BIOCHEMICAL STUDIES ON THE CYTOSINE PERMEASE OF *SACCHAROMYCES CEREVISIAE*

N. PARLEBAS and M. R. CHEVALLIER

Laboratoire de Génétique Physiologique, I.B.M.C. 15, rue R. Descartes, 67000 Strasbourg, France

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1. Introduction

A functional characterization of the cytosine-purine permease from *Saccharomyces cerevisiae* has been reported in previous papers [1,2]. It was shown that permease-less mutants are readily isolated. Furthermore, genetic analysis has shown that all the permease mutants belong to the same complementation group, thus suggesting that only one specific protein is involved in this particular transport system. In this report we demonstrate that the plasma membranes of cytosine-purine permease-less mutants have significantly fewer polypeptides migrating at one specific position in SDS-polyacrylamide gel electrophoresis (PAGE). This strongly suggests that the cytosine-purine permease migrates at this position and enabled us, firstly, to evaluate its mol. wt. at approx. 80 000 and secondly, to estimate that this protein represents 1% of the membraneous protein fraction isolated.

2. Materials and methods

2.1. *Strains and media*

The strains used were *Saccharomyces cerevisiae* FL 100 (haploïd, *a*, mating type) and two cytosine-purine permease-less derivatives : strains FL 480-1B (*a*, *fcy* 2–3) and NP 2 sp3 (*a*, *fcy* 2–18). These mutants have been selected as presumptive frameshift mutation by their pattern of reversion frequency in response to several mutagens according to the method described by Pittman and Brusick [3]. Among twenty independently obtained mutants (19 ICR_{170} induced and 1 u.v. induced) the two strains FL 480-1B and NP 2 sp3 assumed to be devoid of purine-cytosine permease protein of significant size were studied [4]. Cultivation medium used throughout was Yeast Nitrogen Base (YNB) without amino acids (Difco) supplemented with 4% glucose.

2.2. *Cell labelling*

In each experiment permease$^+$ and permease$^-$ strains were independently labelled with [^{3}H]leucine or [^{14}C]leucine as indicated in the text. 20 μCi of [^{14}C]leucine (56 mCi/mM) were added to 500 ml of YNB medium and 100 μCi of [^{3}H]leucine (208 mCi/mM) to 500 ml of YNB medium during the last generation of growth. The cultures were grown until late exponential growth phase (OD $\simeq$ 150 Klett units as monitored in Klett-Summerson colorimeter using a blue filter). The cells were harvested, mixed, washed three times with cold water, then resuspended in 0.5 M sorbitol.

2.3. *Membrane preparation*

The method used for the preparation of membraneous fractions was an adaptation of the method described by Matile ([5] and personal communication). The cell pellet was resuspended in an equal volume of 0.5 M sorbitol, glass beads (diam. 0.5 mm) were added until the total volume was occupied by the beads. The suspension was then homogenized in a Braun Homogeneizer (Braun Melsungen) at full speed for two periods of 30 sec. The homogeneized sample was then sedimented for 10 min at 10 000 *g*. Sucrose (39% w/w) was added to the supernantant so as to obtain a final density approx. 1.21 g/cm^3. This supernatant was then centrifuged 60 min at 150 000 *g* in a MSE Superspeed 50 centrifuge, angle rotor 10 × 10

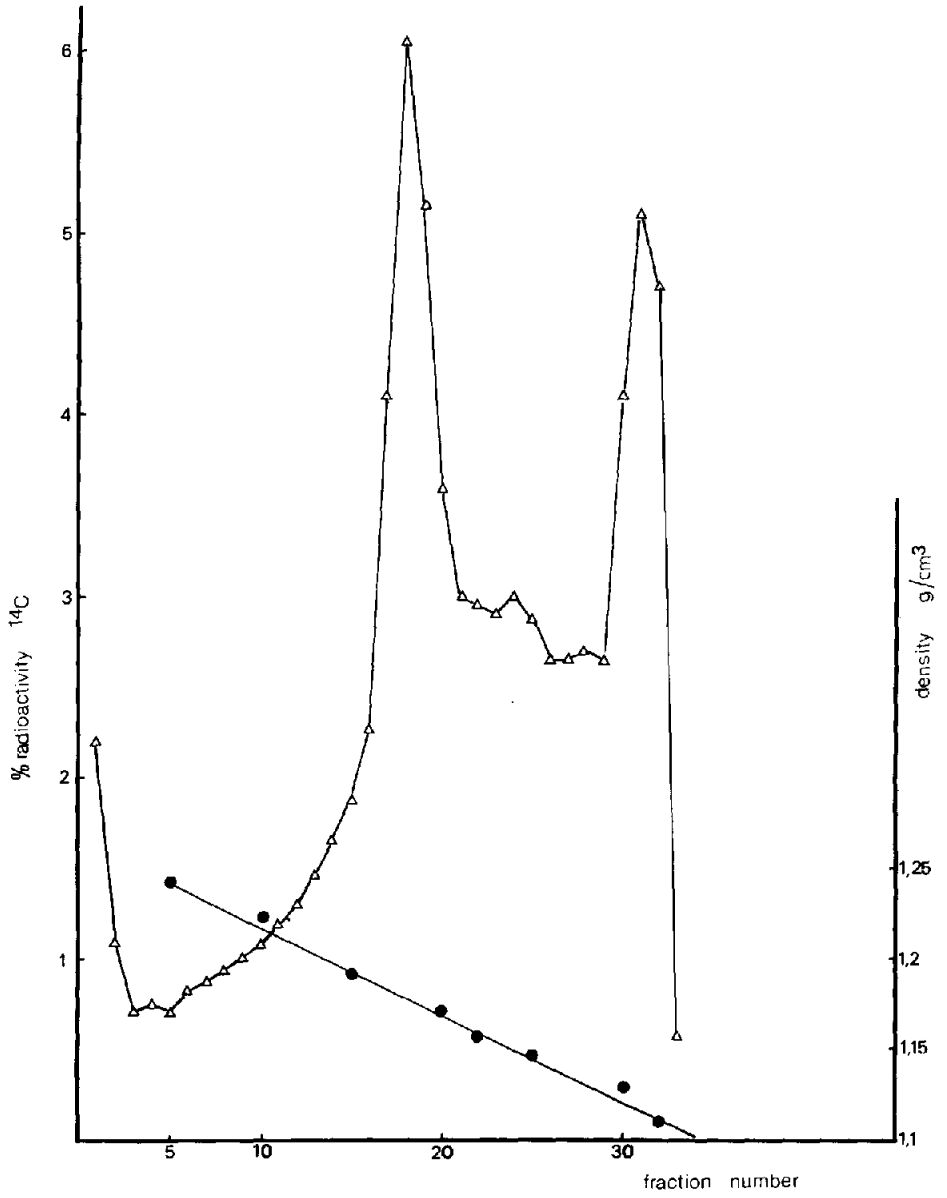

Fig.1. Typical separation obtained by density gradient centrifugation of the crude membrane fraction. Left hand scale : % of input radioactivity of [^{14}C]leucine in each fraction (△——△); Right hand scale : density of renografin in g/cm^3 (●——●); For details, see Materials and methods under membrane preparation. Peak 1 corresponds to the top of the tube. Peak 2 centered at density 1.18 g/cm^3 corresponds to the purified plasmalemma.

ml. The supernatant (S) was usually discarded, and the pellet resuspended in 55% w/w sucrose and centrifuged 60 min at 150 000 *g*. The resulting pellet, resuspended in 0.5 M sorbitol was again centrifuged for 20 min at 150 000 *g* and resuspended in 0.5 M sorbitol, usually 0.5 ml. This constitutes the crude plasmalemma. Further purification was achieved by centrifugation of this fraction in a linear density gradient of urografin* (Schering A. G., Berlin) ranging from 1.10–1.22 g/cm^3. Tubes with 12 ml of urografin gradient were loaded with 0.1–0.3 ml of the crude fraction and centrifuged 2.5 h at 39 000 rev/min, in a Spinco LS 75, rotor SW 41. After the centrifugation two bands were clearly visible, one band near the top (peak 1) and a second band near the middle of the gradient (peak 2). The gradient was collected by carefully introducing a capillary pipet at the bottom of the tube and aspirating with a peristaltic pump (Desaga). Fractions of 0.4 ml were collected. The plasmalemma corresponds to the turbid fraction which is located at a density of 1.175 g/cm^3 [4]. In our experiments, the radioactive leucine of each fraction was analysed, and the peak 2 fractions were pooled, diluted one in four with 0.5 M sorbitol, centrifuged 15 min at 150 000 *g* and resuspended in 0.5 M sorbitol (0.5 ml). This fraction constitutes the purified plasmalemma (See fig.1 for a typical result of this purification procedure).

2.4. *Polyacrylamide gel electrophoresis*

Slabs of gel (18 × 14 × 0.3 cm) containing 5% (w/v) polyacrylamide, were made as described by De Wachter and Fiers [6]. The gels were prepared in the absence of SDS from acrylamide and bis-acrylamide (19:1, w/w) in TEB buffer (90 mM) tris-hydroxymethylaminomethane-borate, (2.5 mM ethylenediamine-tetraacetate, pH 8.3). *N,N,N',N'*-tetramethylethylenediamine (0.033 ml/g of acrylamide) and freshly dissolved ammonium persulfate (3.3% w/v; 0.1 ml/g of acrylamide) were used as catalysts. Polymerization was allowed to proceed overnight at 20°C, after which the gels were prerun at 250 V for 1 h at 4°C, using an electrophoresis buffer of TEB with 0.1% (w/v) SDS. This prerun is necessary to eliminate unpolymerized acrylamides and catalysts from the gels and enables the introduction of SDS into the gel. After the prerun the buffer was replaced with fresh TEB containing 0.1% SDS. The crude or purified plasmalemma fractions were precipitated with one volume of 10% trichloroacetic acid. The precipitate was collected by centrifugation and washed three times with 1 ml of ether. The resulting pellet was resuspended in 50 µl TEB buffer with 2% (w/v) SDS, 10% (w/v) sucrose, 0.05% (w/v) Brom-

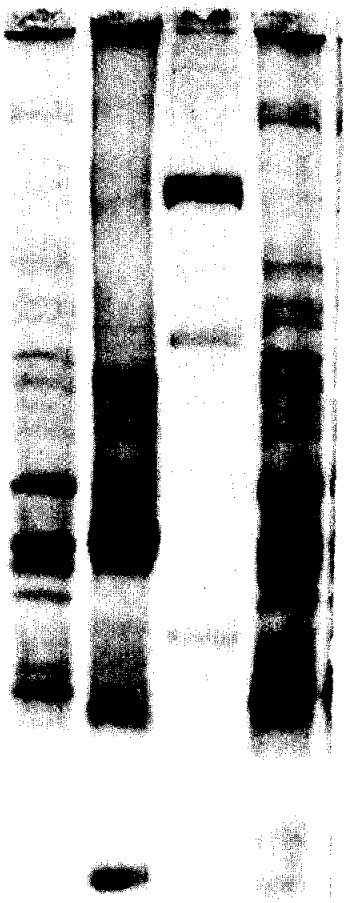

Fig.2. Polyacrylamide gel electrophoresis of the supernatant fraction S (band 1 and 4), crude membrane (band 2) and *E. coli* RNA polymerase stained with Coomassie blue. The crude plasmalemma protein pattern is significantly different from the protein pattern of the supernatant fraction.

* Urografin: Na^+ and methylglucamine salt of *N,N'*-diacetyl-3.5-diamino-2,4,6 triiodobenzoic acid in Na citrate, pH 7, containing 10^{-3} M ethylenediamintetracetate (EDTA).

phenol blue and 5% (v/v) β-mercaptoethanol and heated for 2 min in boiling water just before application. Samples up to 15 μl were applied to the gel, electrophoresis was run at 250 V (about 20 mA/gel) at 4°C, until the Bromphenol blue marker had migrated about 10 cm from the origin. Immediately after electrophoresis, gels were immersed into staining solution consisting of 50% (v/v) methanol, 7.5% (v/v) glacial acetic acid, 0.05% (w/v) Coomassie blue in distilled water and agitated gently overnight. Destaining was achieved by three successive washings in 25% methanol–7.5% acetic acid. The gels were then dried down onto filter paper in a press, by incubation at 80°C in a vacuum oven (See fig.2 for illustration of gel electrophoresis). To determine the distribution of radioactivity in a gel, bands of the dried gel (2.5 mm cut with scissors) were incubated overnight at 60°C in 1 ml of a solution containing H_2O_2 (110 vol) and 5% vol of NH_4OH 25%.

To compensate for evaporation, 0.4 ml of water was added to this solution which was then mixed with 10 ml Triton–toluene–PPO scintillation fluid. Radioactivity measurements were made in an Intertechnique LS 30 Spectrometer using appropriate channels to minimize cross-contamination of the two isotopes. Samples of hydrolysed gel containing only one of the isotopes served as a standard to evaluate the counts in each channel. The ratio of $^{14}C/^{3}H$ counts was then estimated using the appropriate cross corrections.

2.5. *Chemicals*

[^{14}C]Leucine, specific activity 56 mCi/mmole, and [^{3}H]leucine, specific activity 208 mCi/mmole were obtained from CEA-France. Acrylamide (purum) was purchased from Fluka A. G. *N,N'*-methylene-bis-acrylamide (Fluka, practicum) was crystallized from acetone before use. All other reagents were of analytical grade.

3. Results and discussion

Typical results obtained by electrophoresis of a crude plasmalemma preparation where the cytosine permease$^+$ and cytosine permease$^-$ strains were labelled with [^{14}C]leucine and [^{3}H]leucine respectively are given in fig.3. It may be seen that the $^{14}C/^{3}H$ ratio is significantly higher in a band which is located closely to the σ polypeptide of *E. coli* RNA polymerase. Fig.4B shows the radioactivity distribution obtained after electrophoresis of purified membrane fraction with inverted ^{14}C and ^{3}H markers for the two strains. As expected from the preceding results a modification of the $^{3}H/^{14}C$ ratio occured at the same position in this gel. Fig.4A shows the radioactivity recovered in each fraction and indicates that the purified plasmalemma contains 4 major bands; three of which were also clearly visible in the profile obtained from crude plasmalemma. Moreover it had previously been shown in this laboratory that distribution of polypeptides found in crude and purified plasmalemma clearly differed from the distribution in a total protein extract [7] and differs from the distribution that we found in the supernatant fraction S (See fig.2).

Eight independent experiments were done. For each experiment the total number of counts in a gel for each isotope was calculated; in experiments where the wild type strain was labelled with [^{14}C]leucine, the ratio $^{14}C/^{3}H$ was then used to normalize the ratios found in each individual fraction. In experiments with the markers inverted (i.e. wild type labelled with [^{3}H]leucine) the ratio $^{3}H/^{14}C$ was used to normalize the ratios. This procedure allowed a direct comparison between different experiments even when the marker ratios differed to some extent. The molecular weight of the bands were assigned according to their position in the gel using *E. coli* RNA

Fig.3. (A) Profile of the radioactivity of the PAGE bands obtained from crude plasmalemma using double label, as explained in Materials and Methods. (- - - - -) [^{14}C]leucine; (— —) [^{3}H]leucine. (B) Upper curve ratio of $^{14}C/^{3}H$ counts. In this experiment the permease$^+$ strain was labelled with [^{14}C]leucine and the permease$^-$ strain with [^{3}H]leucine. a, b, c, represent respectively the location of the peptides of the *E. coli* RNA polymerase. a corresponds to the β'β subunits (165 000 and 155 000 dalton) which are not separated in our conditions, b corresponds to the σ peptide (90 000 dalton) and c to the α subunit (39 000 dalton). Note the higher ratio in the fraction behind band b of *E. coli* RNA polymerase and that some of the scattering of the ratios $^{14}C/^{3}H$ counts is explained by the low radioactivity.

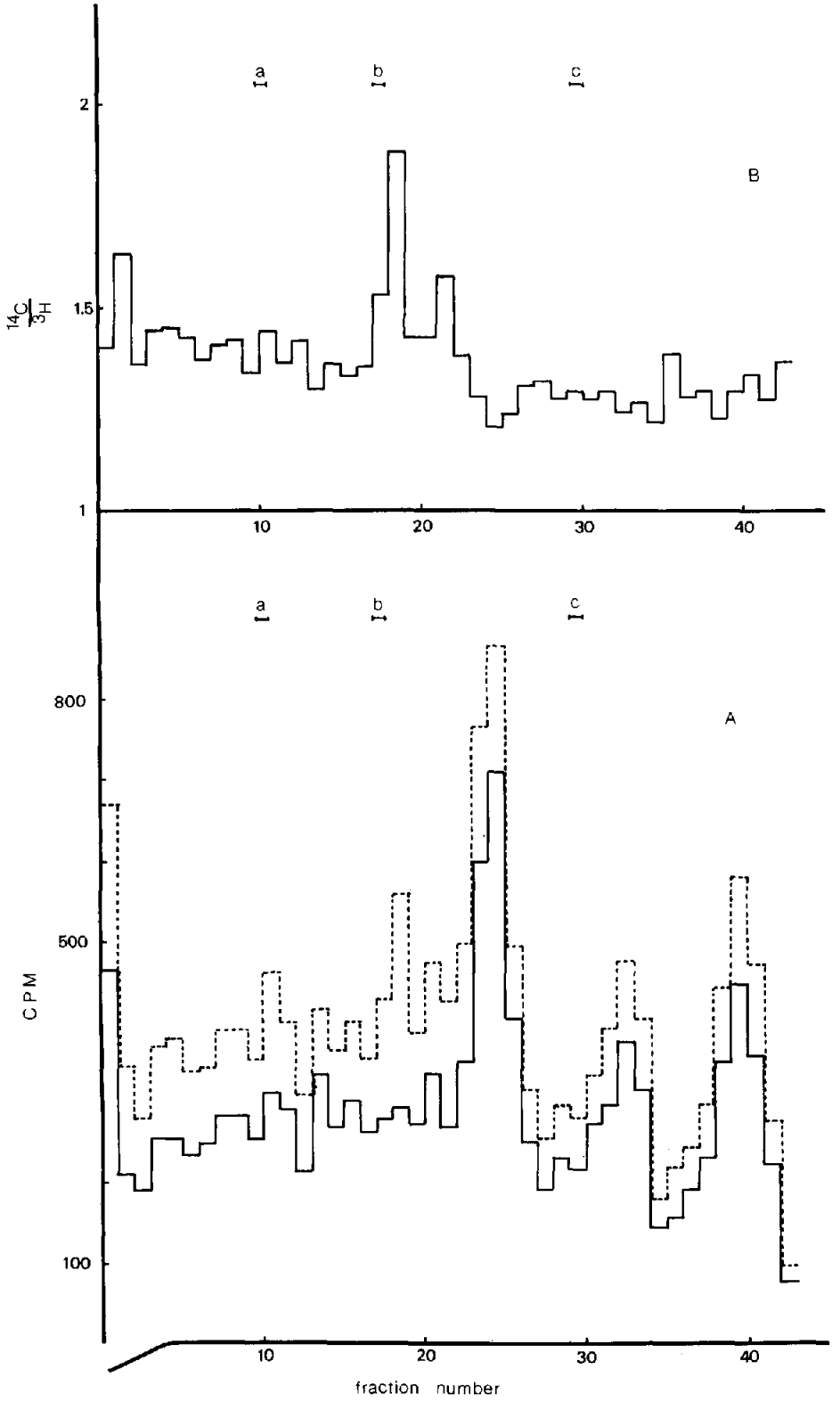
a
b
c
2
1.5
1
B
$^{14}C/^{3}H$
10
20
30
40
a
b
c
800
500
100
A
CPM
10
20
30
40
fraction number

Fig. 3

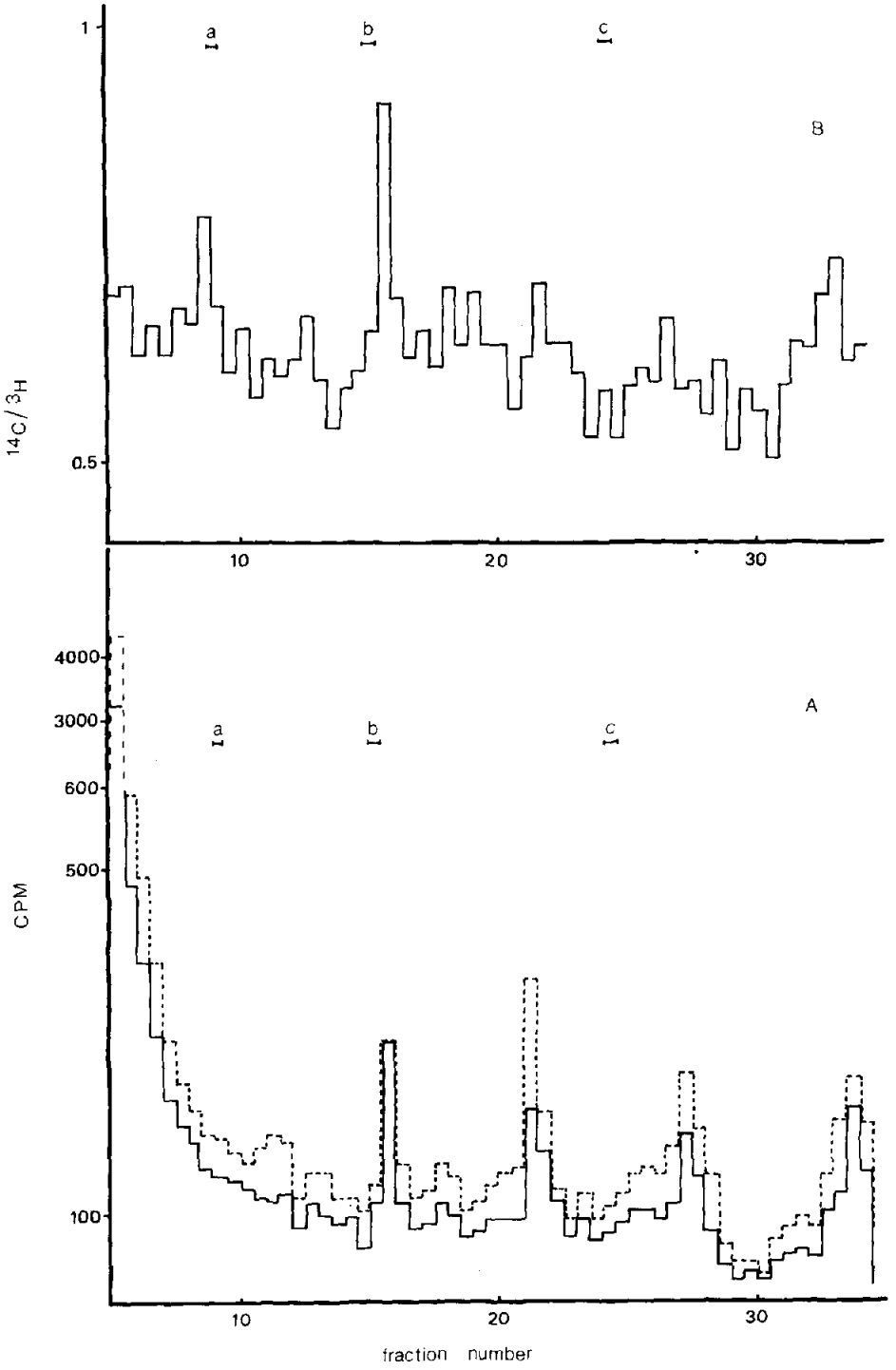

Fig.4. (A) Profile of the radioactivity of the PAGE bands obtained from purified plasmalemma using double label as explained in Materials and methods. (- - - -) [^{14}C]Leucine; (——) [^{3}H]leucine. (B) Upper curve ratio of $^{3}H/^{14}C$ counts. In this experiment the permease^{+} strain was labelled with [^{3}H]leucine and the permease^{-} strain with [^{14}C]leucine. a, b and c; as in fig.3.

Table 1
Comparison of normalized marker ratios for three fractions of the polyacrylamide-SDS gel

		Experiment number, $^{14}C/^{3}H$								
	Mol. wt.	1	2	3	4	5	6	7	8	Average ratio
Crude	160 000	0.998	0.999	0.946	0.979	1.026	1.074	1.167	1.050	1.030
plasma-	39 000	0.944	1.188	1.011	1.055	0.963	1.034	0.868	0.895	0.995
lemma	80 000	1.163	1.314	1.491	1.224	1.400	1.503	1.312	1.412	1.353
Purified	160 000				0.760	1.011	0.946	1.100	1.086	0.981
plasma-	39 000				1.015	1.191	0.886	0.963	0.965	1.004
lemma	80 000				1.542	1.583	1.316	1.048	1.316	1.361

Experiments 1 to 6 compare strains FL 100 and *fcy* 2–3 as wild type and cytosine permease$^-$ respectively. In experiments 7 and 8 the presumed frameshift mutant *fcy* 2–18 was used instead of strain *fcy* 2–3.

In experiments 1 to 5 and 7 the wild type was labelled with [^{14}C]leucine and the cytosine permease$^-$ strain with [^{3}H] leucine, inverted marker were used for experiments 6 and 8.

polymerases subunits which were run simultaneously as calibration polypeptides. To appreciate the significance of the deviation which was observed in the 80 000 fraction, the ratio observed in each experiment in two other fractions were grouped in table 1. We have arbitrarily chosen to compare the fractions corresponding to the mol. wt. of the $\beta\beta'$ and α subunits of *E. coli* RNA polymerase. It appears that some variability of the markers ratios occurs in these fractions, but these variations occur in both directions from the expected one and the average ratios observed for the two bands considered do not differ greatly from 1, whereas the ratio observed in the 80 000 dalton fraction is greater than 1 in all but one case. It should be noted that in the same experiment the 80 000 dalton fraction from crude plasmalemma showed an increased ratio even though the purified plasmalemma did not. From these data it appears that a polypeptide of 80 000 dalton is missing in the membraneous fractions of the two cytosine permease$^-$ strains analysed.

Mutants specifically resistant to 5-fluorocytosine (and not to other fluorinated pyrimidines) have been shown to belong to two complementation groups: one group involves the cytosine deaminase gene and the other group shows impaired cytosine uptake [8]. This suggests that only one gene is specifically involved in cytosine transport. An intensive search for intragenis complementation among cytosine permease mutants was made in our laboratory [4]. In eight hundred possible combinations no intragenic complementation was found. This strongly suggests that the permease protein is not an homomultimere. Therefore the polypeptide of 80 000 daltons which is missing in the two permeaseless strains analysed very probably represents the permease specific protein itself.

The number of c.p.m. corresponding to the permease protein relative to the total protein counts was calculated for the crude and purified membrane fractions (see table 2). The radioactivity of permease/total protein was 0.93 for crude, and 1.29 for purified plasmalemma, even if the protein pattern indicates that the purified plasmalemma membranes contain qualitatively more heavy polypeptides than the crude membranes. This indicates that some of the permease polypeptides are lost during the purification suggesting either that the permease is also an integral part of membraneous components which are eliminated during the purification procedure (e.g. endoplasma reticulum, 'light' membranes), or that some preferential solubilization occurs which releases permease molecules from the membrane structure.

We have estimated the number of permease molecules per haploïd yeast cell using the following estimated values:

(1) Surface of one haploïd cell: 56.5 μm^2. This

Table 2
Percentage of cytosine permease in the crude and purified plasmalemma

	Crude plasmalemma	Purified plasmalemma
	1.09	
% of excess counts	0.84	
in the 80 000 dalton	1.37	
fraction relative	0.33	1.02
to the total number	0.67	
of counts recovered	0.94	1.08
from the gel	1.43	1.42
	0.80	1.62
% average	0.93	1.29

value has been calculated using data from F. Lacroute which were obtained by direct measurements of cell size and assuming cell volume to be best represented by an ellipsoid with a long axis of 4.65 μm and a short axis of 4.04 μm.

(2) Yeast plasmalemma thickness: 80 Å, value taken from Matile et al. [9].

(3) Protein weight percentage in the plasmalemma: 26% [5].

(4) Density of plasmalemma: 1.18 g/cm^3 as found in the urografin isopycnic sedimentation (see Materials and methods).

(5) Quantity of cytosine permease in the plasmalemma: 1.3% of the proteins as estimated table 2.

(6) Mol. wt. of the permease: 80 000. With these values it can be estimated that one haploid cell contains about 1.5×10^4 cytosine permease molecules in the plasmalemma. This value may be underestimated since as already mentioned some solubilization possibly occurs during the purification procedure. This value has the same order of magnitude as the 2×10^4 found by Medweczky [10] for the phosphate binding protein from *E. coli* but is significantly lower than the value of 3×10^6 found by Horak and Kotyk [11] for the glucose transport protein from *Saccharomyces cerevisiae*. This difference is perhaps related to the fact that cytosine is transported by an active system with high affinity whereas glucose is transported by a facilited diffusion system with low affinity.

Acknowledgements

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References

[1] Grenson, M. (1969) Eur. J. Biochem. 11, 249–260.
[2] Chevallier, M. R., Jund, R. and Lacroute, F. (1975) J. Bacteriol. 122, 629–641.
[3] Pittman, D. and Brusick, D. (1971) Molec. Gen. Genet. 111, 352–356.
[4] Parlebas, N. (1976) Thèse de Spécialité en Biochimie, Université Louis Pasteur, Strasbourg.
[5] Matile, Ph. (1970) in: Membranes: Structure and Function, FEBS Vol. 20, pp. 39–49, (Villanueva, J. R. and Pouz, F., eds.) Academic Press, New York, London.
[6] De Wachter, R. and Fiers, W. (1971) in: Methods in Enzymology (Moldave, K. and Grossman, L., eds.), Vol. 21, pp. 167–178, Academic Press, New York, London.
[7] Waldron, C., Jund, R. and Lacroute, F. (1974) FEBS Lett. 46, 11–16.
[8] Jund, R. and Lacroute, F. (1970) J. Bacteriol. 102, 607–615.
[9] Matile, Ph., Moor, H. and Robinow, C. F. (1969) in: The Yeast, Vol. 1, pp. 237, (Rose, A. H. and Harrison, J. S., eds.) Academic Press, New York, London.
[10] Medweczky, N. and Rosenberg, H. (1970) Biochem. Biophys. Acta 211, 158.
[11] Horak, J. and Kotyk, A. (1973) Eur. J. Biochem. 32, 36–41.