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AMINO ACID UPTAKE BY YEASTS

IV. EFFECT OF THIOL REAGENTS ON L-LEUCINE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* *

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(1) *N*-Ethylmaleimide (a penetrating SH- reagent) inactivated L-[^{14}C]leucine entrance (binding and translocation) into *Saccharomyces cerevisiae*, the extent of inhibition depending on the time of preincubation with *N*-ethylmaleimide, *N*-ethylmaleimide concentration, the amino acid external and internal concentration, and the energization state of the yeast cells. With D-glucose-energized yeast, *N*-ethylmaleimide inhibited L-[^{14}C]leucine entrance in all the assayed experimental conditions, but with starved yeast and low (0.1 mM) amino acid concentration, it did not inhibit L-[^{14}C]leucine binding, except when the cells were preincubated with L-leucine. With the *rho*$^-$ respiratory-deficient mutant (energized cells), *N*-ethylmaleimide inhibited L-[^{14}C]leucine entrance as with the energized wild-type, though to a lesser extent. (2) Analysis of the *N*-ethylmaleimide effect as a function of L-[^{14}C]leucine concentration showed a significant decrease of J_{max} values of the high- (S_1) and low- (S_2) affinity amino acid transport systems, but K_T values were not significantly modified. (3) When assayed in the presence of D-glucose, *N*-ethylmaleimide inhibition of D-glucose uptake and respiration contributed significantly to inactivation of L-[^{14}C]leucine entrance. Pretreatment of yeast cells with 2,4-dinitrophenol enhanced the effect of L-[^{14}C]leucine binding and translocation. (4) Bromoacetylsulfanilic acid and bromoacetylaminoisophthalic acid, two non-penetrating SH- reagents, did not inactivate L-[^{14}C]leucine entrance, while *p*-chloromercuribenzoate, a slowly penetrating SH-reagent, inactivated it to a limited extent. When compared with the effect of *N*-ethylmaleimide, these negative results indicate that thiol groups of the L-[^{14}C]leucine carrier were not exposed on the outer surface of the yeast cell permeability barrier.

Introduction

Amino acid entrance into yeasts *Saccharomyces* is a complex process involving multiple transport systems, as shown by genetic, kinetic and energy requirement data [1–8]. L-Leucine transport in *S. cerevisiae* involves a high-affinity, low-velocity system (S_1) and a low-affinity, high-velocity system (S_2), which are characterized by the different values of their kinetic parameters and also by their different response to specific inhibitors [9–11]. The general amino acid permease and the yeast plasmalemma ATPase should be part of the high-affinity system [10,11]. Despite the information

* Paper III in the series is Ref. 11.

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Abbreviations: BASA, bromoacetylsulfanilic acid; BAAPA, bromoacetylaminoisophthalic acid; PCMB, *p*-chloromercuribenzoic acid; CMPS, *p*-chloromercuriphenylsulfonic acid; FMA, fluorescein mercuric acetate.

available on the role of membrane proteins associated with amino acid transport by yeasts [12–14], the role of protein thiols has not been established. The point is relevant because thiol proteins are involved in amino acid transport into prokaryotic [15] and eukaryotic cells [16].

In the present study we have examined the effect of several thiol reagents on L-[^{14}C]leucine transport in *S. cerevisiae*. These reagents were selected on the basis of their ability to penetrate the yeast cell membrane and could be classified as (a) a non-penetrating reagent (BASA or BAAPA) [17]); (b) a slowly penetrating reagent (PCMB), and (c) a fast penetrating reagent (*N*-ethylmaleimide). The results presented here show that the non-penetrating or slowly-penetrating reagents did not significantly affect L-leucine transport, while *N*-ethylmaleimide caused extensive inactivation, depending essentially on the time of preincubation of *N*-ethylmaleimide with the yeast cells, L-leucine concentration in the external medium, and the energization state of the amino acid transport apparatus. Our observations extend preliminary studies by Ramos et al. [11] on iodoacetate inactivation of L-[^{14}C]leucine transport in *S. cerevisiae*.

Materials and Methods

Unless stated otherwise, the yeast employed was *S. cerevisiae* diploid, strain 207, wild type. The corresponding cytoplasmic *rho*$^-$ mutant ('petite colonie'), *S. carlsbergensis* haploid, strain 0646α(50), wild type, and the corresponding *rho*$^-$ mutant were used as indicated under Results. The latter yeast was kindly provided by Dr. M. Claisse, Centre de Génétique Moleculaire, Gif-sur-Yvette. Characteristic features of yeasts and culture conditions, as well as the preparation of 'starved' and 'energized' yeast, have been described before [9–11]. 'Starved' yeast means that before incubation the yeast was suspended in distilled water and aspirated with air for 18–20 h at 20–25°C under sterile conditions. 'Energized' yeast means starved yeast cells preincubated with D-glucose as described below.

L-[^{14}C]Leucine and D-[^{14}C]glucose were purchased from Amersham International, UK. The first was, on request, specially prepared in solid state. *N*-Ethylmaleimide, PCMB and D-glucose were from Sigma Chemical Company, St. Louis, MO; BASA and BAAPA were gifts from Dr. M. Löffler (Physiologisch-Chemisches Institut II der Universitat Marburg/L, F.R.G.); dinitrophenol was from British Drug Houses Ltd., London, U.K. Other chemicals were of analytical grade.

Incubation techniques. The kinetics of L-[^{14}C]leucine and D-[^{14}C]glucose uptake were carried out at 30°C, in a New Brunswick Giratory Water Shaker (Model T-76). The incubation mixture (generally 3 ml) contained yeast (2 mg/ml), 20 mM potassium phthalate buffer, pH 4.5, and additions as specified in each case ('standard' experimental conditions). In every case, T_0 was the time of addition of L-[^{14}C]leucine to the yeast suspension, placed in the water bath under agitation.

In order to study the effect of *N*-ethylmaleimide on L-leucine entrance into energized cells, two experimental procedures were used. With procedure A the effect of *N*-ethylmaleimide was investigated in the presence of D-glucose. Accordingly, D-glucose was added to starved yeast suspension at $T_0 - 15$ min (final concentration 5.0 mM), L-[^{14}C]leucine was added at T_0, and *N*-ethylmaleimide at the times indicated under Results. With procedure B, the effect of *N*-ethylmaleimide was investigated in the absence of external D-glucose, and, accordingly, the yeast cells were incubated with 5.0 mM D-glucose in 20 mM potassium phthalate buffer (pH 4.5), for 15 min, at 30°C. After incubation, the cells were centrifuged and the pellet was resuspended in distilled water, at 0°C. Samples of these cells were suspended in 20 mM potassium phthalatate buffer, pH 4.5. L-[^{14}C]leucine was added at T_0, and *N*-ethylmaleimide was added at the times indicated under Results.

Analytical methods. Assay of radioactive samples was performed as described in Ref. 11 except that Metricel membrane filters 25 ea, HA 0.45 μm were used throughout. Oxygen uptake was assayed in a Gilson Oxygraph (Model K-1C); the incubation medium was the same as used for the transport experiments.

Experimental design and expression of results. Samples were taken immediately after T_0, the time for taking and filtering the samples not exceeding

5 s. These samples ('very early uptake samples' in previous studies) will be termed 'T_0 samples' and the corresponding L-[^{14}C]leucine values are deemed to represent the amino acid binding to the corresponding carrier [11]. After T_0, samples were taken at $T_0 + t$ (unless stated otherwise, $t = 3$ min), the corresponding L-[^{14}C]leucine values being termed 'entrance' values. The $(T_0 + t) - T_0$ values are termed 'translocation' (T_t) values. L-[^{14}C]Leucine values are expressed as μmol/g cells (dry weight) and those for ^{14}C-labelled compounds resulting from D-[^{14}C]glucose uptake are expressed as cpm/g cells. The concentration of yeast suspensions is expressed by the weight after drying at 104°C for 24 h. In each experiment all measurements were repeated three times and the values presented are the average of three experiments. The deviation of these latter values from the mean was less than 5%.

Results

Preliminary inhibition experiments

Fig. 1(a) shows the effect of *N*-ethylmaleimide on L-[^{14}C]leucine entrance in cells energized by pretreatment with D-glucose (procedure A). It is to be seen that the inhibition varied as a function of the time the yeast was preincubated with *N*-ethylmaleimide. Thus, when *N*-ethylmaleimide was added at $T_0 - 10$ s, the inhibition increased from 0% after T_0 to $62 - 67$% at $T_0 + 3$ min, but when *N*-ethylmaleimide was added at $T_0 - 15$ min (at the same time as D-glucose), L-[^{14}C]leucine values were reduced to the level of the starved, non-energized control (95–99% inhibition). Similar results were obtained using 0.1 or 1.0 mM L-[^{14}C]leucine, or starved cells in the absence of external D-glucose (Fig. 1(b)). This latter experiment shows that, despite substrate limitation, *N*-ethylmaleimide effectively inhibited L-[^{14}C]leucine entrance from 0% at T_0 to 60% at $T_0 + 3$ min (experiment with 1.0 mM L-[^{14}C]leucine).

Fig. 2 shows the results of an experiment using the energized *rho*$^-$ mutant. Since the aerobic respiratory mechanism was not operating in this yeast, energization of L-leucine transport resulted exclusively from aerobic glycolysis. It is to be seen that, when added at $T_0 - 10$ s, *N*-ethylmaleimide decreased L-[^{14}C]leucine entrance ($T_0 + 3$ min) val-

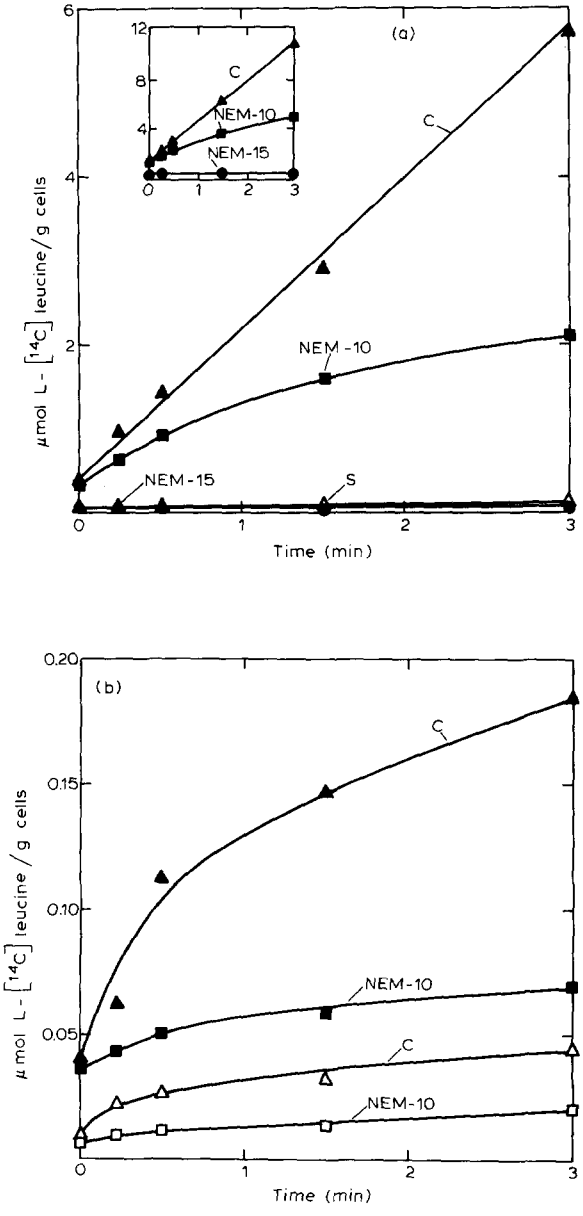

Fig. 1. Effect of *N*-ethylmaleimide on L-[^{14}C]leucine entrance into *S. cerevisiae*. (a) Energized cells. 5 mM D-glucose was added at $T_0 - 15$ min, 0.1 mM L-[^{14}C]leucine at T_0 and 1.0 mM *N*-ethylmaleimide as indicated. C (▲), yeast plus D-glucose; NEM-10 (■), same, plus *N*-ethylmaleimide added at $T_0 - 10$ s; NEM-15 (●), same, plus *N*-ethylmaleimide added at $T_0 - 15$ min; S (△), starved yeast. Inset: same experimental conditions except L-[^{14}C]leucine (1.0 mM). (b) Starved cells; 1.0 mM L-[^{14}C]leucine. C (▲), control yeast; NEM-10 (■), same, plus *N*-ethylmaleimide added at $T_0 - 10$ s; △, □, same experimental conditions, except L-[^{14}C]leucine (0.1 mM). Other conditions were as described under Materials and Methods. The incubation time after T_0 is indicated on the abscissa.

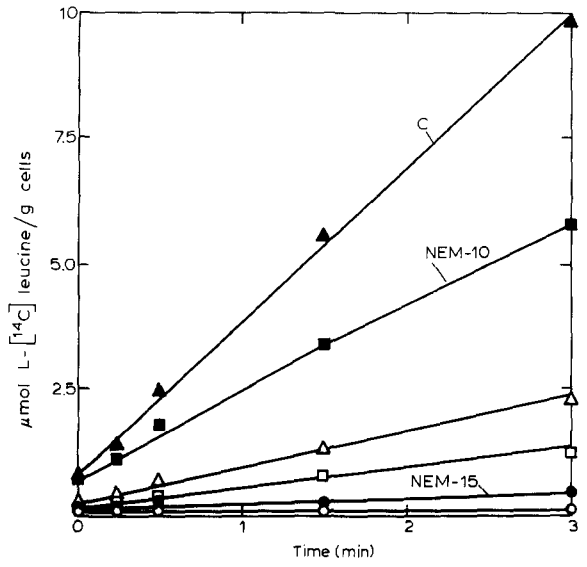

Fig. 2. Effect of *N*-ethylmaleimide on L-[^{14}C]leucine entrance into *S. cerevisiae*, *rho*$^-$ mutant (energized). 5 mM D-glucose was added at $T_0 - 15$ min and L-[^{14}C]leucine at T_0. Experiment with 1.0 mM L-[^{14}C]leucine: C (▲), yeast plus D-glucose; NEM-10 (■), same, plus 1.0 mM *N*-ethylmaleimide added at $T_0 - 10$ s; NEM-15 (●), same, plus 1.0 mM *N*-ethylmaleimide added at $T_0 - 15$ min. △, □, ○, same as above, except L-[^{14}C]leucine (0.1 mM). Other conditions were as described in Fig. 1.

ues by 40% and when added at $T_0 - 15$ min, by 98%. No significant differences were observed using 0.1 or 1.0 mM L-[^{14}C]leucine (Fig. 2). Similar results were obtained using *S. carlsbergensis* (experimental data omitted).

Observations by Kotyk and Rihová [18] showed that preincubation of yeast cells with amino acids in the absence of an energy source increases the subsequent uptake of α-aminoisobutyric acid, an effect attributed to an inductive synthesis of the amino acid carrier [18]. In good agreement with these observations, preincubation with L-leucine increased L-[^{14}C]leucine entrance by starved yeast cells (Table I). *N*-Ethylmaleimide inhibited L-[^{14}C]leucine translocation in L-leucine-preincubated and control cells to almost the same extent (Table I). Interestingly enough, with 0.1 mM L-[^{14}C]leucine and L-leucine-preincubated cells, the amino acid binding (T_0 value) was significantly decreased by *N*-ethylamleimide, in contrast with the lack of inhibition observed with the control yeast.

BASA and BAAPA, two non-penetrating SH reagents, did not inactivate L-leucine uptake. Thus, with the D-glucose-energized wild-type yeast and 5.0 mM L-[^{14}C]leucine (other experimental conditions as in Fig. 1), total uptake values at $T_0 + 3$ min (in μmol L-[^{14}C]leucine/g yeast) were 11.7 for the control yeast and 11.4 for the yeast treated with BASA (1.0 mM, added at $T_0 - 3$ min). BASA (1.0 or 10 mM) was also ineffective when using (a) 0.1 mM L-[^{14}C]leucine and 5 mM propionaldehyde as energizing substrate, or (b) the D-glucose-

TABLE I

EFFECT OF PREINCUBATION WITH L-LEUCINE ON SUBSEQUENT L-[^{14}C]LEUCINE UPTAKE AND *N*-ETHYLMALEIMIDE INHIBITION

Starved *S. cerevisiae* suspensions were divided into two aliquots, one of which was used as control while the other was preincubated for 5 min, at 30°C, with 0.1 (Expt. A) or 1.0 (Expt. B) mM L-leucine. Control and L-leucine preincubated samples were centrifuged, resuspended in potassium phthalate buffer, pH 4.5, and assayed for L-[^{14}C]leucine transport. 1.0 mM *N*-ethylmaleimide was added at $T_0 - 30$ s and L-[^{14}C]leucine entrance was measured at T_0 and $T_0 + 3$ min. Other experimental conditions were as described under Materials and Methods. The figures in parenthesis indicate percentage inhibition of L-[^{14}C]leucine entrance. $T_t = (T_0 + 3 \text{ min}) - T_0$ L-[^{14}C]leucine values.

L-Leucine during preincubation (mM)	*N*-ethylmaleimide (mM)	L-[^{14}C]Leucine entrance (μmol/g)			
		Expt. A		Expt. B	
		T_0	T_t	T_0	T_t
None	None	0.064 [a]	0.24	0.71	1.39
None	1.0	0.069	0.15(40)	0.51(28)	0.80(43)
0.1(A); 1.0(B)	None	0.148 [b]	0.35	1.00	1.74
0.1(A); 1.0(B)	1.0	0.103(30)	0.23(36)	0.66(34)	0.96(45)

b/a = 2.3: stimulation of L-[^{14}C]leucine entrance after preincubation.

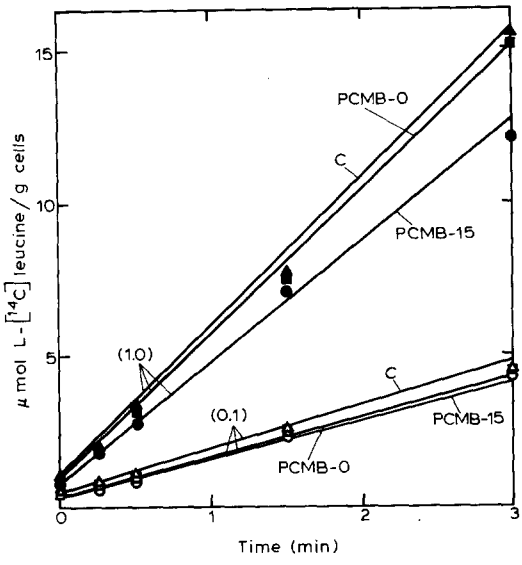

Fig. 3. Effect of PCMB on L-[^{14}C]leucine entrance into *S. cerevisiae*, *rho*$^-$ mutant. 5 mM D-glucose was added at $T_0 - 15$ min; L-[^{14}C]leucine (concentration (mM) as indicated by the figures in parenthesis) was added at T_0. C (▲, △), control yeast; PCMB-0 (■, □), same, plus 0.1 mM PCMB added at T_0; PCMB-15 (●, ○), same, plus 0.1 mM PCMB, added at $T_0 - 15$ min. Other conditions were as described in Figs. 1 and 2.

energized *rho*$^-$ mutant, with 0.1 or 5.0 mM L-[^{14}C]leucine. Similar negative results were obtained with 1.0–10 mM BAAPA.

Fig. 3 shows the effect of 0.1 mM PCMB on L-[^{14}C]leucine entrance. The mercurial was ineffective, except when added at $T_0 - 15$ min and only with 1.0 mM L-[^{14}C]leucine, in which case PCMB inactivated L-[^{14}C]leucine entrance by 23–25%.

Effect of N-ethylmaleimide on D-[^{14}C]glucose uptake and respiration

Ramos et al. [8,10,11] were able to observe that iodoacetate, a typical inhibitor of glycolysis, significantly decreases L-[^{14}C]leucine uptake into *S. cerevisiae* and *S. ellipsoideus*. It seemed reasonable, therefore, to assume that *N*-ethylmaleimide could inactivate L-[^{14}C]leucine transport by preventing D-glucose entrance (or catabolism). In order to test this hypothesis, the effect of *N*-ethylmaleimide was examined using D-[^{14}C]glucose, in experiments in which the time-schedule strictly imitated that used in the L-[^{14}C]leucine transport experiments. The results in Table II can be described as follows. (a) When *N*-ethylmaleimide was added at $T_0 - 10$ s and D-[^{14}C]glucose was added at T_0, the inhibition of D-glucose entrance at $T_0 + 15$ s was 60% (with the wild-type yeast), or 30% (with the *rho*$^-$ mutant). Similar inhibitions were observed at $T_0 + 30$ s. (b) When D-[^{14}C]glucose was added at $T_0 - 15$ min and *N*-ethylmaleimide at $T_0 - 10$ s, ^{14}C activity in the *N*-ethylmaleimide-treated yeasts (wild type or *rho*$^-$ mutant) decreased by 16% (at $T_0 + 15$ s) or 40% (at $T_0 + 3$ min). However, if the inhibition is

TABLE II

EFFECT OF *N*-ETHYLMALEIMIDE ON D-[^{14}C]GLUCOSE UPTAKE BY *S. CEREVISIAE*

Starved cells suspended in 20 mM potassium phthalate (pH 4.5); 5.0 mM D-[^{14}C]glucose (1 μmol = $1.9 \cdot 10^5$ (A) or $9 \cdot 10^4$ cpm (B)) was added as indicated. *N*-Ethylmaleimide was added at $T_0 - 10$ s. Samples were taken at the times indicated and ^{14}C values were measured. Other experimental conditions were as described under Materials and Methods. The figures in parenthesis indicate percentage inhibition of D-[^{14}C]glucose entrance.

Expt.	Yeast	Time of D-[^{14}C]glucose addition	*N*-ethylmaleimide (mM)	^{14}C assimilation (cpm/g cells) ($\times 10^3$)	
				$T_0 + 15$ s	$T_0 + 30$ s
A	Wild type	T_0	None	1.37	2.40
			1.0	0.56(60)	0.92(62)
	Rho$^-$ mutant	T_0	None	0.12	0.14
			1.0	0.09(30)	0.10(24)
				T_0	$T_0 + 3$ min
B	Wild type	$T_0 - 15$ min	None	15.8	21.3
			1.0	13.3(16)	12.7(40)
	Rho$^-$ mutant	$T_0 - 15$ min	None	17.8	22.4
			1.0	15.2(15)	12.6(40)

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE ON *S. CEREVISIAE* RESPIRATION

Yeast cells (1.7 mg/ml) were suspended in 20 mM potassium phthalate buffer (pH 4.5); D-glucose and *N*-ethylmaleimide were added as indicated below; final volume, 2 ml. The respiration rate was measured polarographically. Yeast preparations were as follows: A and C, starved cells; B, starved cells preincubated with 5.0 mM D-glucose and then washed. Other experimental conditions were as described under Materials and Methods. Values in parenthesis indicate percentage inhibition of respiration.

Yeast sample	Additions (mM)		Time of incubation with *N*-ethylmaleimide before measuring respiration(s)	Rate of respiration (atom O/min per mg yeast cells)
	D-Glucose	*N*-Ethylmaleimide		
A	5.0	None	—	62
		1.0	6	35(44)
			60	15(76)
			140	9(86)
B	None	None	—	95
		1.0	15	18(82)
			135	6(94)
C	None	None	—	6.2
		1.0	45	2.8(55)

calculated on the basis of ($T_0 + 3$ min) minus T_0 values, it follows that, with *N*-ethylmaleimide added at $T_0 - 10$ s, D-glucose entrance was completely inhibited.

Table III shows the effect of *N*-ethylmaleimide on D-glucose oxidation. It is to be seen that addition of 1.0 mM *N*-ethylmaleimide to yeast cells respiring D-glucose produced, within a short time, significant inhibition of oxygen uptake (76–86% in 1–2 min; Expt. A). The highest and fastest inhibitions were observed when using cells which had been preincubated with D-glucose for 15 min (Expt. B), where, after 15 s preincubation, *N*-ethylmaleimide decreased the respiration rate by 82%. *N*-Ethylmaleimide also decreased starved-cell respiration, but to a lesser extent (58%; Expt. C). With PCMB as inhibitor (0.1 and 1.0 mM), no effects on either D-glucose entrance or respiration were observed (experimental data omitted).

Kinetics of N-ethylmaleimide inactivation of L-leucine entrance

In order to determine the kinetics of *N*-ethylmaleimide inhibition, the following parameters were examined: (a) the time of preincubation of the yeast cells with *N*-ethylmaleimide, before T_0; (b) the time after T_0 at which amino acid binding and translocation values were measured; (c) the amino acid external concentration; (d) the energization state of yeast cells, and (e) *N*-ethylmaleimide concentration. In these experiments, the energized cells were pretreated with D-glucose according to procedure B and, therefore, the effect of *N*-ethylmaleimide on D-glucose transport was avoided. The results are presented in Figs. 4 and 5, where L-[^{14}C]leucine values are expressed as percentage of the non-inhibited control yeast. Fig. 4 show the effect of time of preincubation with *N*-ethylmaleimide. It is to be seen (Fig. 4(a)) that with starved cells and 0.1 mM L-[^{14}C]leucine, T_0 values were not affected by *N*-ethylmaleimide but T_t values decreased from 80% (when *N*-ethylmaleimide was added at T_0) to 50% (when *N*-ethylmaleimide was added at $T_0 - 60$ s). In the same experimental conditions, but using 1.0 mM L-[^{14}C]leucine, T_0 values decreased from 100 to 79%, and T_t values, from 48 to 39%. Fig. 4(b) shows the results obtained with energized yeast cells. With 0.1 mM L-[^{14}C]leucine, T_0 and T_t values decreased significantly as a function of the time of preincubation, particularly T_t (from 22 to 17%). Similar results were observed using 1.0 mM L-[^{14}C]leucine (Fig. 4(b)).

Fig. 5 shows the influence of increasing con-

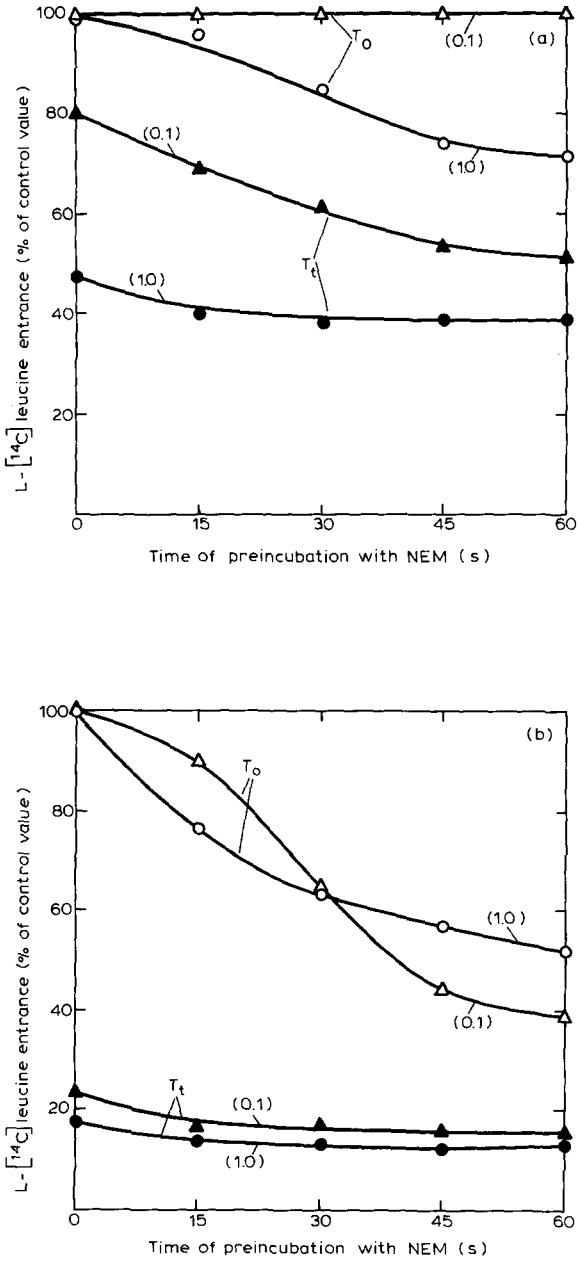

Fig. 4. Inhibition of L-[^{14}C]leucine entrance as a function of the time of preincubation with *N*-ethylmaleimide (NEM) at fixed *N*-ethylmaleimide concentration. 1.0 mM *N*-ethylmaleimide was added at the times indicated on the abscissa. L-[^{14}C]Leucine values were measured at T_0 and $T_0 + 3$ min; $T_t = (T_0 + 3\ \text{min}) - T_0$. In Expt. b the yeast cells were energized by preincubation with D-glucose, according to procedure B. External L-[^{14}C]leucine concentration (mM) as indicated by the figures in parenthesis. Other experimental conditions were as described under Materials and Methods. (a) Wild-type, starved yeast; (b) wild-type, energized yeast.

centrations of *N*-ethylmaleimide on L-leucine entrance, at fixed time (40 s) of preincubation. In good agreement with the results in Fig. 4, when using starved yeast and 0.1 mM L-[^{14}C]leucine, binding (T_0) values were not affected by *N*-ethylmaleimide, whatever its concentration (Fig. 5(a)). On the other hand, when using energized cells

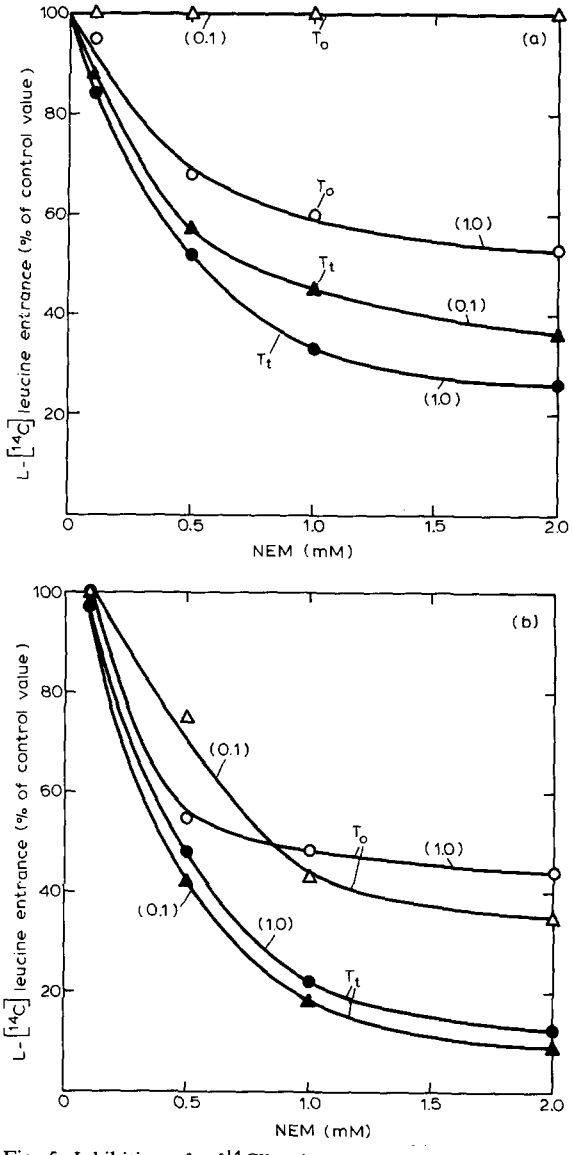

Fig. 5. Inhibition of L-[^{14}C]leucine entrance as a function of *N*-ethylmaleimide (NEM) concentration, at fixed preincubation time. Conditions were as in Fig. 4, except for *N*-ethylmaleimide, which was added at $T_0 - 40$ s, at the concentrations indicated on the abscissa. (a) Wild-type, starved yeast; (b) wild-type, energized yeast.

L-[^{14}C]leucine binding was decreased by *N*-ethylmaleimide, either with 0.1 or 1.0 mM L-[^{14}C]leucine (Fig. 5(b)). The concentration-effect curves were parabolic, showing maximal variation in the 0.1–1.0 mM range and approaching a plateau in the 1.0–2.0 mM range. Remarkably enough, when using energized wild-type yeast, 0.1 mM *N*-ethylmaleimide did not inhibit L-[^{14}C]leucine binding, an unexpected result that was confirmed with protoplasts of the same yeast (unpublished data).

Effect of N-ethylmaleimide on kinetic parameters of L-[^{14}C]leucine entrance

Fig. 6 shows the effect of *N*-ethylmaleimide on the kinetics of L-leucine entrance as a function of the amino acid concentration. Since saturation isotherms were similar to those previously reported with *S. cerevisiae* and *S. ellipsoideus* [9–11], the primary data are omitted. The Hofstee plot for L-[^{14}C]leucine translocation values (T_t) was obtained with the wild-type yeast, energized by pretreatment with D-glucose. The concave-upwards curves confirm the existence of two kinetic systems, namely S_1 and S_2, characterized by parameters J_{max} and K_T. J_{max} expresses the maximum flux that the yeast cells can exhibit towards the amino acid, and K_T formally expresses the substrate concentration at which the flux is one half the limited flux J_{max}. J_{max} and K_T were calculated from the influx data, using the graphical method, and secondary parameters A–D were calculated from the corresponding J_{max} and K_T values. Parameter values are presented in Table IV, where it can be seen that in every case *N*-ethylmaleimide decreased J_{max} but did not significantly affect K_T. Inequalities $A/B + BC/A < D$ and $4C < D^2$ did not vary as a result of *N*-ethylmaleimide treatment, in good agreement with the inhibition of both L-[^{14}C]leucine transport systems.

Effect of dinitrophenol on N-ethylmaleimide inhibition

Combination of *N*-ethylmaleimide and uncouplers (e.g., dinitrophenol) has provided interesting information on the mode of action of *N*-ethylmaleimide on membrane thiols [19]. On this basis we investigated the combined effects of dinitrophenol and *N*-ethylmaleimide on L-[^{14}C]-leucine entrance. The energized *rho*$^-$ mutant was used for this experiment, to avoid the effect of dinitrophenol on energy-yielding reactions at the mitochondrial membranes, since *rho*$^-$ mutant yeasts do not have a coupled, functional mitochondrial ATPase [20]. The cells were incubated with dinitrophenol, the excess dinitrophenol was removed, and then *N*-ethylmaleimide and L-[^{14}C]leucine were added as indicated in Table V. The results presented show that accumulation [21] of dinitrophenol into the yeast cells significantly enhanced the effect of *N*-ethylmaleimide, with either 0.1 or 1.0 mM L-[^{14}C]leucine. T_o and T_t values reflected the increased effect of *N*-ethylmaleimide after treatment of cells with dinitrophenol, the larger dif-

TABLE IV

EFFECT OF *N*-ETHYLMALEIMIDE ON KINETIC PARAMETERS OF L-[^{14}C]LEUCINE TRANSPORT

Yeasts cells were energized by pretreatment with D-glucose (procedure B). Experimental conditions were as described in Fig. 6, and under Materials and Methods. $A = K_{T,1}\,J_{max,2} + K_{T,2}J_{max,1}$; $B = J_{max,1} + J_{max,2}$; $C = K_{T,1} \cdot K_{T,2}$, and $D = K_{T,1} + K_{T,2}$. Units, as follows: A, mM·μmol·min^{-1}·g^{-1}; B, μmol·min^{-1}·g^{-1}; C, mM^2; D, mM. Values in parenthesis indicate percentage inhibition of J_{max}.

Yeast	*N*-Ethylmaleimide (mM)	$K_{T,1}$ (mM)	$J_{max,1}$ (μmol/min per g cells)	$K_{T,2}$ (mM)	$J_{max,2}$ (μmol/min per g cells)	$(A/B)+(BC/A)$	$4C$	D^2
Wild type	0	0.048	2.70	0.41	6.60	0.33	0.08	0.21
	1.0	0.049	0.34(88)	0.95	0.64(90)	0.46	0.15	0.98
Rho$^-$ mutant	0	0.027	0.48	0.80	2.34	0.29	0.08	0.68
	1.0	0.031	0.17(64)	0.44	0.48(79)	0.23	0.06	0.22

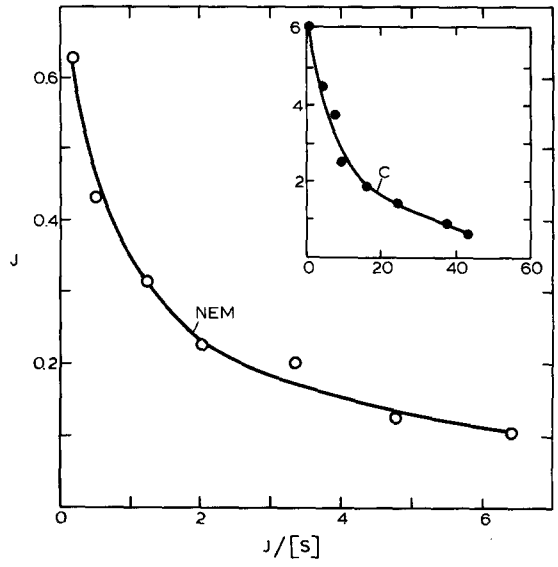

Fig. 6. Effect of *N*-ethylmaleimide on the kinetic of L-[^{14}C]leucine entrance into *S. cerevisiae* (wild-type); energized cells (procedure A). C, control yeast; NEM, yeast added 1.0 mM *N*-ethylmaleimide at $T_0 - 35$ s. Samples were taken at T_0 and $T_0 + 2$ min (T_2). J (μmol L-[^{14}C]leucine/g cells per min) = 0.5 ($T_2 - T_0$ L-[^{14}C]leucine values); S, L-[^{14}C]leucine (mM). Other experimental conditions were as described under Materials and Methods.

TABLE V

EFFECT OF DINITROPHENOL ON *N*-ETHYLMALEIMIDE INHIBITION OF L-[^{14}C]LEUCINE ENTRANCE

Rho$^-$ mutant cells were energized by pretreatment with D-glucose (procedure B). The cell suspension in 20 mM potassium phthalate buffer, pH 4.5, was divided into two aliquots, one of which was used as control while the other was incubated with dinitrophenol for 5 min. Control and incubated cells were centrifuged, washed and used for the transport experiment; *N*-ethylmaleimide was added at $T_0 - 35$ s. Other experimental conditions were as described under Materials and Methods. Values indicate percentage inhibition of L-[^{14}C]leucine entrance, as compared with the non-inhibited control. $T_t = (T_0 + 3$ min$) - T_0$ L-[^{14}C]leucine values.

Dinitrophenol during preincubation (mM)	*N*-Ethylmaleimide during transport experiment (mM)	L-[^{14}C]Leucine entrance			
		L-[^{14}C] Leucine: 0.1 mM		L-[^{14}C] Leucine: 1.0 mM	
		T_0	T_t	T_0	T_t
None	None	0	0	0	0
	1.0	4	9	10	25
0.05	None	11	17	10	20
	1.0	37	52	35	67

ferences being observed with 0.1 mM L-[^{14}C]leucine.

Discussion

Thiol groups play an important role in the activity of many functional proteins and, accordingly, characterization of thiol groups in amino acid transport proteins may constitute an interesting approach towards elucidating the mechanism of the transport process. In this connection, there is evidence for a role of thiol groups in amino acid transport in bacteria [15], erythrocytes [16] and other biological systems [17]. BASA and BAAPA are very effective SH alkylating agents and their limited membrane penetrability makes these compounds advantageous for the study of membrane systems in intact living cells [17]. On this basis, the insensitivity of L-leucine transport to these reagents argues against the presence of an SH-dependent transport-protein on the outer surface of the cell permeability barrier, and a similar conclusion may be inferred from the effect of PCMB. In contrast with these negative results, *N*-ethylmaleimide effectively inactivated L-leucine transport, both in *S. cerevisiae* (the results described here) and in *S. carlsbergensis* (unpublished observations). These inhibitions fit in well with several properties of *N*-ethylmaleimide, namely (a) good penetration through membranes because of the uncharged nature of the *N*-ethylmaleimide molecule; (b) relatively high selectivity for SH groups, and (c) reaction with only certain accessible groups on proteins, making specific metabolic effects possible. Taking into account the negative effects of the non-penetrating SH reagents it seems reasonable to assume that the *N*-ethylmaleimide-sensitive protein(s) involved in L-leucine transport should be deeply embedded in the yeast cell plasmalemma.

L-[^{14}C]Leucine entrance rates at 0.1 and 1.0 mM L-[^{14}C]leucine can be taken as indicative measurements of the transport activity of systems S_1 and S_2, respectively [11]. Accordingly, the different effects of *N*-ethylmaleimide at these different amino acid concentrations provide additional evidence for the characterization of L-leucine transport systems. Generally speaking, the effect of *N*-ethylmaleimide may be due to inhibition of (a)

formation of the amino acid carrier complex; (b) the amino acid translocation step; (c) the energy coupling mechanism (including the H^+ pump); (d) the metabolic, energy-yielding reactions, that drive the amino acid transport (e.g., D-glucose oxidation); (e) combination of these mechanisms. Concerning point (a), it is worth noting that T_0 values were previously interpreted [11] as the expression of L-leucine binding to a carrier protein and, accordingly, in the case of S_1, the effect of *N*-ethylmaleimide (after preincubation with L-leucine or D-glucose) may well signify the presence of essential SH groups in this protein, in all probability the general amino acid permease. The lack of protection afforded by excess external or internal L-leucine (Table I), as well as the absence of significant modification of K_T values by *N*-ethylmaleimide (Table IV), support the view that the *N*-ethylmaleimide-reactive thiols should not be located at the carrier binding-site. Interestingly enough, when using non-energized yeast and 0.1 mM L-[^{14}C]leucine (Figs. 4 and 5), *N*-ethylamleimide did not affect the amino acid binding, despite the carrier reactivity to *N*-ethylmaleimide, as proved by pretreating the yeast with L-leucine (Table I). The modulation of the S_1 carrier component by the energy load implies a special feature of the high-affinity, low-velocity transport systems, since, when using 1.0 mM L-[^{14}C]leucine, *N*-ethylmaleimide inhibited binding irrespective of the energization state of the yeast cells. In this connection, other differences between the L-[^{14}C]leucine entrance systems are worth recalling, namely, the selective inhibition of S_1 by DCCD, quercetin and diethylstilbestrol [11].

N-Ethylmaleimide inactivation of L-[^{14}C]leucine translocation was always more extensive than the inhibition of binding (Figs. 3 and 4) and, accordingly, the former inhibition should imply mechanisms other than *N*-ethylmaleimide interaction with the amino acid carrier. In support of this assumption there stand the following facts: (a) in experimental conditions resembling those used in the transport experiments, *N*-ethylmaleimide rapidly inhibited D-glucose uptake (Table II) and respiration (Table III), either with exogenous (D-glucose) or endogenous substrates; (b) measurement of the *N*-ethylmaleimide effect on L-[^{14}C]leucine translocation involved at least 3 min incubation with the yeast cells (Figs. 4 and 5), a time interval that, according to results in Tables II and III, was sufficient to ensure extensive inhibition of glycolysis and other reactions yielding high-energy compounds. On this basis, it seems reasonable to assume that the inactivation of translocation also involved that of the metabolic process driving L-[^{14}C]leucine transport. In this regard, it is interesting to note that with the respiratory-deficient *rho*$^-$ mutant, where aerobic generation of high-energy compounds could be excluded, the effect of *N*-ethylmaleimide was less than with the respiratory-competent yeast (Fig. 2). Finally, the possible role of the plasmalemma ATPase in L-[^{14}C]leucine transport inactivation can be ruled out, since ATPase activity was not affected by *N*-ethylmaleimide, in experimental conditions approaching those used for the transport experiments (see Addendum).

Any interpretation of the effect of energization on *N*-ethylmaleimide inhibition must taken into account the fact that, in the cell membrane, the availability of protein SH groups for specific reagents might be modulated by complex mechanisms including (a) the concentration of internal and external ions, (b) the membrane potential, (c) the phosphate potential and (d) the intracellular amino acid concentration. Regulatory processes (a) and (b) are consistent with the results in Table V, since dinitrophenol modifies the proton gradient at the cell membrane (yeast loses K^+ and gains H^+ during exposure to dinitrophenol), while mechanisms (c) and (d) are supported by the effect of preincubation with D-glucose (Figs. 4 and 5) and L-leucine (Table I), respectively. Variations of parameters (a)–(d) as a function of the metabolic conditions in the yeast cell might determine conformational changes of carrier proteins, by exposing hidden SH groups or by facilitating the dithiol-disulfide interconversion, with generation of the free SH [22]. In this connection it seems pertinent to recall previous observations by other workers showing that energization (or de-energization) modifies the effect of *N*-ethylmaleimide on membrane thiols [22]. Thus, the reactivity of the *Escherichia coli* membrane proteins to *N*-ethylmaleimide increases after membrane energization with phenazine methosulfate, whereas pretreatment with uncouplers enhances the inhibitory ef-

fect of *N*-ethylmaleimide on proline transport in membrane vesicles from the same organism [19]. *E. coli* and yeast cell membrane differ in composition and function, a fact which makes it hazardous to extrapolate the results obtained with one organism to the other. Nevetheless, the results in Table V are in good agreement with the observations of Janick et al. [15] on the combined effect of uncouplers and *N*-ethylmaleimide on amino acid transport in *E* coli.

Addendum

Effect of N-ethylmaleimide on the rho⁻ mutant ATPases

(Esther M. De Pahn and A.O.M. Stoppani)

L-Leucine entrance in *Saccharomyces* can be explained in terms of H^+-dependent cotransport driven by an H^+ pump, the plasmalemma ATPase [11,23–25]. Accordingly, *N*-ethylmaleimide inhibition of L-leucine entrance might involve the H^+ pump, since plasmalemma ATPases are thiol enzymes [24–28]. In order to test this hypothesis, the effect of *N*-ethylmaleimide on yeast ATPases was investigated. Toluene-treated yeast cells [24] were used for these experiments, since the toluene treatment, by altering the cell membrane, allowed measurement of intracellular ATPases in situ, under conditions closely similar to those operating in vivo. ATPase activity was measured as described by Serrano [24]. Duplicate samples of permeabilized cells (*S. cerevisiae*, strain 207, *rho⁻* mutant) were preincubated with the inhibitors for 5 min before starting the reaction with ATP. After further incubation for 10 min, the hydrolysed P_i was measured. The experiments were performed at 30°. *N*-Ethylmaleimide, CMPS and FMA were purchased from Sigma Chemical Company, St. Louis, MO. *N*-Ethylmaleimide solutions were prepared immediately before use.

Table VI shows that at pH 5.6 (optimal pH for plasmalemma ATPases [24,26,28,29]), *N*-ethylmaleimide stimulated ATP hydrolysis, whereas the organic mercurials and NaF inhibited it to a significant extent. These results recall those reported for other plasmalemma ATPases [24–29], especially the effect of *N*-ethylmaleimide, which, under experimental conditions closer to those described here, did not inhibit the *Neurospora* enzyme [26].

TABLE VI

EFFECT OF INHIBITORS ON THE *Rho⁻* MUTANT ATPase

The experimental conditions were as described in the text. Toluene-treated yeast cells: 4–1 mg/ml reaction medium. The figures represent percentage inhibition of activity. ATPase control activities (mU/mg protein): 2.1–0.7 (pH 5.6) and 5.6–2.7 (pH 8.5).

Inhibitor	Concentration (mM)	ATPase inhibition (%)	
		pH 5.6	pH 8.5
N-Ethylmaleimide	1.0	−27; −25	56
	2.0	−36; −25	48
CMPS	0.20	90	70
FMA	0.10	67;75	−19
NaF	2.5	92	9

ATP hydrolysis at pH 8.5 largely reflected the mitochondrial ATPase activity, since (a) 'petite' strains contain F_1-ATPase [20]; (b) pH curves [24,28] indicate that, at pH 8.5, about 80% of the ATPase activity was due to the mitochondrial enzyme [24]; (c) in accordance with the lesser sensitivity of the mitochondrial ATPase for organic mercurials and NaF [29], these inhibitors were less effective at pH 8.5 than at pH 5.6 (Table VI).

Relevant to the purpose of this investigation was the effect of *N*-ethylmaleimide at pH 8.5, which proved that this reagent was effective in inhibiting intracellular enzymes in the toluene-treated cells. The inhibition of the mitochondrial ATPase, together with the lack of inhibition of the plasmalemma enzyme, supports the view that in *S. cerevisiae* *N*-ethylmaleimide inhibited L-leucine transport by modifying the amino acid carrier protein. On the other hand, organic mercurials migh inhibit L-leucine transport, at the level of the plasmalemma ATPase (Refs. 27 and 29, and Table V) but the enzyme location inside the cell membrane [24], as well as the low penetrability of PCMB, prevented the inhibition.

Note added in proof (Received May 3rd, 1983)

After submission of this paper, Brooker and Slayman [30] reported that *N*-ethylmaleimide inhibits purified plasma membrane ATPase of *N.*

crassa. This inhibition does not necessarily contradict previous observations by Scarborough [26] and those described here, since the time-course of the ATPase inhibition is relatively slow and Mg nucleotides provide significant protection against *N*-ethylmaleimide.

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