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A rapid method for the reconstitution of Na^+-dependent neutral amino acid transport from bovine renal brush-border membranes

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1. A simple and rapid method for the reconstitution of Na^+-dependent neutral amino acid transport activity from bovine renal brush border membranes is described. 2. The neutral detergent decanoyl-*N*-methylglucamide ('MEGA-10') was employed to solubilize the membrane protein. This obviated the necessity for a prolonged dialysis step. 3. The properties of amino acid transport in these vesicles were similar to those observed in native membranes. 4. This should be a useful procedure in the eventual identification and isolation of amino acid transport proteins.

INTRODUCTION

The transport of neutral amino acids in brush-border membranes prepared from the kidney cortex of several species has been studied in detail (Evers *et al.*, 1976; Fass *et al.*, 1977; Hammerman & Sacktor, 1977, 1982; McFarlane-Anderson & Alleyne, 1979; Goldman *et al.*, 1979; McNamara *et al.*, 1979, 1981; Alleyne *et al.*, 1980; Mircheff *et al.*, 1982; Medow *et al.*, 1983; Biber *et al.*, 1983; Steiger *et al.*, 1983). It is found that in all cases neutral amino acid transport is to a large extent dependent on an electrochemical gradient of Na^+ ions. However, very little work has been undertaken to identify and ultimately to isolate the transport proteins involved. Identification of amino acid transport proteins is hindered by the lack of specific tight-binding inhibitors. The affinity of the carriers for their substrate amino acids is also relatively low, and this does not facilitate the use of approaches based on affinity chromatography or substrate protection against non-specific inhibitors.

A possible approach to the identification of these carriers is to incorporate membrane protein fractions into artificial phospholipid membranes and reconstitute functional transport activity. Using rabbit renal brush-border membranes solubilized with Triton X-100, Kinne & Faust (1977) demonstrated the incorporation of glucose, phosphate and alanine transport activity into artificial membranes prepared from soya-bean phospholipids. The reconstituted alanine transport in this system did not, however, show the same Na^+-dependence as that observed in native membrane vesicles. More recently, Koepsell *et al.* (1984) have described a detailed method for the incorporation of proteins from pig kidney-cortex brush-border membranes into artificial membranes and have convincingly demonstrated the functional reconstitution of a number of Na^+-dependent transport systems. Glutamate transport was characterized in detail in that study. Reconstitution of alanine transport by a similar method has also been reported by Parker & Wheeler (1986).

Neutral amino acid transport activity from ascites cells has been reconstituted (Cecchini *et al.*, 1978; McCormick *et al.*, 1984). In particular, McCormick *et al.* (1984) have described a method involving solubilization of the protein with cholate, mixing with soya-bean phospholipids and formation of proteoliposomes by freezing and thawing. Using this procedure they were able to demonstrate the incorporation of transport activity corresponding to systems A, ASC and L, which are known to exist in ascites cells.

All of these methods, however, include a prolonged dialysis step, which may potentially lead to inactivation of the transport protein. Such a lengthy procedure is also disadvantageous for approaches to the isolation of carriers based on fractionation and reconstitution of membrane proteins. In this paper, a rapid and simple method for the reconstitution of neutral amino acid transport activity from bovine renal brush-border membrane vesicles is described. This method employs the neutral detergent decanoyl-*N*-methylglucamide, which is readily removed from proteins by gel filtration and dilution. This obviates the need for a prolonged dialysis step and allows the entire reconstitution procedure to be completed in 3–4 h. A preliminary report of some of this work has been presented (McGivan & Lynch, 1986).

METHODS

Preparation of membranes, lipids and detergent

Brush-border membrane vesicles were prepared from bovine kidney cortex by the $MgCl_2$-precipitation method of Booth & Kenny (1974) as modified by Biber *et al.* (1981). The vesicles were suspended in a medium containing 0.25 M-sucrose, 10 mM-Hepes/KOH and 0.2 mM-$CaCl_2$, pH 7.4, frozen in liquid N_2 and stored at −20 °C until use.

Phospholipids were prepared from egg yolk as described by Dawson (1963). The yolks were homogenized with acetone and the insoluble material was extracted with chloroform/methanol (1:1, v/v). After removal of the solvents by evaporation, the solid material was dissolved in light petroleum (b.p. 60–80 °C), and the phospholipids were precipitated with acetone. The lipids were dissolved in chloroform containing 0.1% butylated hydroxytoluene as antioxidant and stored under N_2 at −20 °C for not more than 3 weeks. Avoidance of oxidation of the lipids appears to be critical for the success of this reconstitution procedure. The phospholipid preparation was analysed by t.l.c. (Skipski *et al.*, 1964), and was found to contain predominantly

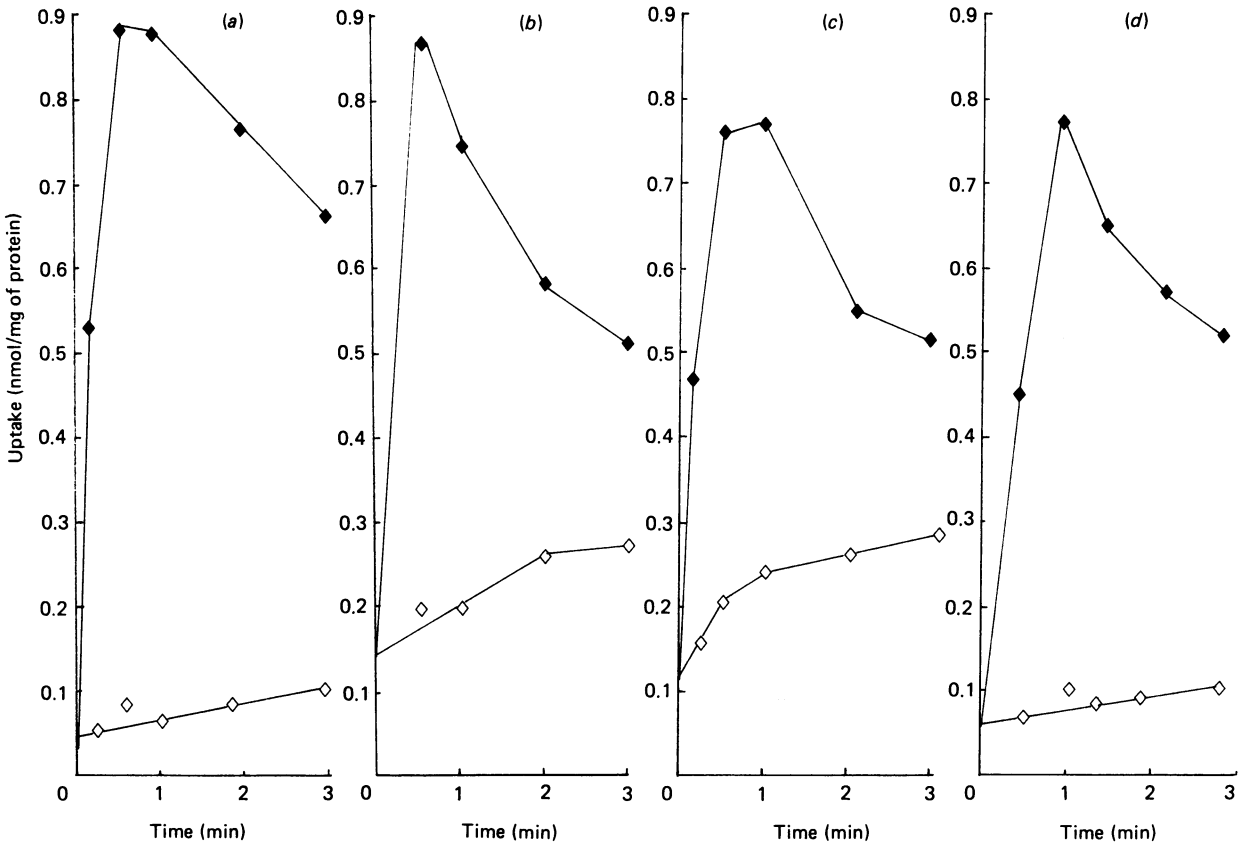

Fig. 1. Transport of amino acids and glucose in native brush-border membrane vesicles from bovine kidney cortex

Membrane vesicles were incubated in a medium containing 0.25 M-sucrose, 10 mM-Hepes, 0.2 mM-$CaCl_2$, 5 mM-$MgCl_2$ and the substrates shown at a final concentration of 0.1 mM, together with either 100 mM-NaCNS (◆) or 100 mM-KCNS (◇) as shown. The time courses shown relate to the uptake of (*a*) alanine, (*b*) leucine, (*c*) phenylalanine and (*d*) glucose.

phosphatidylcholine, with some phosphatidylethanolamine.

The detergent decanoyl-*N*-methylglucamide (MEGA-10) was prepared by the method of Hildreth (1982) and was used after recrystallization from methanol/diethyl ether (1:9, v/v).

Reconstitution procedure

Brush-border membrane vesicles at a concentration of 5 mg/ml were thawed at room temperature and diluted with an equal volume of a medium containing 100 mM-NaCl, 10 mM-Tris/HCl, 2 mM-phenylmethanesulphonyl fluoride and 1 mM-dithiothreitol. The appropriate volume of a 5% (w/v) solution of MEGA-10 was added to give a final concentration of 0.5%. After mixing, the solubilized membranes were centrifuged at 150000 *g* for 20 min at 4 °C. The small pellet was discarded, and the protein content of the supernatant was measured.

A sample of the chloroform solution of phospholipids was placed in a round-bottomed flask and the chloroform evaporated under a stream of N_2. The lipid was suspended in a solution containing 0.1 M-potassium cyclamate/10 mM-Hepes/KOH, pH 7.5 (cyclamate buffer), by gentle agitation and then sonicated on ice until translucent. The dispersion was centrifuged at 150000 *g* for 20 min and any pellet discarded. The content of lipid phosphate in the dispersion was measured at this stage as described below.

Protein and lipid were mixed in a ratio of 15 μmol of lipid phosphate/mg of protein, and the detergent concentration was decreased by passage of 1 ml samples through a column (27 cm × 1 cm) of Sephadex G-50 (coarse grade) equilibrated in cyclamate buffer. The turbid fraction eluted in the void volume was collected, diluted a further 4-fold with cyclamate buffer and immediately frozen in liquid N_2.

After thawing at room temperature, the proteoliposomes were collected by centrifugation at 45000 *g* for 20 min at 4 °C. The pellet was resuspended in 40 ml of cyclamate buffer and re-centrifuged. The final pellet was then resuspended in cyclamate buffer at approx. 2 mg of protein/ml.

Transport assays were performed by the technique of rapid filtration through a nitrocellulose filter (0.45 μm). The reconstituted vesicles were mixed with an equal volume of a solution containing 0.2 M-NaCNS or -KCNS, 10 mM-Hepes/KOH and 0.2 mM radiolabelled amino acid at room temperature (20 °C). Samples containing 20–30 μg of protein were removed at the appropriate time, and the transport reaction was stopped by the addition of 1 ml of ice-cold 'stop' solution (0.1 M-NaCl/10 mM-Hepes/KOH, pH 7.4). The vesicles suspension was immediately filtered under pressure and the filter washed with 3 × 1 ml of stop solution. The filters were removed into scintillation fluid, allowed to dissolve for a minimum of 30 min and assayed for radioactivity by liquid-scintillation counting.

Protein was assayed by the method of Bradford (1976); the lipid in the vesicles did not interfere with this assay. For the rapid estimation of lipid phosphate, the following procedure was used: samples of the lipid dispersion containing 0.05–0.3 μmol of lipid phosphate were digested by heating with 0.2 ml of H_2SO_4 plus 0.05 ml of $HClO_4$. Phosphate in the digest was determined by the method of Chen *et al.* (1956).

^{14}C- and ^{3}H-labelled amino acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

Transport in native brush-border membrane vesicles from bovine kidney

The characteristics of transport of a number of substrates into bovine renal brush-border vesicles have not been reported previously and are shown in Fig. 1. The transport of alanine and phenylalanine is Na^+-dependent, in agreement with previous findings with renal vesicles from other species (Fass *et al.*, 1977; Evers *et al.*, 1976); the transport of leucine in these vesicles is also Na^+-dependent. There is evidence that these amino acids and glutamine are transported on a single common carrier in this membrane system (Lynch & McGivan, 1987). The transport of glucose is also Na^+-dependent, as shown in other species (see, e.g., Hopfer, 1977).

Reconstitution of amino acid transport

The reconstitution of amino acid transport activity in vesicles prepared as described in the Methods section was highly dependent on the final ratio of lipid to protein in the vesicles, as previously shown for other systems (Eyton, 1982). Fig. 2 shows that optimum alanine transport was obtained at lipid/protein ratios of greater than 8 μmol of lipid phosphate/mg of protein. At lower lipid/protein ratios, the initial rate of transport declined, and little transport was obtained at 4 μmol/mg. In order to obtain vesicles composed of greater than 8 μmol of lipid phosphate/mg of protein, it was found to be necessary to mix the lipid and protein in a ratio of approx. 15 μmol of lipid phosphate/mg of protein in the original detergent solution, and this ratio was used throughout in forming the vesicles. It is of interest that the lipid/protein ratio required for transport in reconstituted vesicles is higher than that in native brush-border membrane vesicles (1 μmol of lipid phosphate/mg of protein).

Electron microscopy was performed on the reconstituted membrane vesicles prepared by this method. Fig. 3 shows the resultant photograph, in which closed vesicles of diameter 0.3–0.6 μm can be observed. These vesicles are considerably smaller than native bovine renal brush-border membrane vesicles or the reconstituted vesicles of Koepsell *et al.* (1984), but are similar in size to the reconstituted vesicles prepared from ascites cells by McCormick *et al.* (1985).

Characteristics of transport activity in reconstituted membrane vesicles

Fig. 4 illustrates the time course of alanine uptake in reconstituted vesicles in the presence of different Na^+ salts. The apparent uptake extrapolated to zero time was independent of Na^+ ions and was temperature-insensitive (results not shown), and this was taken to represent non-specific binding of the amino acid to the lipid and to the filters. The initial rate in the presence of thiocyanate, which is known to penetrate membranes rapidly, was faster than that in the presence of the less penetrant anion cyclamate. These results are consistent with electrogenic Na^+–amino acid co-transport similar to that which occurs in native vesicles.

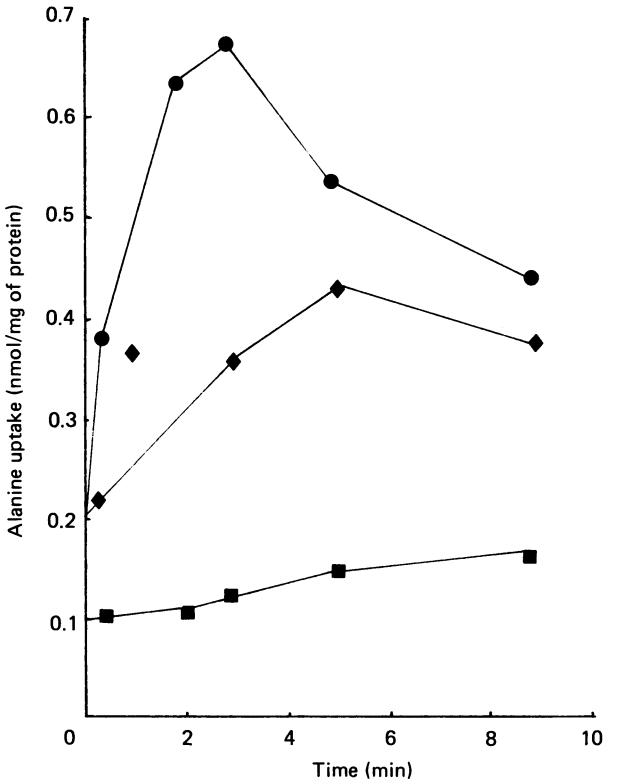

Fig. 2. Dependence of alanine transport on the ratio of phospholipid to protein in reconstituted membrane vesicles

Phospholipids and protein were mixed in various ratios in the detergent solution, membrane vesicles were reconstituted and the uptake of 0.1 mM-alanine was measured as described in the Methods section. The lipid/protein ratios used in the reconstitution procedure were (■) 2.5, (◆) 10 and (●) 15 μmol of lipid phosphate/mg of protein. The lipid/protein ratios in the final reconstituted membranes were 4.4, 6 and 8.4 μmol of lipid phosphate/mg of protein respectively.

Fig. 5 shows the time courses for the uptake of alanine, glutamine, leucine and phenylalanine in vesicles reconstituted as described in the Methods section. The results shown are corrected for the extrapolated uptake at zero time, which represents binding. The transport was carried out in the presence of media containing NaCNS or KCNS as indicated. In the presence of Na^+, there was an uptake of amino acids which was maximal after 3–5 min and then declined. This overshoot is characteristic of transport driven by a Na^+ electrochemical gradient. In the presence of K^+ instead of Na^+, no overshoot was observed. As in native brush-border membrane vesicles, the transport of alanine and glutamine was almost entirely Na^+-dependent. The transport of the hydrophobic amino acids leucine and phenylalanine was largely Na^+-dependent, but also exhibited a significant rate in the absence of Na^+. No glucose transport activity was reconstituted into these

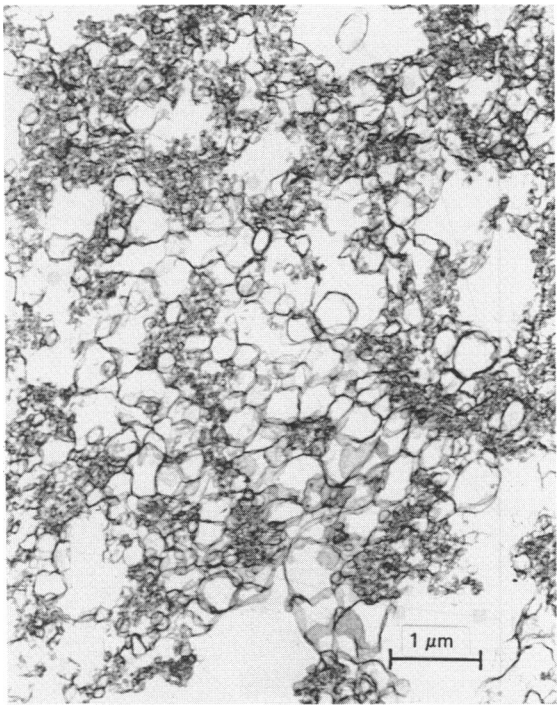

Fig. 3. Typical electron micrograph of reconstituted membrane vesicles

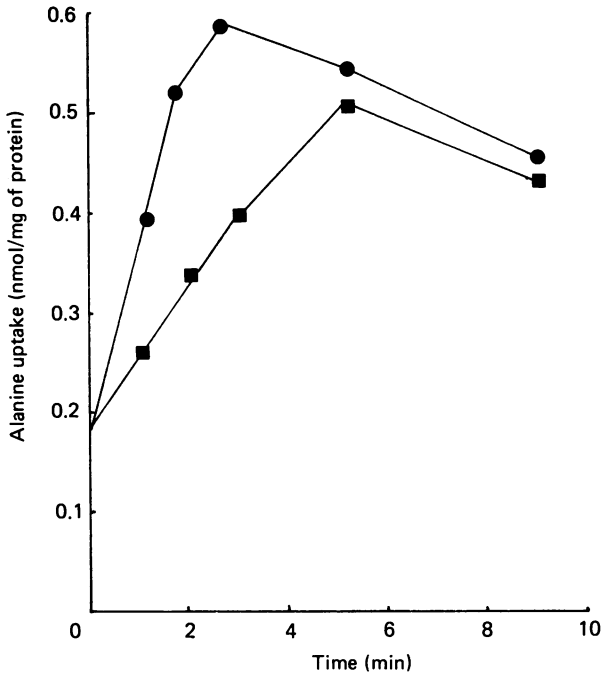

Fig. 4. Anion-dependence of alanine transport in reconstituted membrane vesicles

The uptake of 0.1 mM-alanine in the presence of a final concentration of (●) 100 mM-NaCNS or (■) 100 mM-sodium cyclamate (replacing NaCNS) in the incubation medium was measured as described in the Methods section.

vesicles. This may be due to the incorporation of an inactive carrier or the non-incorporation of glucose carrier molecules into the artificial membrane system.

Fig. 6 shows a comparison between the SDS/polyacrylamide-gel-electrophoresis patterns of the native brush-border membrane vesicles, the MEGA-10-solubilized protein fraction and the reconstituted membrane vesicles. There was no selective extraction of membrane proteins by the detergent. However, there was a selective incorporation of polypeptides into the reconstituted vesicles when compared with the solubilized fraction. In particular, three bands showed a major enhancement: their M_r values were 121000 ± 900, 92300 ± 1300 and 46800 ± 1100 (mean $\pm$ S.E.M. for nine determinations). This is consistent with the previous findings by Koepsell *et al.* (1984), who, using a different reconstitution method, showed selective enhancement of bands of M_r 152000, 120000, 94000 and 45000 in reconstituted pig renal membrane vesicles. There is no evidence at present to correlate the presence of any individual band with amino acid transport activity.

DISCUSSION

The primary object of this work was to develop a rapid method for the reconstitution of functional transport activity from renal brush-border membrane vesicles into artificial phospholipid membranes. The above method appears to achieve this objective. The major advantage of the present method over those published previously is the use of the detergent MEGA-10, which has not to our knowledge been used previously for the reconstitution of membrane transport activity. This detergent has many properties in common with the more widely used detergent octyl glucoside (Baron & Thompson, 1975), but is far less expensive.

The method used here is similar to that previously described by McCormick *et al.* (1984, 1985) for reconstitution of amino acid transport from ascites cells, except that those authors used cholate to solubilize the membrane proteins and found it necessary to include an overnight dialysis step. It is of interest that the size of vesicles formed by both methods is similar. The Na^+-dependence of amino acid transport in vesicles formed by the present method is, however, much more apparent than that of α-aminoisobutyrate in vesicles formed by the method of McCormick *et al.* (1984).

The characteristics of the transport of amino acids into vesicles formed by the method in the present paper are qualitatively similar to, but quantitatively rather different from, those reconstituted by Koepsell *et al.* (1984) from pig renal brush-border membranes. The initial rates of alanine transport are the same for vesicles formed by both methods. However, the time course varies considerably. Maximum uptake of alanine as determined by Koepsell *et al.* (1984) occurred after 2 h and reached a value of 11 nmol/mg of protein, as opposed to corresponding values of 3 min and 0.6 nmol/mg respectively in the present study. This discrepancy can possibly be explained by the relative size of the reconstituted vesicles prepared by the two methods. Vesicles exhibiting maximum alanine transport activity as determined by the earlier workers had a diameter of approx. 1 μm, which corresponds to a much larger internal volume.

The procedure of Koepsell *et al.* (1984) involves a long and complex solubilization procedure, which has, however, been thoroughly characterized. The vesicles formed are very suitable for the study of the kinetic

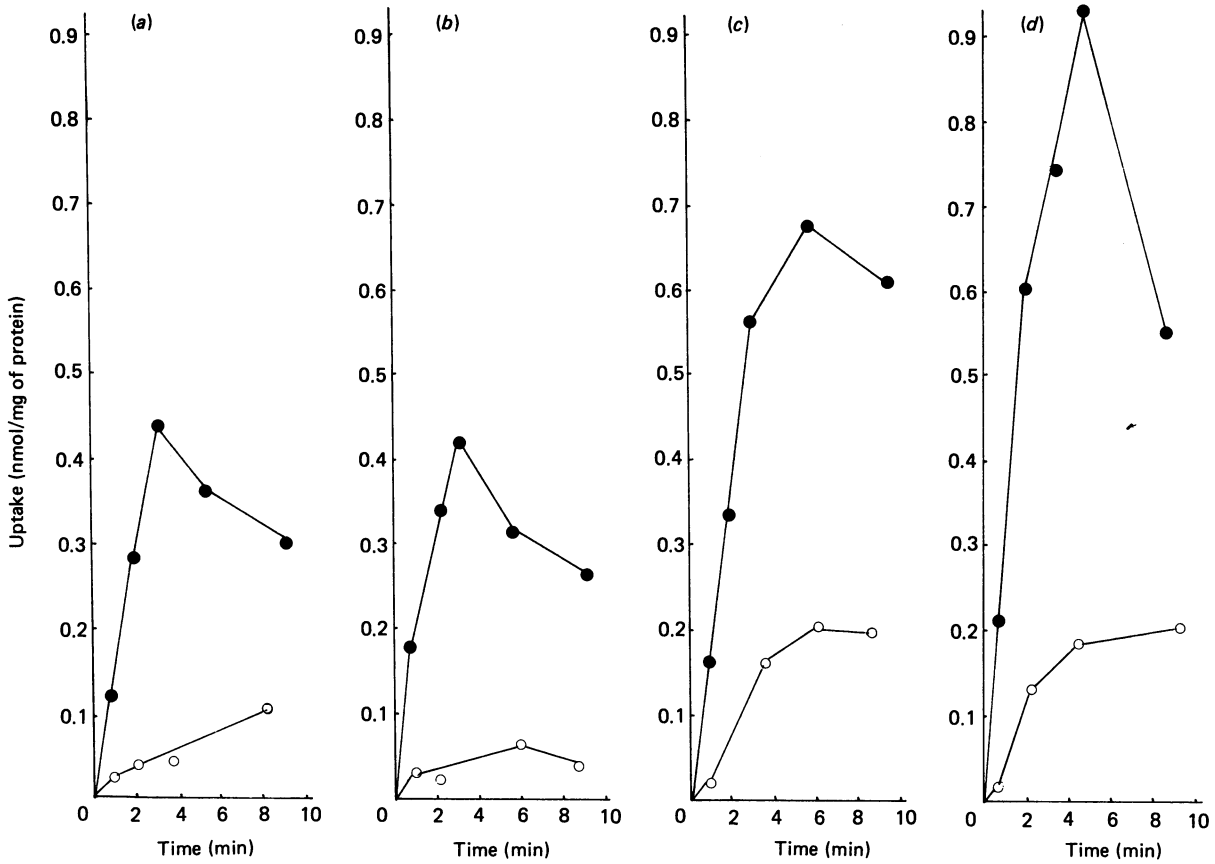

Fig. 5. Time courses for the uptake of amino acids in reconstituted membrane vesicles

The transport of various amino acids at 0.1 mM final concentration was measured as described in the Methods section. The results shown were obtained by subtracting the zero-time uptake values (representing binding) from the total uptake value. The apparent binding at zero time for alanine, glutamine and the lipophilic amino acids leucine and phenylalanine was 0.2, 0.2, 1.0 and 1.55 nmol/mg respectively. The Figure shows the uptake of (*a*) alanine, (*b*) glutamine, (*c*) leucine and (*d*) phenylalanine in the presence of either (●) NaCNS or (○) KCNS.

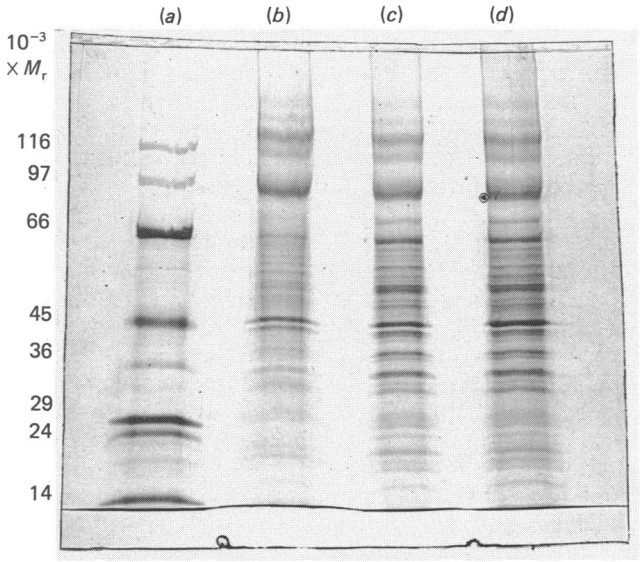

Fig. 6. SDS/polyacrylamide-gel electrophoresis of native and reconstituted membrane vesicles

Gel electrophoresis was performed by the method of Laemmli (1970). The tracks shown are: (*a*) M_r ($\times 10^{-3}$) standards; (*b*) reconstituted membrane vesicles; (*c*) MEGA-10-solubilized brush-border membranes; (*d*) native brush-border membrane vesicles. The loading was 150 μg of protein in each case.

properties and stoichiometry of Na^+–amino acid co-transport. It is of interest that the pattern of protein incorporation into artificial membranes by the method in this paper is similar to that obtained by Koepsell *et al.* (1984). The present method offers no advantages over that of Koepsell *et al.* (1984) in terms of kinetic or mechanistic studies, but has the merit of rapidity and simplicity. This is a considerable advantage in approaches to the identification of carrier molecules, which involve protein fractionation followed by a reconstitution step.

In this context a particular feature of the present procedure is the use of an uncharged detergent to solubilize the membrane protein. This should allow preliminary fractionation of the proteins by ion-exchange chromatography before reconstitution of various fractions into liposomes and determination of transport activity. This procedure was not possible in previous reconstitution methods using cholate and deoxycholate. It is also possible that this method could be extended to the reconstitution of transport activity from the membranes of other cell types. In summary, the method described here should be a useful tool in the eventual identification and isolation of amino acid transport systems in cell membranes.

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