DISCUSSION

Both leucine and tyrosine were taken up by *C. fusca* cells via high affinity uptake mechanisms which were not general amino acid permeases as uptake was not inhibited by a 10-fold higher initial concentration of phenylalanine, threonine or serine. The amino acids were both taken up to give a final pool concentration very much greater than the residual concentration in the medium. When 0.2 μM -leucine was supplied to growing cells the concentration in the pool fraction was 222 times greater than in the medium after 10 min of incorporation (Table 3) if the calculation was based only on the radioactive leucine supplied. The chemical determination of free leucine in *C. fusca* cells (Table 2) suggested that this was a considerable underestimate.

Under the same conditions tyrosine was concentrated 920 times inside the cells (again taking no account of the extra endogenously synthesized free tyrosine). The exact concentrations of free leucine and tyrosine in the pool fraction during uptake experiments were not determined and could not be calculated from these results as it was not determined whether amino acid taken up from the medium expanded an existing pool or replaced endogenously synthesized amino acid, thus conserving a constant pool size.

In the same way the increase in the amount of radioactive leucine found in the pool fraction as growth rate declined (Table 1) could be due to an absolute increase in pool size and/or to replacement of a decreased proportion of the pool by endogenously synthesized leucine.

Both amino acids were, nevertheless, taken up against a substantial concentration gradient by an energy-requiring process. We are consequently unable to explain why the uncoupling agent 2,4-dinitrophenol did not inhibit uptake. This inhibitor was expected to prevent ATP

synthesis by the processes of photophosphorylation and oxidative phosphorylation leaving substrate level phosphorylation as the sole source of ATP. Syrett (1958) has shown a marked drop in ATP levels when nitrogen-starved *C. fusca* cells were given 0.2 mM-2,4-dinitrophenol. As protein synthesis was completely stopped by the inhibitor, we conclude that either uptake uses energy which is not acquired via ATP, or the ATP requirement can be satisfied by very low ATP levels which are insufficient for protein synthesis.

The intracellular pools of leucine and tyrosine did not readily exchange with the same amino acid added to the medium as a 'trap'. With growing cells leucine did not exchange out of the cells to a measurable extent, and when the cells were nitrogen-starved exchange was slow with a half-time of about 1 h. About 30 % of the tyrosine pool of growing *C. fusca* cells exchanged relatively quickly with tyrosine added to the medium, but the residual 70 % did not.

These results suggest that the pool fraction detected here is composed of several metabolically distinct pools. When the leucine pool fraction was loaded with radioactive amino acid and then a high exogenous concentration of unlabelled amino acid was supplied, the pool radioactivity was conserved (Fig. 4a), so that within the limits of these measurements all of the radioactivity was in a pool which was not consumed by protein synthesis, nor was it able to exchange with the leucine in the medium. When no trap was supplied but cycloheximide was added, about a third of the labelled leucine of the pool was incorporated into protein. If this labelling was due to organellar protein synthesis (which would not be expected to be inhibited by the antibiotic), it is noteworthy that no such comparable incorporation was observed with tyrosine. Although the present results are insufficient to formulate a detailed kinetic model, they are consistent with the presence in *C. fusca* of storage pools of amino acids which are not always in rapid equilibrium with the precursor pools for protein synthesis nor with amino acids being taken up from the medium. The existence of storage pools has been suggested for a number of other eukaryotic organisms, for example *Neurospora* (Zalokar, 1961), maize (Oaks, 1965) and mammals (Mortimore *et al.*, 1972; Kipnis *et al.*, 1961). In *C. fusca* it has been demonstrated that there are at least two pools of amino acids, one of which is rapidly labelled with ^{14}C during photosynthesis with labelled carbon dioxide; this pool represents only 20 to 50 % of the total pool for several amino acids (Smith *et al.*, 1961).

In conclusion, the leucine and tyrosine pool fractions in *C. fusca* are complex and in growing cells these amino acids do not readily exchange from the pool fraction into the medium. Trapping of radioactive amino acids released from prelabelled protein is therefore not a suitable method for measuring overall rates of protein degradation (turnover) in this organism. The results of Halvorson (1958a, b) for *Saccharomyces cerevisiae* and of Davies & Humphrey (1978) for *Lemna minor* and *Schizosaccharomyces pombe* suggest that this restriction is common in eukaryotic organisms.

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