GLUCOSE PROTECTION AGAINST [^{14}C]*N*-ETHYLMALEIMIDE LABELLING OF A PROTEIN IN GALACTOSE-TRANSPORTING MEMBRANE VESICLES OF *ESCHERICHIA COLI*

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Received 17 March 1980

1. Introduction

Rapid progress towards an understanding of the energetics of active transport of substrates across the cytoplasmic membrane of bacteria has been made using whole cells and derived vesicles [1–4], yet further insight into the mechanisms of active transport would be greatly facilitated by the isolation of solubilized transport proteins and their subsequent incorporation into artificial lipid membranes.

Much attention has focussed on the lactose 'permease' (LacY, or M protein) of *Escherichia coli*, with recent publications highlighting its susceptibility to genetic manipulation [5–7] but relative intractability with regard to solubilization [8]. In an effort to gain parallel information on another membrane protein we have undertaken the identification of the galactose-specific transport system (GalP of *E. coli* [9–12], already demonstrated in this laboratory to function as a galactose-proton symporter [11–13] in accordance with the chemiosmotic theory [1]. The epithet 'galactose-specific' in historical [4,9], while its substrate specificity spans a range of hexoses including D-galactose, D-fucose and D-glucose, with the latter showing the best rates of transport [13].

This report describes the identification, in a GalP constitutive strain, of a membrane protein which is specifically protected by glucose against inactivation by NEM. Its mobility under SDS-PAGE indicates an apparent MW of 34 000 ± 1000 and it is tentatively identified as the GalP transport protein.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; KP, potassium phosphate buffer, pH 6.6; NEM, *N*-ethylmaleimide; PMS, phenazine methosulphate; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

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2. Materials and methods

E. coli strain S183-27T (*mglB, mglC, mglD, lac* (zya) deletion; MglP$^-$, GalP constitutive), a kind gift of Dr B. Rotman, Brown University, Providence, RI 02912, USA, was maintained and grown under conditions described previously [11], with additions of L-histidine, L-methionine, L-leucine and L-threonine to final concentrations of 90, 80, 80 and 80 μg/ml, respectively. Cells were harvested in late log phase after overnight growth on 20 mM glycerol as carbon source. Membrane vesicles were prepared as in [14] after preparing spheroplasts by the method in [15], and either used fresh, or frozen in liquid N_2 and stored at −20°C prior to use; such frozen preparations consistently showed only marginal losses in transport activity when rapidly thawed at 37°C.

Transport assays of GalP activity vesicles energized by ascorbate PMS followed the methods in [11] and [16], with manual sampling onto cellulose acetate filters (Oxoid, 0.45 μm pore size); 15 s time points were taken as comparative estimates of initial rates.

Proteins were estimated in the presence of 0.5% SDS by the method in [17], using bovine serum albumin (Sigma, Fraction V) as standard.

SDS-PAGE followed the method in [18], using slab gels comprising stacking and resolving gels of 5% and 15% acrylamide plus 0.13% and 0.08% *NN'*-methylenebisacrylamide, respectively. After develop-

Elsevier/North-Holland Biomedical Press

ment at a constant 75 V gels were fixed and stained for 3 h, in each of the Coomassie solutions described in [19], destained in 10% acetic acid and finally perfused with water; 0.1 cm wide slices (2 cm long, 0.16 cm deep) were solubilized in sealed vial inserts overnight at 37°C in 40 μl 30% w/v hydrogen peroxide, and radioactivity counted as in [11].

[^{14}C]NEM, D-[1-^{3}H]galactose and D-[1-^{3}H]fucose were purchased from The Radiochemical Centre, Amersham, UK; all other chemicals were of the highest grade commercially available. Dialysis tubing (Medicell, size 1-8/32) was prepared for use as in [20]. Lysozyme, DNase and RNase were purchased from Sigma Chemical Co.

3. Results

In common with lactose transport [6,8,21] and several other systems [22–24], galactose transport was inactivated by the sulphydryl reagent NEM (fig.1). That the decrease in galactose uptake by NEM-treated vesicles was attributable to interaction at the transport site of GalP, and was not simply an impairment of the capacity of vesicles to generate a proton motive force is indicated by the results in fig.2, where counterflux of the non-metabolizable GalP substrate fucose in whole cells was examined under conditions independent of respiration-driven transport [25]. The 'overshoot' uptake of [^{3}H]fucose was delayed by 14 min in NEM treated whole cells; equilibrium was approached at much longer incubation times (not shown). This indicates that a proportion of the GalP sites have been inactivated by NEM in a similar manner to that described for LacY [25]. In addition, it has been reported elsewhere that NEM does not affect the magnitude of the proton motive force generated in membrane vesicles oxidizing ascorbate-PMS [26].

Results of protection studies on GalP transport activity (table 1) reflected data established for its substrate specificity. Glucose, which is transported at least as rapidly as galactose [11] afforded significant protection to GalP in the presence of NEM; arabinose, which is not transported on GalP [11] offered little or no protection to inactivation. The apparent inhibition of transport activity by glucose treatment on its own reflects the difficulty in reducing the concentration of intra- and extravesicular glucose (added during protection) to levels which do not compete with subsequent energized accumulation of labelled galactose.

The ability of glucose to protect GalP transport activity against NEM inactivation suggested that the transport protein might be identified by substrate protection during 'cold' NEM treatment followed by removal of the substrate and incubation with [^{14}C]-NEM [21]. Using this strategy one would expect to find proportionally enhanced labelling in GalP compared with controls pre-incubated in the absence of protective substrates either with or without 'cold'

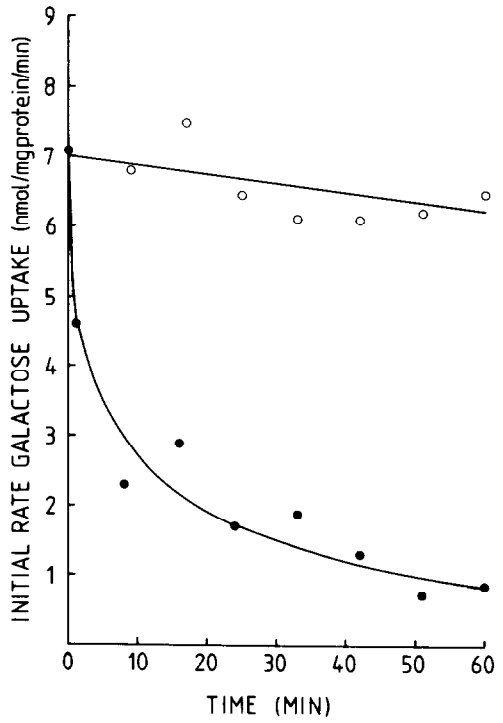

Fig.1. Time course for inactivation of GalP by NEM. Membrane vesicles in 0.1 M KP pH 6.6 were incubated at 25°C with (•) or without (○) 1 mM NEM. At the times indicated 0.25 ml samples were quenched with 0.22 ml 10 mM mercaptoethanol and stored on ice until assayed for galactose transport. Quenched samples were then incubated at 25°C with stirring and gentle bubbling (compressed air), with 10 mM $MgSO_4$ final. After 2.5 min and 2.8 min, PMS and potassium ascorbate (pH 6.5) were added in sequence to final concentrations of 0.1 mM and 20 mM, respectively. Transport was initiated by the addition of [^{3}H]galactose to a final concentration of 0.04 mM after 3 min incubation. 0.2 ml samples were withdrawn and filtered 15 s after addition of the labelled sugar, washed immediately with a total of 4 ml 0.1 M LiCl, and counted as in [11]. Final protein concentration, 1.22 mg/ml.

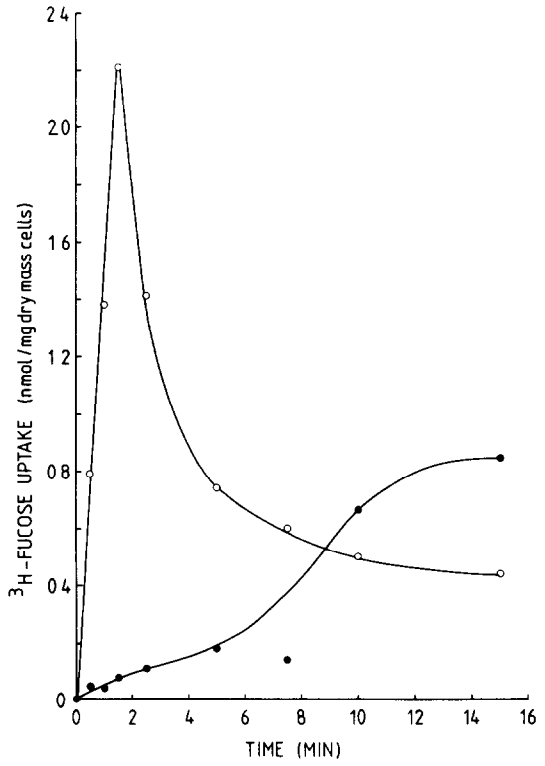

Fig.2 Inhibition of fucose counterflux in NEM-treated whole cells. S183-27T cells were harvested at dry mass 0.54 mg/ml, resuspended in 150 mM KCl, 5 mM HEPES, 30 mM sodium azide, pH 6.5 and incubated at 37°C for 1 h. Cells were again harvested, and resuspended in the above buffer to 1.92 mg/ml. 1.0 ml aliquots of this suspension were incubated with (•) or without (○) 1 mM NEM at 25°C for 15 min; fucose was then added to each cell suspension to a final concentration of 20 mM, and left to equilibrate at 25°C for 30 min. Cells were harvested, resuspended in 1.0 ml 150 mM KCl, 5 mM HEPES, 20 mM sodium azide pH 6.5 plus 0.5 mM [^{3}H]fucose (6.25 μCi/μmol) and uptakes of label assayed at 15°C, when 0.1 ml samples were withdrawn, filtered, washed with fucose-free buffer at the times indicated, and counted as in [11].

Table 1
Substrate protection of GalP from inactivation by NEM

Vesicle treatments	GalP activity (nmol/mg membrane protein/min)	Activity % control
H_2O	11.34	100
NEM	1.42	12.5
Glucose	6.64	58.6
Glucose + NEM	3.24	28.6
Arabinose + NEM	1.52	13.4

Vesicle suspensions were pre-incubated at 25°C for 10 min with 50 mM sugar (or H_2O), 10 mM $MgSO_4$ in 50 mM KP pH 6.6 prior to addition of NEM (1 mM final, as required; total reaction volume 1.0 ml). After a further 30 min at 25°C in the presence of NEM samples were transferred to ice, quenched by slow addition of 20 μl 0.5 M mercaptoethanol and dialysed overnight against several changes in 50 mM KP pH 6.6, 0–4°C (8 l total). Samples were diluted with 0.2 ml buffer before carrying out duplicate energized assays at 25°C on 0.5 ml aliquots, as in fig.1. Final protein concentrations 0.66 mg/ml

NEM. Indeed such a procedure for [^{14}C]NEM labelling of membrane vesicle proteins subsequently separated by SDS-PAGE revealed a single peak of proportionally enhanced labelling in samples which had received prior substrate protection (fig.3a,b; gel slices 50–52, MW range 33–35 000). In all other regions the profiles for radioactive incorporation overlap closely, although there are slight discrepancies (1 to 3 gel slices) due to the difficulty in aligning different tracks precisely during slicing. The failure of 'cold' NEM to prevent completely the subsequent interaction of [^{14}C]NEM with proteins at unprotected sites is inter-

Fig.3. Relative enhancement of [^{14}C]NEM labelling of vesicle membrane protein after substrate protection. (a) Vesicles (200 μg protein) were pre-incubated for 10 min at 25°C with 50 mM glucose (as required), 10 mM $MgSO_4$, in 50 mM KP pH 6.6, prior to addition of NEM (1 mM final, as required; total reaction volumes 0.5 ml). After 30 min or 60 min further pre-incubation at 25°C free 'cold' NEM was removed by sedimenting and washing vesicles in 50 mM KP pH 6.6, 0–4°C. Vesicles were then resuspended in 1 mM [^{14}C]NEM, 50 mM KP pH 6.6 (7.3 mCi/mmol), incubated 60 min at 25°C before washing as above, and solubilization in dissolving buffer [18] by incubation at 100°C for 1 min. All sample loadings were 120 μg protein; one of the four identical gel tracks is shown adjacent to profiles of radioactivity. Preincubation conditions and total counts per track after subtraction of backgrounds: 60 min, 25°C, no glucose, no NEM, 10 851 cpm (○); 60 min, 25°C, no glucose, 1 mM NEM, 2477 cpm (△); 60 min, 25°C, 50 mM glucose, 1 mM NEM, 2848 cpm (▲); 30 min, 25°C, 50 mM glucose, 1 mM NEM, 3990 cpm (•). (b) As in (a), but 280 μg protein initially. Samples solubilized at 60°C for 10 min, with 200 μg protein loaded per sample track. Preincubation treatments and legends as for (a), with total counts per profile after subtraction of all backgrounds in the order given for (a): 13 202 cpm; 3893 cpm; 4387 cpm; 5537 cpm. The MW of [^{14}C]NEM-labelled protein bands were estimated using the following protein markers: Bovine serum albumin 68 000; glutamate dehydrogenase, 56 000; ovalbumin, 43 000; lactate dehydrogenase, 35 500; carbonic anhydrase, 30 000; soyabean trypsin inhibitor, 21 000; ribonuclease, 14 000. The position of markers adjacent to each treatment is shown by the appropriate symbol on the lower axes.

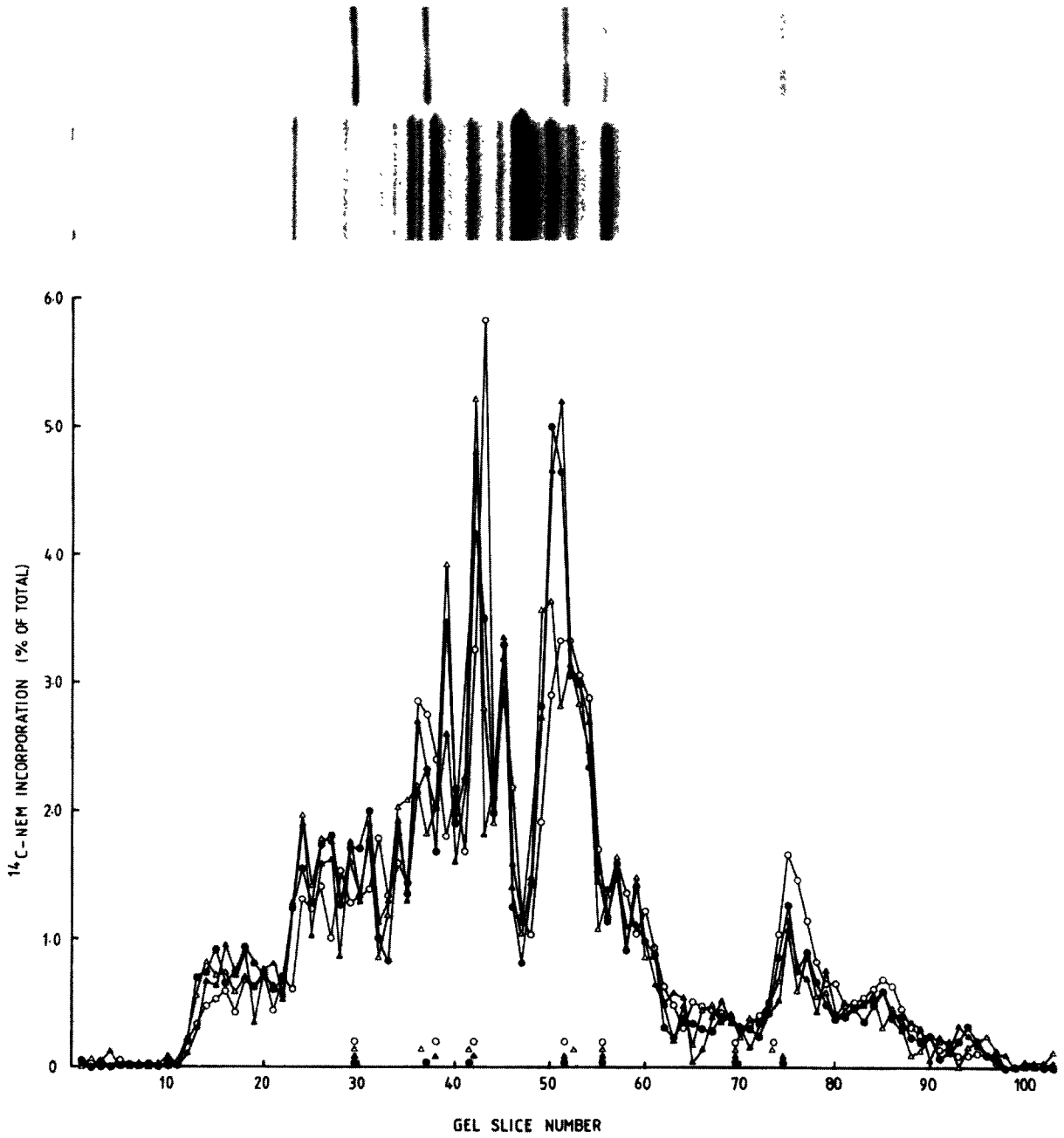
6·0
5·0
4·0
3·0
2·0
1·0
0
^{14}C-NEM INCORPORATION (% OF TOTAL)
10
20
30
40
50
60
70
80
90
100
GEL SLICE NUMBER

Fig.3a

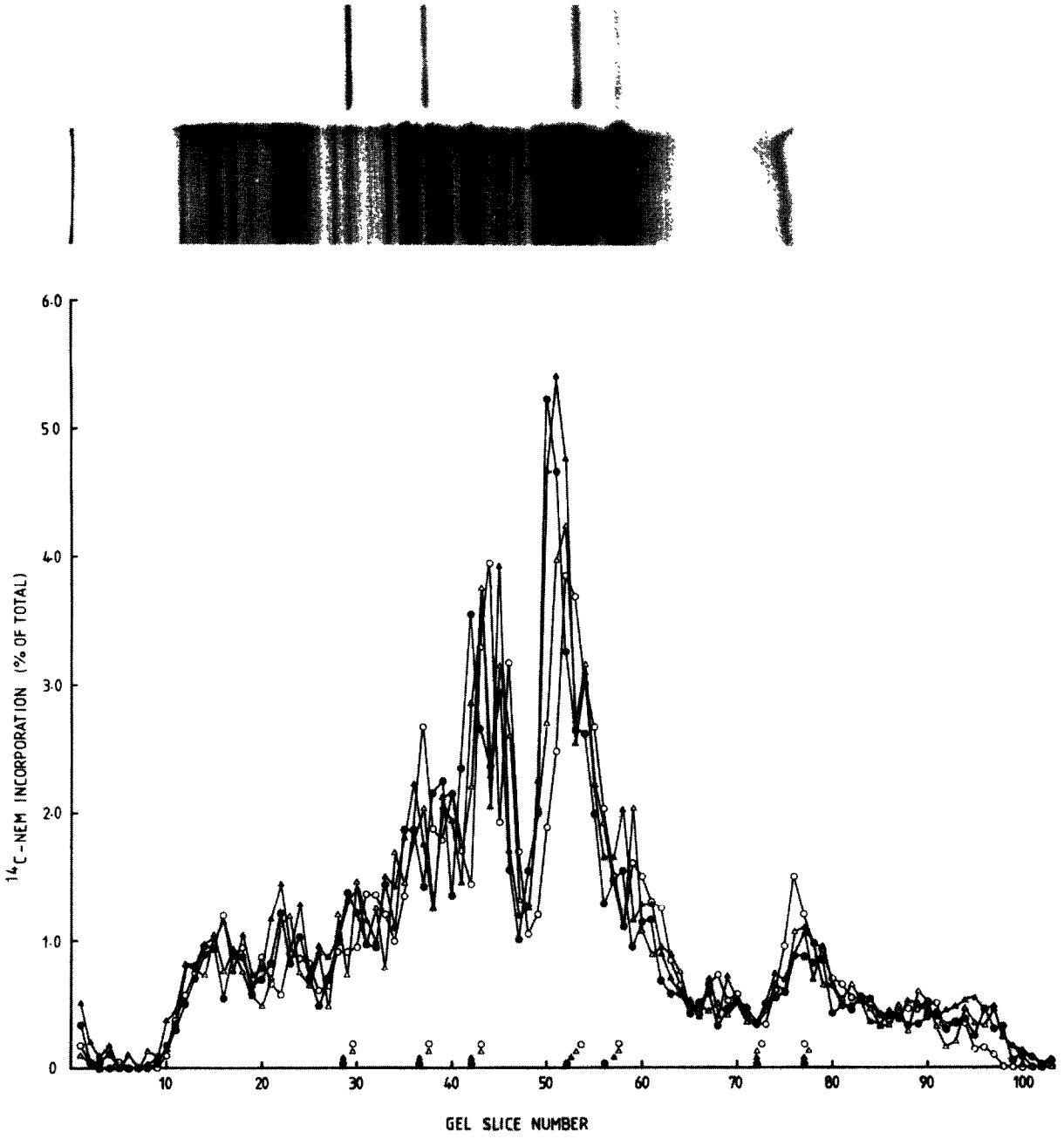
6·0
5·0
4·0
3·0
2·0
1·0
0
^{14}C-NEM INCORPORATION (% OF TOTAL)
10
20
30
40
50
60
70
80
90
100
GEL SLICE NUMBER

Fig.3b

preted as being due to the time dependence of the reaction.

That these results are not an artifactual enhancement of labelled bands due to reduction in total [^{14}C]NEM incorporation (caused by prior incubation with 'cold' NEM) is indicated by the manner in which the profiles for samples pretreated without protective agent in the presence of 'cold' NEM (open triangles) match closely these for samples pre-incubated with neither 'cold' NEM nor substrate (open circles), despite a 2–3-fold difference in total counts.

The peak showing enhanced labelling has an apparent MW close to that reported for one outer membrane protein of *E. coli* [27]. Fig.3b shows that identical profiles were obtained under conditions where the outer membrane protein did not enter the gel matrix during electrophoresis (samples prepared by incubation in dissolving buffer at 60°C for 10 min, rather than 100°C for 1 min in fig.3a [27]), suggesting that this protein was not responsible for the enhanced labelling observed.

4. Discussion

There are seven systems known to be capable of transporting galactose in *E. coli* which must be considered in interpreting the results reported here, i.e. GalP, MglP, AraE, AraF, LacY, MelA and PtsG [28]. The lactose permease (LacY) can be eliminated because the strain chosen has a *lac* (zya) deletion; the melibiose (MelA) and arabinose transport systems (AraE and AraF) can also be disregarded since whole cells and vesicles of S183-27T do not transport methyl β-D-thiogalactoside or arabinose [12,13]. None of the respiration-driven uptake data reported in fig.1 or table 1 or the fluxes in fig.2 are consistent with MglP or PtsG transport activity; the strain is phenotypically MglP-negative, and the preparation of vesicles ensures loss of essential cytoplasmic phosphotransferase components or their poisoning in azide-treated whole cells. Yet protection by glucose membrane-bound MglP or PtsG components account for the results of fig.3, only if it is assumed that the demonstrated substrate protection of GalP (table 1) makes a negligible contribution to [^{14}C]NEM incorporation. The transport data suggest that it is GalP which is preferentially labelled by [^{14}C]NEM after substrate protection from 'cold' NEM.

Acknowledgements

We thank Drs P. F. J. Henderson, M. C. Jones-Mortimer and I. S. Hunter for valuable suggestions and critical appraisal of this manuscript. This work was financed under SRC Grant G/RA/4472.5 to PJFH, and an MRC Research Studentship to PH.

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