LIMITATIONS TO THE USE OF RADIOACTIVELY LABELLED SUBSTRATES FOR STUDYING PEPTIDE TRANSPORT IN MICROORGANISMS

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1. Introduction

Solute transport in microorganisms is normally studied with radioactively-labelled substrates, the counts accumulated within the cells being taken as a measure of solute uptake during the incubation. However, the general lack of commercial, radioactively-labelled peptides has led us to develop alternative ways to study peptide transport. In the first method [1], peptide uptake is determined by difference, the peptide remaining in solution being quantitated from the fluorescent intensity of its dansyl derivative after separation by thin-layer chromatography. Besides being able to measure simultaneously the uptake of several peptides from a mixture, this approach revealed that selective exodus of peptide-derived amino acid residues could occur within seconds of peptide uptake [1,2]. Subsequently [3], fluorescamine derivatives were formed with the peptide remaining in the medium, direct quantitation of these being possible simply from the fluorescence of the solution.

We wished to investigate the stoicheiometry of energy coupling to peptide transport in whole cells of several organisms, a study that requires accurate measurements of the rate and amount of peptide translocation. Here we show that using radioactively-labelled substrates can lead to severe miscalculation of these parameters and produce misleading data on the kinetics of uptake. These conclusions are based on comparative studies using the fluorescamine and dansyl procedures.

2. Materials and methods

2.1. *Microbial strains*

Escherichia coli W M2626*lys* [1] and *Saccharomyces cerevisiae* Σ1278b [3] were grown as described. *Streptococcus faecalis (faecium)* ATCC 9790 was grown at 37°C in RST2 medium [4].

2.2. *Transport assays*

For the fluorescamine assay [3], samples (0.5 ml) of the incubation medium (see section 3) are removed periodically, filtered immediately to remove microorganisms, 0.05 ml filtrate is added to 2.5 ml 0.1 M disodium tetraborate (pH 6.2), and 0.5 ml fresh fluorescamine solution (0.15 mg/ml^{-1} in acetone) is added with vortex mixing. After 2–30 min, peptide concentration is determined from the fluorescence of the solution (excitation 390 nm; emission 485 nm). For radioactive assays, samples (0.5 ml) of the same incubation medium as above are removed periodically, filtered under vacuum using glass-fibre discs (Whatman GF/C, 25 mm diam.), and washed with 5 ml saline (0.9%, w/v). For *S. faecalis*, filters were dried at 80°C for 90 min before counting in 5 ml scintillant solution (toluene containing 0.4% 2,5-bis[2-(5-*tert*-butyl-benzoxazolyl)]thiophene (BBOT) (Fisons Sci.). For *E. coli* and *S. cerevisiae*, the filters were added to 2 ml Soluene 350 (Packard), incubated overnight at 45°C, 10 ml of NE260 micellular scintillant (Nuclear Enterprises), was added and samples counted after 6 h.

2.3. *Materials*

Fluorescamine, and Ala–[U^{14}C]Ala (1.93 μCi/mg^{-1}) were gifts from Dr P. S. Ringrose and Dr W. J. Lloyd, respectively, both of Roche Products Ltd, Welwyn Garden City, Herts. Gly–[U^{14}C]Phe (58 μCi/mg^{-1}), and [1^{14}C]Gly–Phe (74 μCi/mg^{-1}) were purchased from the Radiochemical Centre, Amersham.

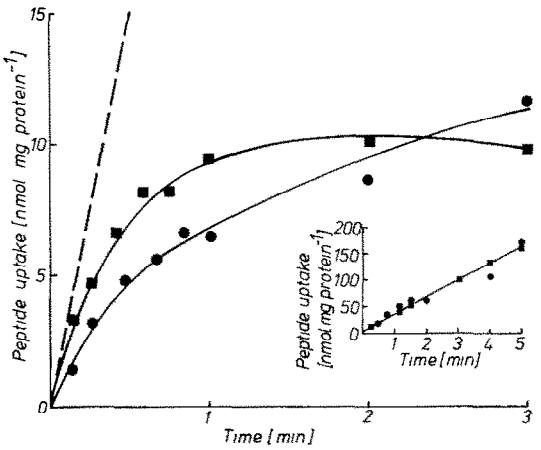

Fig.1. Uptake of Gly–Phe by *E. coli* W M2626*lys*. Exponential-phase bacteria were filtered, washed, and resuspended at 0.2 mg protein/ml in 50 mM potassium phosphate (pH 6.9) with 0.2% (w/v) glucose, at 37°C. After 10 min preincubation, peptide (0.1 mM) was added. Uptake was measured by radioactive counting, (■——■) is Gly–[^{14}C]Phe, (●——●) is [^{14}C]Gly–Phe, or with fluorescamine (– – –). Inset, extended analysis of peptide uptake from both incubations using fluorescamine, (■——■) and (●——●) distinguish the two, as above.

3. Results

3.1. *Uptake of Gly–Phe by* E. coli

Gly–Phe was chosen for study because it was available labelled in either residue. By using the dansyl procedure [1] we first showed Gly–Phe was rapidly accumulated by *E. coli*, and Gly and Phe were concomitantly excreted, as is normally observed with peptide uptake in this organism [1,2]. When the uptake of label from [^{14}C]Gly–Phe and Gly–[^{14}C]Phe was assayed, different kinetic curves were obtained (fig.1). The apparent initial rates of uptake are 12 and 23 nmol . min^{-1} . mg protein^{-1}, respectively, and uptake appears to depart from linearity in both cases within 15 s, and, for Gly–[^{14}C]Phe at least, to approach a plateau value at ~1 min. In contrast, when samples of the same incubation media are analysed by using fluorescamine, an identical initial rate of uptake is found for both samples (30 nmol . min^{-1} . mg protein^{-1}), and this rate is maintained until the peptide is fully absorbed (fig.1 and inset). The possibility that here, and with the other organisms, external deamination or hydrolysis of peptide could contribute to the progressive decrease in fluorescamine

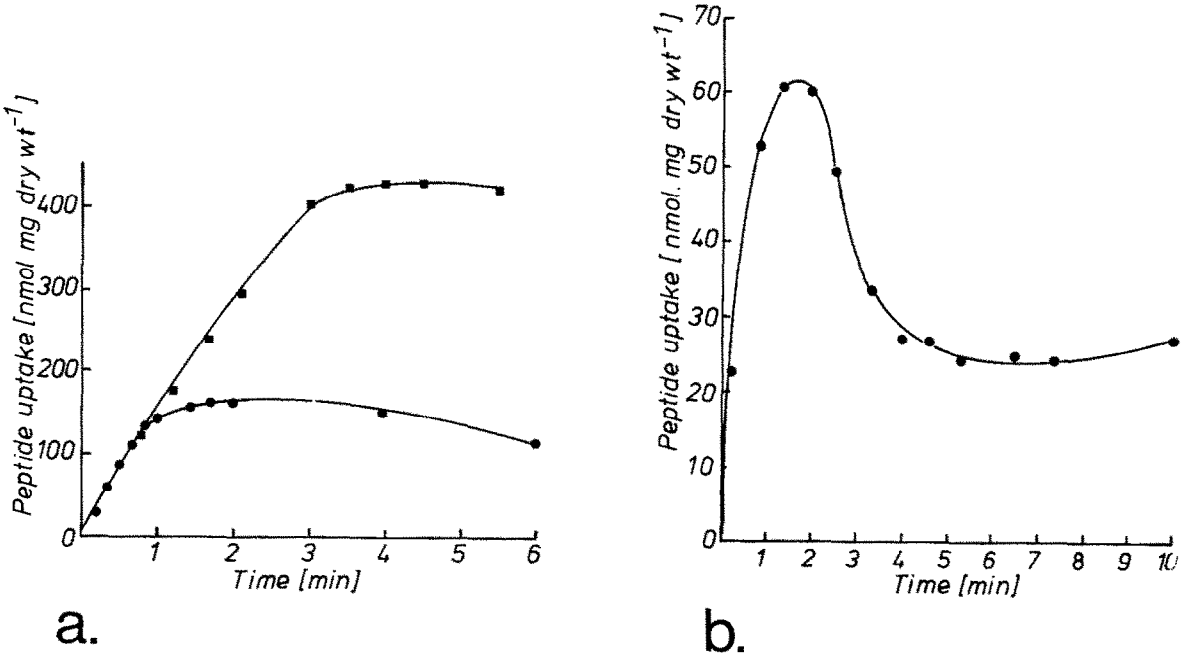

Fig.2. Uptake of Ala–[^{14}C]Ala in *S. faecalis* ATCC9790. (a) Exponential-phase bacteria were filtered, washed, and resuspended at 0.22 mg dry wt/ml in 50 mM potassium phosphate (pH 6.9) with 0.2% (w/v) glucose, at 37°C. After 10 min preincubation. peptide (0.1 mM) was added. Uptake was measured by radioactive counting (●——●), or by fluorescamine (■——■). (b) As above, except bacteria were resuspended at 0.36 mg dry wt/ml in RST2 medium. Uptake was measured by radioactive counting.

yield, thereby causing an overestimate of transport rate, is negated by various controls [1], including the complete abolition of transport by specific energy inhibitors. At ~15 min, when peptide uptake is approaching completion, the label accumulation curve for Gly–[^{14}C]Phe falls from its plateau value, whereas that for [^{14}C]Gly–Phe continues to rise because of incorporation of counts into cellular material [5] (not shown).

3.2. *Uptake of Ala–Ala in* S. faecalis

We find using the dansyl technique that exodus of amino acid residues accompanies peptide uptake by *S. faecalis* as it does in *E. coli*. The kinetics of Ala–Ala uptake based on radiotracer measurements and fluorescamine assay are shown in fig.2a. A constant transport rate, continuing until essentially all peptide is accumulated, is revealed by fluorescamine assay. In contrast, accumulation of label slows within 1 min before reaching a steady state. To get the same initial rate (148 nmol . min^{-1} . mg protein^{-1}) from both assays only data obtained within the first 30 s of the radiotracer study can be used. When *S. faecalis* is resuspended in a complex medium containing amino acids, accumulation of label from Ala–Ala is less after 1 min and there follows a rapid exodus of label (fig.2b). We interpret these effects as being due to rapid exodus of [^{14}C]Ala caused by trans-stimulation by exogenous amino acids [6,7].

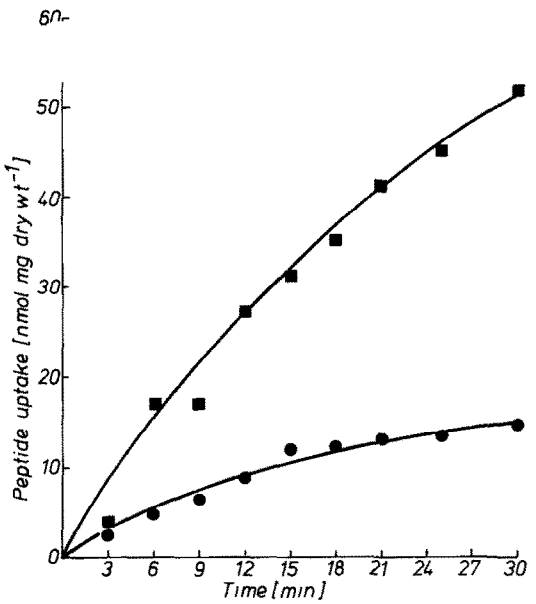

Fig.3. Uptake of Ala–[^{14}C]Ala in *Sacc. cerevisiae* Σ1278b. Cells were filtered, washed, and resuspended at 1.5 mg dry wt/ml in 25 mM citrate–potassium phosphate (pH 4.5), with 1% (w/v) glucose, at 28°C. After 10 min preincubation, peptide (0.1 mM) was added. Uptake was measured by radioactive counting (●——●), or by fluorescamine (■——■).

3.3. *Uptake of Ala–Ala by* Sacc. cerevisiae

It was reported [3] that although peptides are rapidly cleaved following uptake by *Sacc. cerevisiae* there is no associated exodus of amino acid residues. Nevertheless fig.3 shows that the kinetics of Ala–Ala uptake based on radiotracer and fluorescamine methods are significantly different.

4. Discussion

Using whole cells of three diverse microbial species we find that erroneous (under) estimates of the rate and extent of peptide transport are obtained from radiotracer studies. The degree of error depends on two essentially unquantifiable features: the extent to which the labelled amino acid undergoes exodus and is metabolised. In *E. coli*, differential amino acid exodus can accompany peptide uptake [1,2,8], and the greater exodus of Gly than Phe (shown by dansylation) can in part explain the differences in the kinetic curves for [^{14}C]Gly–Phe and Gly–[^{14}C]Phe (fig.1). However, in addition, loss of counts as $^{14}CO_2$ through decarboxylation will be greater with [^{14}C]Gly–Phe [5]. Using appropriate controls we have shown that with metabolisable amino acids, the bulk of the counts are lost not during the actual incubation period, but during the subsequent period when cells are on the filter before inactivation by solubilization.

In *S. faecalis* [^{14}C]Ala exodus similarly distorts the overall kinetics of Ala–Ala uptake (fig. 2) but because Ala exodus is relatively slow initially, and metabolic losses slight, accurate initial rates can be obtained from the first 30 s of the radiotracer curve. In contrast, in *E. coli*, radiotracer results are in error within 8 s due to metabolic losses and exodus [9]. The discrepancies found in *Sacc. cerevisiae* (fig.3) may arise partly from metabolic loss as $^{14}CO_2$ as discussed above, and partly from exodus of deamination products of amino acids [10]. Amino acid exodus and metabolism was shown to influence measurements of peptide uptake in *Staphylococcus*

aureus, but no comparative methods were available to evaluate the effects [5].

An aspect more fundamental than errors in rate calculation is the possible interpretation of the kinetic curves obtained from such radiotracer data. These exponential curves (fig.1–3) appear to show that uptake slows within a few seconds and quickly reaches a steady state. Considered on their own these (quite typical) curves might well lead to models in which it was considered that uptake reached a saturating level, feedback regulation were operative, or a dynamic equilibrium occurred between peptide uptake and exodus, etc. That all such speculations are wrong is clear from the fluorescence assays that show peptide uptake to be proceeding as fast under apparent steady state conditions as it is initially. A fuller discussion of the implications of these findings is given in [11].

Analogous effects to those found for peptides may attend the transport of other substrates, and when such studies are based solely on measurements of radioactive counts and ion (e.g., H^+, K^+) movements, it seems prudent to us to establish whether metabolic losses and fluxes of other solutes are occurring before proposals are made for molecular mechanisms of transport.

Acknowledgements

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