a rather advanced vertebrate, although the reasons for the absence of parenchymal vascularization and the mechanism by which gas exchange is achieved have not been satisfactorily elucidated at present.

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Transport of a low molecular weight extracellular esterase into membrane vesicles of *Candida lipolytica*

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Summary. The low mol. wt extracellular esterase of *Candida lipolytica* is actively transported into membrane vesicles. In the absence of metabolic energy, a proton gradient can drive the transport process. The transport system does not accumulate the enzyme at peak levels due to the presence of a leak pathway.
Key words. Candida lipolytica; peptide; active transport proton gradient; leak pathway.

Isolated membrane vesicles from fungal and bacterial sources are used extensively to study transport at the cellular level, as they appear to retain the biological properties of membranes in intact cells[1]. The dependence of peptide transport on energy sources has been demonstrated not only for simple peptides[2,3], but also for oligopeptides[4–6]. The addition of energy uncouplers has been shown to prevent the uptake of peptides[7].

Candida lipolytica produces a low mol. wt (5700 ± 100) esterase which can be detected intra- and extracellularly. This esterase accounts in part for the observed lipolytic activity of the cells and culture medium[8]. Previous studies on the transport of this enzyme into membrane vesicles, prepared from young cultures of *Candida lipolytica*, showed that initially there was binding to the membranes followed by translocation into the vesicles[9]. This translocation process, which lead to a transport overshoot peak, required metabolic energy. Binding to the membrane vesicles was differentiated from transport into the vesicles by sonication and nystatin treatment, both of which disrupted the membrane vesicles. Both treatments abolished transport into the vesicles but not binding[9]. This paper shows that an alternative source of energy, a proton gradient, maintains the active transport of this esterase in the absence of metabolic energy.

Materials and Methods. Candida lipolytica (CMI 93743) was obtained from the Commonwealth Mycological Institute, Kew, Surrey and *Trichoderma viride* from the Central Bureau, UAST, Schimmel Cultures, Casterstraat I Baarn, The Netherlands. The snail gut juice of *Helix pomatia* was purchased from Pharmindustrie (l'Industrie Biologique Française).

Other chemicals and reagents were obtained from BDH and SIGMA and as far as possible, ANALAR grade reagents were used.

Preparation of radioactive esterase. *C. lipolytica* was grown in batch cultures in the modified medium of Fukumoto et al.[10] containing [^{14}C] glycine (10 μCi/100 ml of medium). Growth conditions were as previously described[8]. Extracellular proteins in the cell-free medium were precipitated, without prior concentration, by $(NH_4)_2SO_4$. The precipitate was dissolved, filtered through a Sephadex G-100 column and the active esterase fractions were pooled and concentrated as described earlier[8]. The final protein concentration was 0.2 mg/ml.

Preparation of membrane vesicles of *C. lipolytica*. Snail gut juice of *Helix pomatia* was used without any further purification or dilution. Lytic enzyme was obtained by growing *T. viride* in batch cultures on a rotary shaker at 28 °C for 10 days. The medium contained 3 g glucose; 1.4 g $(NH_4)_2SO_4$; 2 g KH_2PO_4; 0.3 g $MgSO_4 \cdot 7H_2O$; 0.3 g $CaCl_2$; 0.3 g urea; 1.5 g *C. lipolytica* cell walls and 1 ml of trace metal solution per l. The trace metal solution contained 500 mg $FeSO_4$; 150 mg $MnSO_4$; 167 mg $ZnCl_2$; 200 mg $CoCl_2$ and 1 ml 19% HCl per 100 ml. The mycelium was harvested by centrifugation at 5000 × g. The cell-free medium was filtered and freeze-dried. This enzyme was used without further purification.

To about 1.5 g of 24-h-old *C. lipolytica* cells was added 1.4 ml of 0.1 M EDTA and 0.05 ml of 2-mercaptoethanol. Distilled water was added to a final volume of 5 ml and the suspension incubated for 90 min at 35 °C. After washing, 1 g of the cells was suspended in 10 ml 0.1 M KH_2PO_4 buffer, pH 6.4, containing 1 M $MgSO_4$ as an osmotic stabilizer. The freeze-dried lytic enzyme (200 mg), and 0.05 ml of undiluted *Helix pomatia* gut juice were added and the incubation was continued at 35 °C with minimal shaking. Protoplast formation was complete within 3–4 h of incubation. Protoplasts were purified by differential centrifugation at 4 °C.

The suspension of protoplasts was centrifuged at 10,000 × g and approximately 0.5 g of the resulting pellet was suspended in 20 ml distilled water at room temperature for 30 min. In the absence of an osmotic stabilizer, the protoplasts of *C. lipolytica* lysed immediately. Buffer (0.1 M KH_2PO_4, pH 6.4 containing 10 mM $MgSO_4$) was added and the suspension was centrifuged at low speed and then at 14,000 × g to sediment membrane vesicles. The membrane vesicles were washed three times with the same buffer. Vesicles were resuspended in the 0.1M KH_2PO_4 buffer or

0.25 M sucrose to give a final concentration of 25 mg dry wt/ml.
General procedure for transport studies. Incubations were performed at 28 °C or 37 °C. Radioactive esterase solution (9 ml) and membrane vesicle suspension (1 ml) were equilibrated separately for 10 min in a shaking water bath. At zero time, the esterase solution was added to the membrane vesicle suspension and incubation continued. Samples of 1 ml were removed at intervals and rapidly filtered through Whatman grade GF/C glass fiber discs. The discs were washed once with 1 ml of buffer or 0.025 M sucrose solution. They were dried and radioactivity was counted in 2,5-diphenyloxazole scintillator containing Triton X-100.
The investigation of the effects of metabolic inhibitors and antibiotics followed the same pattern. The substance was added to both the membrane vesicle suspension and to the esterase solution to give the same desired final concentration. The test substance was allowed to equilibrate with the esterase and membrane vesicles for at least 30 min. The remaining steps of the experiments were as described above.
Results and Discussion. Membrane vesicles. The lytic enzyme of *Helix pomatia* gut juice converted less than 5% of the cells from young cultures of *C. lipolytica* to protoplasts unless used with the lytic enzyme from *T. viride*. Possibly, the hydrolytic enzyme from *T. viride* contained what Nagasaki et al.[11] called the 'PR-factor' which acts like the thiols in altering the nature of the cell walls, making them more susceptible to the lytic activity of the snail enzyme.
The yield of protoplasts from 24-h-old cells of *C. lipolytica* using the lytic enzyme preparation from *T. viride* was between 85 and 90%. The incubation of *C. lipolytica* cells with the lytic enzyme alone, without pre-incubation with 2-mercaptoethanol and EDTA, required 6–7 h incubation for the maximum yield of protoplasts. Pre-incubation of *Candida* cells for 1 or 2 h with 2-mercaptoethanol and EDTA reduced the time for protoplast formation to between 3 and 4 h. The addition of snail gut juice had no effect on this reaction. 2-Toluidononaphthalene-6-sulphonic acid was used as previously described[9] to show that the membrane vesicles obtained by this method were intact.
Transport studies. Early transport studies[9] showed that two processes take place sequentially, namely binding to, and transport into the membrane vesicles. Whereas nystatin (200 U.S.P. units/mg) and 2,4-dinitrophenol (1 mM) were shown to inhibit transport into the membrane vesicles, binding, which requires neither the integrity of the membranes nor metabolic energy, was not inhibited (fig. 1). When membrane vesicles disrupted by sonication were tested, a similar result was obtained (fig. 1). The binding peak was at a maximum at about 5 min and returned to a constant level by 15 min. The transport peak occurred from 15 min onwards depending on the concentration of the enzyme and the pH. The level of the binding and transport overshoot peaks were dependent on the concentration of the radioactive esterase. The Michaelis constant (K_m) for binding at 2 min was 6.7×10^{-5}M and V_{max} of 9.09×10^{-3} ^{14}C radioactivity (cpm), which suggests a relatively high affinity for the enzyme. At concentrations of 3.24 and 2.28 mg protein of esterase, the transport peaks were obtained at 15 min while they were unattainable at concentrations of 1.32 mg and lower (fig. 2).

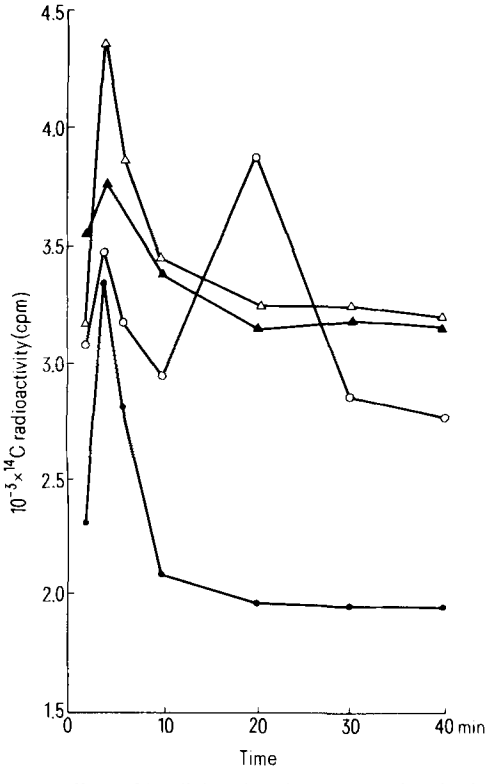

Figure 1. The effects of 2,4-dinitrophenol, nystatin and sonication on the uptake of radioactive esterase by membrane vesicles. Membrane vesicles suspension (1 ml containing about 25 mg dry wt), in 0.1 M KH_2PO_4 buffer, pH 6.4, and 9.0 ml of radioactive esterase solution (approx. 2 mg protein), adjusted to pH 6.4 were equilibrated at 37 °C. At zero time, the esterase solution was added to the membrane suspension. Incubation was continued and 1 ml samples were removed at intervals and rapidly filtered through a Whatman grade GF/C glass fiber disc. The paper was washed once with 0.1 ml 0.01 M phosphate buffer and dried. Radioactivity was counted in 2,5-diphenyloxazole (PPO) scintillator containing Triton X-100 for 30 min. ○, control; ●, uptake in the presence of 2,4-dinitrophenol; △, uptake by membrane vesicles treated with nystatin; ▲, uptake by sonicated membrane vesicles.

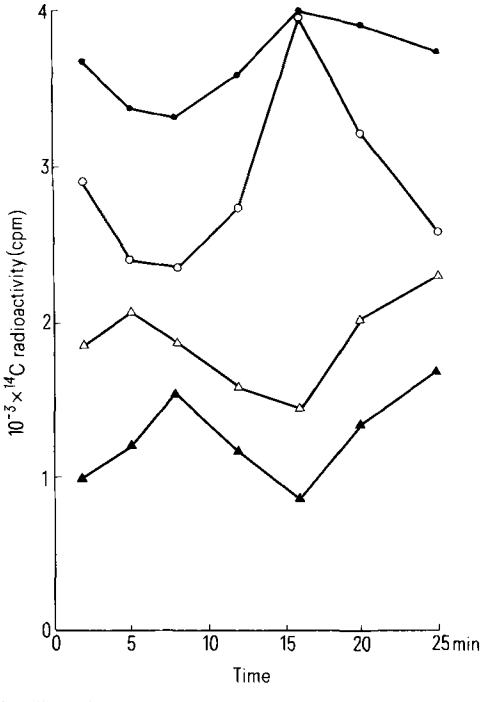

Figure 2. Effect of concentration of radioactive esterase on its uptake by membrane vesicles. A suspension of membrane vesicles (1.5 ml) in 0.25 M sucrose, pH adjusted to 2.0 and 13.5 ml of esterase solution (3.24 mg protein), pH 2.0 were equilibrated at 28 °C. At time zero, the esterase was added to the membrane vesicles and experiment performed as for figure 1, taking 2 ml samples at intervals for filtration and washing the glass fiber disc with 1 ml 0.025 M sucrose solution. The experiment was repeated with different concentration of the esterase solution. ●, 3.24 mg esterase; ○, 2.28 mg esterase; △, 1.32 mg esterase; ▲, 0.60 mg esterase.

pH-Dependence of transport of esterase. Binding to, and transport into membrane vesicles were particularly sensitive to pH, with a maximum value at pH 2 and a minimum at neutral pH (fig. 3). Although some cytoplasm still adheres to the vesicles, this result is unlikely to be due to the increased availability of metabolic energy since the rate of glycolysis actually decreases with increase in acidity of the extracellular medium[12-14].

The effect of 1 mM ATP showed that at pH 2, addition of ATP enhanced transport into the vesicles (the binding peak was eliminated), giving an apparent maximum at 10 min (fig. 4a) while at pH 7, ATP had no effect on transport (fig. 4b). The addition of sodium azide (1 mM) with 1 mM ATP at pH 2 had little effect on the transport of the radioactive esterase into the membrane vesicles (fig. 4a) but abolished the transport peak at pH 7 (fig. 4b). At pH 2, the pH difference across the vesicular membranes is maximal, while it is minimal at neutral pH. These results suggest that at low pH values, transport of the peptide proceeds normally in spite of the likely absence of metabolic energy to drive it, and this implicates another source of energy for the active process; a proton gradient force. This possibility is supported by the failure of membrane vesicles prepared by lysing protoplasts in 0.2 M KCl/HCl buffer, pH 2 and resuspended in the same buffer, to transport the peptide at pH 2 in the presence of 1 mM ATP and sodium azide.

Esterification of free carboxyl groups and acylation of free amino groups of the esterase by the methods of Fraenkel-Conrat[15] reduced the activity of the enzyme by 3.6% and 93.4.% respectively, suggesting that the free amino groups of basic amino acid residues are involved in binding and catalysis. Also, the pH dependence of the uptake showed that the enzyme was transported largely by a cationic transport system[16]. This might be an indication that the protonated free amino groups of basic amino acid residues play a greater role in binding of the esterase molecules to the membrane vesicles than the ionized free carboxyl groups. It therefore seems that a reverse proton gradient (alkaline pH) would be less effective in driving the transport of the radioactive esterase.

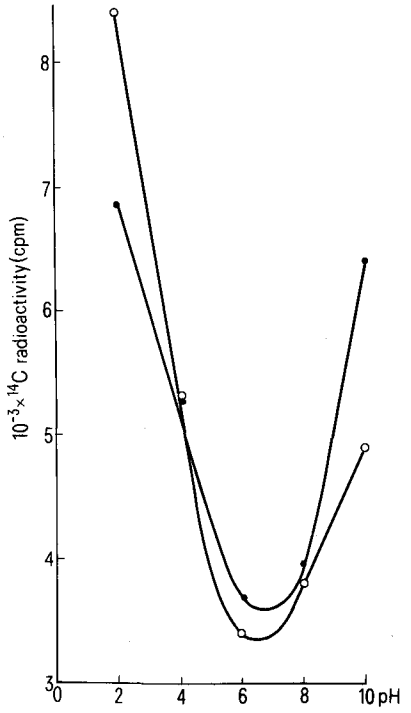

Figure 3. Effect of pH on uptake of radioactive esterase by membrane vesicles. Membrane vesicles at pH 6.4 were sedimented by centrifugation and then suspended in 0.25 M sucrose. The pH of the suspension and radioactive esterase solution were adjusted to the desired values with 0.1 M HCl or NaOH solution. After equilibration at 28 °C, 9 ml of the esterase solution (approx. 2 mg protein) was added to 1 ml of the membrane vesicle suspension (about 25 mg dry wt), at zero time. The experiment was continued as for figure 1, removing 1-ml samples at intervals for filtration and washing with 1 ml 0.025 M sucrose solution. The binding and transport peaks were plotted against their corresponding pH values. ●, binding peak; ○, transport peak.

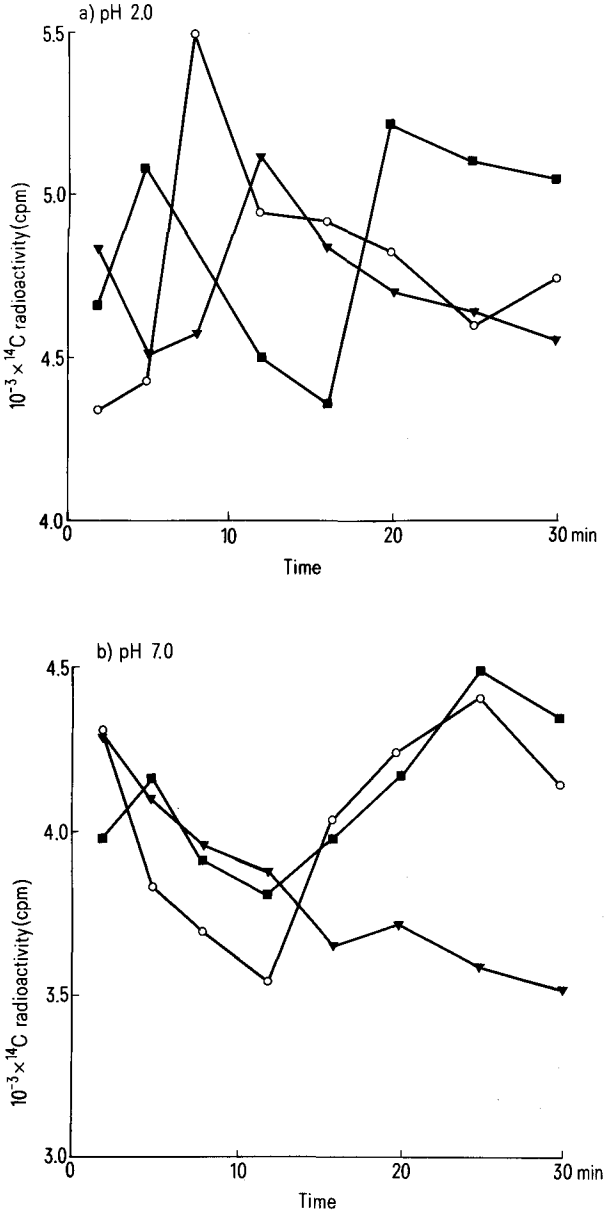

Figure 4. Effects of ATP and sodium azide on uptake of esterase by membrane vesicles at different pHs. ATP standard solution (10 mM) was added to 1 ml of membrane vesicles suspension (about 25 mg dry wt) in 0.25 M sucrose and to 8 ml of radioactive esterase to give a final concentration of 1 mM at the desired pH. Sodium azide (10 mM) in ATP standard solution (10 mM), was similarly added to 1 ml of membrane vesicles suspension and to 8 ml of the esterase solution (approx. 2 mg protein), to a final concentration of 1 mM at the desired pH. The control contains 1 ml distilled water in place of test solutions. The samples were equilibrated at 28 °C and the experiment performed as described earlier for figure 1. ■, control at given pH; ○, sample containing 1mM ATP; ▲, sample containing 1 mM ATP/NaN_3.

Presence of a leak pathway. The failure of the system to retain the esterase at the peak level would suggest that a leak pathway was operating in the reverse direction, transporting the enzyme from inside the vesicles into the bathing medium. In the simplest form of a proton symport coupled to a proton pump being driven by the hydrolysis of ATP, Eddy[17,18] proposed that the solute S, enters the vesicles with n equivalents of protons which are expelled by the proton pump. If m equivalents of protons are inside the vesicles, then as the ratio, n/m increases, so does the value of the ratio $[S]_{in}/[S]_{out}$ at equilibrium. This is consistent with our findings that the value of the transport peaks increased with decrease in the pH of the medium. The ratio, $[S]_{in}/[S]_{out}$ at any given proton concentration is dependent on the concentration of the solute outside the vesicles, $[S]_{out}$[19]. The observation that the ratio $[S]_{in}/[S]_{out}$ increased as the enzyme concentration was increased at pH 2 is in agreement with these proposals. The concept of a leak pathway is also supported by measurements of the mass action ratio for the transport process in whole cells which suggested that the process was close to equilibrium[20].

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Establishment of a regeneration-specific in vivo bioassay for neurotrophic activity in denervated *Ambystoma* forelimbs[1]

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Summary. Use of continuous ^{3}H-thymidine labeling and subsequent assay for cell cycle activity using a novel parameter, mitotic index of a selectively labeled cell population, has led to the development of a regeneration-specific in vivo bioassay for neurotrophic activity. This system is based on the stimulation of cell cycle arrested cells to resume cycling activity after reinnervation in denervated larval *Ambystoma mexicanum* forelimb stumps.
Key words. Regeneration; neurotrophic; bioassay; *Ambystoma.*

Amputated limbs of several species of urodele amphibians fail to regenerate when maintained in a denervated condition[3]. The nervous influence has been hypothesized to be effected through axonal release of a neurotrophic factor (NTF) into the distal limb tip[3,4]. In vivo[5] and in vitro[6] evidence lend support to the existence of a diffusible NTF. Bioassays measuring stimulation of biochemical and/or cell cycle events in denervated limb stumps/blastemas have reported neurotrophic activity associated with dorsal root ganglia[6-8], nerve and brain extracts[5,9-12], fibroblast growth factor[13], and transferrin[14]. The authentication of a putative neurotrophic factor(s) will ultimately require an in vivo bioassay based specifically on stimulation of a regeneration event. We have attempted to develop such an assay with larval *Ambystoma,* one which measures a restimulation of cell cycle blocked cells in denervated limbs.

Unlike denervated adult newt limbs, denervated limb stumps of larval *Ambystoma* regenerate upon subsequent reinnervation without reamputation[15]. There is limited but measurable early cell cycling in denervated limbs (4-6 days post-amputation/denervation) as determined by mitotic index (MI) and pulse labeling with ^{3}H-thymidine (^{3}H-T), but the MI is very low just prior to reinnervation[16-19]. Increases in the blastema MI to control levels occur upon reinnervation[17-19]. The early cycling cells in denervated limbs have been selectively labeled with ^{3}H-T and scored for subsequent mitotic activity with reinnervation[19]. Olsen et al.[19] showed that this labeled population is prevented from cycling after the initial round of activity and is 'rescued' into renewed cycling activity correlated with reinnervation. Therefore, cells which block due to denervation will contribute to blastema formation in response to the reintroduction of a nervous influence. This specific event in the regeneration of a reinnervated limb can be directly characterized as a neurotrophic response. Theoretically, the introduction of the neurotrophic factor into a denervated limb in the continued absence of nerves will produce the same rescue response.

The potential use of the rescue system as an NTF bioassay has previously been compromised by a low percentage of cells labeling with a single ^{3}H-T pulse and an apparent 'loss' of dedifferentiated labeled cells due to label dilution prior to reinnervation[19]. This result provided only a relatively small and lightly-labeled population scored as rescued, a less than satisfactory system for use as a quantitative or qualitative bioassay.