A rapid method for the functional reconstitution of amino acid transport systems from rat liver plasma membranes

Partial purification of System A

Ana R. QUESADA* and John D. McGIVAN†

Department of Biochemistry, University of Bristol, Bristol, U.K.

A rapid method for the functional reconstruction of amino acid transport from liver plasma-membrane vesicles using the neutral detergent decanoyl-*N*-glucamide ('MEGA-10') is described. The method is a modification of that previously employed in this laboratory for reconstitution of amino acid transport systems from kidney brush-border membranes [Lynch & McGivan (1987) *Biochem. J.* **244**, 503–508]. The transport activities termed 'System A', 'System N', and 'System L' are all reconstituted. The reconstitution procedure is rapid and efficient and is suitable as an assay for transport activity in studies involving membrane fractionation. By using this reconstitution procedure, System A transport activity was partially purified by lectin-affinity chromatography.

INTRODUCTION

Amino acid transport into isolated hepatocytes [see Kilberg (1982), Shotwell *et al.* (1983) and Kilberg *et al.* (1985) for reviews] and into liver plasma-membrane vesicles (e.g. Sips *et al.*, 1980*a,b*; Hayes & McGivan, 1983; Dudeck *et al.*, 1987) has been extensively studied. Na⁺-dependent uptake of amino acids is catalysed by three major systems with overlapping specificity which are termed 'System A', 'System ASC', and 'System N'. Branched-chain and aromatic amino acids are transported via a Na⁺-independent system termed 'System L'. These transport proteins have not been identified and very little is known of their molecular properties. Indeed, there is at present no direct evidence that these transport systems represent different proteins in the cell membrane, although this can be inferred from the different behaviour of transport activities towards hormonal induction. No specific tight-binding inhibitors which could be used for the identification of these transport proteins have been found. One possible approach to the identification of liver plasma-membrane transport proteins is to fractionate membrane proteins, reconstitute the various protein fractions into artificial phospholipid membranes and assay for transport activity.

The reconstitution of cell-membrane amino acid transport systems in general has received relatively little attention. One of the first successful investigations reported a method for the reconstitution of a number of transport systems from ascites cells (McCormick *et al.*, 1984). In this procedure, membranes were solubilized with cholate and reconstituted with asolectin by a freeze-thaw procedure. A method for the reconstitution of amino acid transport from rabbit kidney brush-border membranes has been described and characterized in detail by Koepsell *et al.* (1984). Bracy *et al.* (1987) have reported a procedure for the reconstitution of Na⁺-

dependent 2-aminoisobutyrate transport from liver plasma membranes.

All these procedures are time-consuming and involve a prolonged dialysis stage. A major objective in developing a successful reconstitution method is to provide an assay for functional transport systems. Therefore it would be of advantage to devise a reconstitution method which was more rapid and simple than the above procedures. Such a method for the reconstitution of transport activity from kidney brush-border membranes has been reported from this laboratory (Lynch & McGivan, 1987*a*). The distinctive feature of this method was the use of the neutral detergent decanoyl-*N*-glucamide, which can be rapidly removed from proteins by gel filtration and dilution. It was therefore of interest to attempt to extend this method to the reconstitution of amino acid transport from liver plasma membranes.

In the present paper we reported the rapid reconstitution of alanine, glutamine and leucine transport, catalysed by System A, N and L respectively, from liver plasma-membrane vesicles. The procedure is compared with that recently described for the reconstitution of liver plasma-membrane 2-aminoisobutyrate transport by Bracy *et al.* (1987). It is further shown that System A activity, but not that of System N, binds to the lectin concanavalin A.

MATERIALS AND METHODS

Preparation of rat liver plasma-membrane vesicles, phospholipids and detergent

Plasma-membrane vesicles were prepared from the livers of normally-fed female Wistar rats essentially by the method of Van Amelsvoort *et al.* (1978), but with the sucrose gradients described by Samson & Fehlmann (1982). The vesicles were resuspended at a concentration

Abbreviations used: MEGA-10, decanoyl-*N*-glucamide; MeAIB, methylaminoisobutyrate; NEM, *N*-ethylmaleimide; NPM, *N*-phenylmaleimide; AIB, aminoisobutyrate; PCMBs, *p*-chloromercuribenzenesulphonate.

* Permanent address: Departamento de Bioquímica, Facultad de Ciencias, Universidad de Malaga, Malaga, Spain.

† To whom correspondence and reprint requests should be sent.

of 7–10 mg of protein/ml in a medium containing 0.25 M-sucrose, 0.2 mM-CaCl₂ and 10 mM-potassium Hepes at pH 7.5, frozen in liquid N₂ and stored at –20 °C until required. The yield of plasma membranes in a typical preparation using two rat livers was 25–30 mg of protein.

Phospholipids were extracted from egg yolk by the method of Dawson (1963), as described in detail previously (Lynch & McGivan, 1987a), taking precautions to minimize lipid oxidation. The preparation contained predominantly phosphatidylcholine, together with some phosphatidylethanolamine. The phospholipids were stored under N₂ in chloroform containing 0.1% butylated hydroxytoluene at –20 °C and used within a week of preparation. Avoidance of lipid oxidation appeared to be essential for the reconstitution procedure. When transport activity could no longer be reconstituted using a particular batch of lipids, it was consistently found that successful reconstitution could be obtained by using a new lipid preparation. Commercially obtained phospholipids were not found to be suitable for this procedure, possibly because of oxidation. The detergent decanoyl-*N*-methylglucamide (MEGA-10) was synthesized from *N*-methylglucamine and decanoic acid exactly as described by Hildreth (1982) and was used after one recrystallization from methanol/diethyl ether (1:9, v/v). This compound can also be obtained commercially from Sigma.

Reconstitution procedure

Liver plasma membrane vesicles (7–10 mg/ml) were thawed and diluted to a concentration of 5 mg/ml by addition of a medium containing 20 mM-Tris/HCl, 1 mM-dithiothreitol and 1 mM-phenylmethanesulphonyl fluoride at pH 7.4 and 4 °C. Sufficient of a 5% (w/v) solution of MEGA-10 was added to produce a detergent/protein ratio of 3:1 (w/v). The solution was kept on ice for 10 min and then centrifuged at 150 000 *g* for 20 min at 4 °C. The pellet was discarded, and the protein concentration of the supernatant was measured.

To a sample of a chloroform solution of phospholipids containing approx. 150 μmol of lipid phosphate was added cholesterol (5%, mol/mol), and the chloroform was removed under N₂. The lipids were suspended by gentle shaking in 5 ml of 0.1 M-potassium cyclamate/10 mM-potassium Hepes, pH 7.5 (cyclamate buffer). After sonication on ice for 10 × 10 s with intervals for cooling (DAWE Soniprobe at 40 W with a 1 cm diameter probe), the lipid dispersion was centrifuged at 150 000 *g* at 4 °C for 20 min and the pellet was discarded. The concentration of lipid phosphate in the dispersion was measured at this stage.

The solubilized protein was mixed with the lipid dispersion to give a ratio of 40 μmol of lipid phosphate/mg of protein. The lipid/protein mixture was passed down a column (27 cm × 1 cm) of Sephadex G-50 (coarse grade) equilibrated in cyclamate buffer. The turbid fraction eluted from the column was diluted a further 1:4 with cyclamate buffer and frozen in liquid N₂. The preparation could if required be stored overnight at –20 °C at this stage. After thawing at room temperature, the vesicles were sedimented by centrifugation for 20 min at 45 000 *g* in the cold. The pellet was resuspended in cyclamate buffer at a final protein concentration of 1–2 mg/ml.

Transport measurements

For measurement of amino acid transport, reconstituted vesicles (approx. 20 μg of protein) were mixed at 20 °C with an equal volume of a medium containing 0.2 M-NaCNS or KCNS, 10 mM-Hepes, pH 7.5, plus 0.2 mM of the required ³H-labelled amino acid (500 d.p.m./pmol). The transport reaction was stopped by the addition of 1 ml of ice-cold 'stop solution' (0.1 M-NaCl/10 mM-Hepes, pH 7.5) and the vesicles were immediately separated by filtration through a nitrocellulose filter (0.45 μm pore size). After washing with 3 × 1 ml of stop solution, the filters were placed in 10 ml of scintillation fluid, left to dissolve for at least 30 min and assayed for radioactivity. The apparent uptake of amino acids extrapolated to zero time was independent of the presence of Na⁺ and was taken to represent binding. This binding was greater for the hydrophobic amino acid leucine than for alanine and glutamine. The binding component was subtracted from the total uptake to give the time courses shown in Figs. 1, 3 and 4 (below). In Fig. 2 (below) the binding component has not been subtracted.

Lectin-affinity chromatography

Liver plasma-membrane vesicles (approx. 70 mg of protein) were solubilized as described above, and undissolved material was removed by centrifugation. A column containing 5 ml of concanavalin A-agarose (Sigma) was equilibrated in a medium containing 20 mM-Tris/HCl and 0.5% MEGA-10, pH 7.4 at 20 °C. The solubilized protein was applied to this column, and the column was washed through with the same medium until no further protein emerged, as judged by measurements of A₂₈₀. The column was then eluted with a solution containing 150 mM-D-mannose, 0.5% MEGA-10 and 20 mM-Tris/HCl, pH 7.4. The protein peak which emerged was concentrated by dialysis against 30% (v/v) poly(ethylene glycol) in the cold. The yield was approx. 1 mg of protein. This protein fraction was reconstituted as described above, and amino acid transport activity was assayed.

Assays

Protein was measured by the method of Bradford (1976). For the rapid determination of lipid phosphate, samples of the lipid dispersion containing 0.05–0.3 μmol of lipid phosphate were digested by heating with 0.2 ml of H₂SO₄ plus 0.05 ml of HClO₄. Phosphate in the digest was measured by the method of Chen *et al.* (1956). Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

Amino acid transport in native plasma-membrane vesicles

Fig. 1 shows the characteristics of uptake of L-alanine, L-glutamine and L-leucine into plasma-membrane vesicles prepared from rat liver by sucrose-density-gradient centrifugation. In the presence of NaCNS, the uptake of both alanine and glutamine reached a maximum after approx. 20 s and then declined. This overshoot is characteristic of active transport driven by an Na⁺ gradient. In the presence of KCNS the rate of uptake was much slower. The transport of leucine was independent of the presence of Na⁺. These results are in good

Fig. 1. Time courses of amino acid uptake by native plasma-membrane vesicles from rat liver

To 10 μ l of a suspension of liver plasma-membrane vesicles (0.1 mg of protein) was added an equal volume of a medium containing 0.25 M-sucrose, 10 mM-potassium Hepes, 10 mM-MgCl₂, 0.2 mM-CaCl₂, 0.2 M-NaCNS or KCNS and 0.2 mM-[³H]-alanine, -glutamine or -leucine as indicated at pH 7.5 and 20 °C. Transport was terminated after the times shown by addition of 1 ml of an ice-cold medium containing 0.25 M-sucrose, 10 mM-potassium Hepes plus 0.1 M-NaCl. (a) Alanine; (b) glutamine; (c) leucine; $\blacktriangle$, in the presence of NaCNS; $\triangle$, in the presence of KCNS. The values for apparent binding of alanine, glutamine and leucine were 0.02, 0.04 and 0.75 nmol/mg of protein respectively.

agreement with previous data on alanine, glutamine and leucine transport into plasma-membrane vesicles prepared from isolated hepatocytes (Sips *et al.*, 1980a).

Characterization of reconstitution procedure

Transport activity was reconstituted into artificial phospholipid membranes as described in the Materials and methods section. Preliminary experiments (not shown) indicated that optimum activity of alanine transport was obtained when the plasma membranes were solubilized at a detergent/protein ratio of 3 mg of MEGA-10/mg of protein. A minimum ratio of 2 mg of detergent/mg protein was required for the recovery of significant transport activity. It was established also that the inclusion of cholesterol (5 mol/mol of lipid phosphate) gave optimum rates of transport that were up to 70 % higher than those in the absence of cholesterol. The inclusion of higher cholesterol concentrations increased the apparent binding without affecting the rate of transport.

Fig. 2 shows the effect of varying lipid/protein ratio on the time course of alanine uptake by reconstituted vesicles. The rate increased as the lipid/protein ratio increased, and at 40 μ mol of lipid phosphate/mg of protein a characteristic overshoot was observed. At higher lipid/protein ratios (not shown) non-specific binding to the lipids increased and the amount of protein that could be employed for the transport assay was decreased, owing to limitations of the filtration system; consequently the results became scattered and less reproducible. It was concluded that 40 μ mol of lipid phosphate/mg of protein was an optimum ratio for this reconstitution process and all further experiments were performed with this ratio.

Properties of amino acid transport in reconstituted membrane vesicles

Typical time courses for the uptake of alanine, glutamine and leucine by reconstituted vesicles are shown in Fig. 3. The uptake of both alanine and glutamine was clearly Na⁺-dependent. Maximum uptake occurred after 10 min, and an overshoot was observed. Leucine was taken up into the vesicles in an Na⁺-independent process. In this case, the transport of leucine (0.1 mM) was inhibited by > 75 % in the presence of 5 mM-L-isoleucine (results not shown). It was concluded that a large part of leucine uptake was carrier-mediated, presumably via System L. The apparent uptake of leucine was somewhat higher than the Na⁺-independent uptake of alanine or glutamine. Leucine transport is difficult to measure accurately, since the amount of this hydrophobic amino acid binding to the membrane is comparable with, or greater than, the amount transported. Some of the intravesicular leucine may also represent leucine which has been transported but remains bound to the inside of the membrane. The apparent difference between the Na⁺-independent uptake of leucine and that of alanine or glutamine is therefore probably not significant. It is unlikely that the Na⁺-independent uptake of leucine represented H⁺/leucine symport, since replacement of the membrane-permeable thiocyanate anion in the transport medium with the less-penetrant anion cyclamate did not affect Na⁺-independent leucine transport (results not shown).

In intact hepatocytes, alanine transport is mediated by two separate Na⁺-dependent transport systems that have been termed 'System A' and 'System ASC'. System A, but not System ASC, is inhibited by excess concentrations

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

Vesicles were reconstituted by using various ratios of lipid to protein as shown. The time course of transport of alanine (0.1 mM) was measured as described in the Materials and methods section. Lipid/protein ratios were 20 (■), 30 (●) or 40 (▲) μ mol of lipid phosphate/mg of protein respectively.

of *N*-methylaminoisobutyrate (MeAIB). Glutamine is transported by a separate Na^+ -dependent system (System N), which is not inhibited by MeAIB (Kilberg *et al.*, 1980). In order to assess which transport systems had been reconstituted in these experiments, the effect of MeAIB on alanine and glutamine transport was tested (Table 1). The transport of 0.1 mM-alanine was completely inhibited by 5 mM-MeAIB, whereas that of glutamine was unaffected. This result indicates that the present method reconstitutes both System A and System N activity, although System ASC activity may be absent.

Alanine transport in native plasma-membrane vesicles is inhibited by the substituted maleimides *N*-ethylmaleimide (NEM) (Hayes & McGivan, 1983; Sips & van Dam, 1981) and *N*-phenylmaleimide (NPM) and also by mersalyl (Hayes & McGivan, 1983; Sips & van Dam, 1981). The transport of aminoisobutyrate (AIB) is inhibited by NEM, HgCl_2 and *p*-chloromercuribenzenesulphonate (PCMBs) (Dudeck *et al.*, 1987). Table 1 shows that, in reconstituted membrane vesicles, NEM and NPM at a concentration of 2 mM inhibited the transport of both alanine and glutamine to similar extents, and the degree of inhibition in each case was similar to that observed in native membrane vesicles (Hayes & McGivan, 1983). Hg^{2+} (0.1 mM) and PCMBs (0.1 mM) had virtually no inhibitory effect on either alanine or glutamine transport, although at these concentrations these inhibitors produced a large inhibition of AIB transport in plasma-membrane vesicles (Dudeck *et al.*, 1987). Mersalyl (0.5 mM), which is a potent inhibitor of alanine transport in plasma-membrane vesicles, had a lesser inhibitory effect on transport in reconstituted membrane vesicles.

These results suggest that NEM and NPM inhibit

Fig. 3. Time course of transport of (a) alanine, (b) glutamine and (c) leucine in reconstituted membrane vesicles

Transport was determined in the presence of NaCNS or KCNS as described in the Materials and methods section. The apparent binding, which has been subtracted from these values, was 0.4, 0.5 and 1.4 nmol/mg for alanine, glutamine and leucine respectively. ▲, in the presence of NaCNS; △, in the presence of KCNS.

Table 1. Effect of inhibitors on alanine and glutamine transport in reconstituted membrane vesicles

Reconstituted membrane vesicles (15–20 μ g of protein) were preincubated for 5 min at 20 °C with various inhibitors (as indicated) in a total volume of 15 μ l. Transport was initiated by the addition of 10 μ l of medium containing NaCNS or KCNS, potassium Hepes, 3 H-labelled alanine or glutamine as described in the Materials and methods section. The final amino acid concentration was 0.1 mM. Transport was terminated after 10 min. In the case of inhibition by MeAIB, the MeAIB was included in the medium containing radioactive amino acids without preincubation. The uptakes in the presence of NaCNS were corrected for non-specific transport and binding by subtraction of the corresponding values in the presence of KCNS. The results are given as means $\pm$ S.E.M. for the number of observations shown and were in each case performed on at least three different reconstituted membrane preparations.

Inhibitor	Na ⁺ -dependent transport (% of control) of:	
	Alanine	Glutamine
MeAIB (5 mM)	-2 $\pm$ 8 (5)	99 $\pm$ 2 (4)
NEM (2 mM)	63 $\pm$ 2 (6)	56 $\pm$ 6 (6)
NPM (2 mM)	41 $\pm$ 3 (7)	53 $\pm$ 7 (5)
Mersalyl (0.5 mM)	79 $\pm$ 8 (6)	64 $\pm$ 4 (6)
PCMBs (0.1 mM)	84 $\pm$ 12 (6)	96 $\pm$ 7 (5)
HgCl ₂ (0.1 mM)	99 $\pm$ 9 (5)	87 $\pm$ 8 (6)

transport of both alanine and glutamine by attacking an essential thiol group in the respective carrier molecules. Inhibition of transport by mercurials in plasma-membrane vesicles may, however, be a consequence of general membrane damage rather than specific inhibition of transport proteins, since this inhibition is not observed in reconstituted membranes. It has been reported previously that mercurials can induce non-specific membrane permeability (Biber & Hauser, 1979; Will & Hopfer, 1979).

Partial purification of System A

A major objective of the present rapid reconstitution method was to achieve the partial purification of amino acid-transport activity from liver plasma membranes as an approach to the eventual identification of the respective transport proteins. In order to achieve a useful degree of purification, an approach based on affinity chromatography was desirable. It has been reported (Barber *et al.*, 1983) that induction of AIB transport in hepatocytes is blocked by tunicamycin, an inhibitor of glycoprotein synthesis.

Liver plasma-membrane vesicles solubilized with MEGA-10 were applied to a concanavalin A-affinity column, and the fraction retained by the column was eluted with 0.15 M-mannose and reconstituted with phospholipids. Fig. 4(a) shows that enhanced alanine-transport activity was recovered in this fraction. Reconstitution of the protein fraction which did not bind to the column did not lead to recovery of alanine-transport activity (results not shown). Fig. 4(b) shows that glutamine-transport activity could not be reconstituted from the fraction retained by the lectin column. That the lectin column had achieved a specific protein separation

is confirmed by Fig. 5, which shows a densitometric scan of an SDS/polyacrylamide gel of solubilized plasma membranes and of the protein fraction eluted from the affinity column with mannose. The protein pattern of the fraction retained by the lectin column is very different from that of the native membranes, with the specific relative enhancement of several specific protein bands. There is at present no evidence to associate alanine transport with the presence of any particular protein bands on SDS/polyacrylamide gels.

DISCUSSION

The method described here achieves the rapid reconstitution of Na⁺-dependent alanine and glutamine transport into artificial phospholipid membrane vesicles. Transport via both Systems A and N is reconstituted, and a clear Na⁺-dependence is exhibited, together with a characteristic overshoot.

This procedure differs in minor details from that used previously in this laboratory to reconstitute Na⁺-dependent amino acid transport from bovine kidney brush-border membranes (Lynch & McGivan, 1987a). Reconstitution of liver amino acid transport requires higher lipid/protein ratios and is enhanced by the presence of cholesterol, which was without effect on the kidney system. The rate of reconstituted alanine transport per mg of protein from kidney membranes was higher than achieved from liver membranes; uptake in reconstituted kidney membrane vesicles was maximum after 3 min, whereas in reconstituted liver membrane vesicles maximum uptake was achieved only after 10 min. The maximum uptake of alanine in the two systems was comparable, suggesting that the vesicles were of similar dimensions.

The kidney amino-acid-transport system has a substrate specificity from those of systems occurring in liver and is probably a different protein. A single transport system in kidney brush-border membranes catalyses the Na⁺-dependent transport of alanine, glutamine, branched-chain and aromatic amino acids, and this system is not sensitive to inhibition by NEM (Lynch & McGivan, 1987b). The fact that such different amino acid transport systems can be reconstituted by the method described in the present paper suggests that this reconstitution procedure may be of more general application. Indeed, a modification of this procedure has recently been used to reconstitute lactate transport from red blood cells (Poole & Halestrap, 1988).

The only previous report of reconstitution of liver amino acid transport systems is that of Bracy *et al.* (1987). These authors demonstrated the reconstitution of AIB transport by solubilizing plasma membranes in cholate and using a freeze-thaw procedure similar to that used by McCormick *et al.* (1984). Enhanced AIB uptake was demonstrated in vesicles reconstituted from the livers of glucagon-injected rats.

A comparison of these two methods for reconstitution of liver plasma-membrane transport systems is of interest. The procedure described here shows a clear difference between the uptake of amino acids in the presence of Na⁺ and K⁺, and an overshoot was observed. Vesicles reconstituted by the method of Bracy *et al.* (1987) exhibited Na⁺-dependent AIB transport, but no overshoot, and the rate of Na⁺-dependent transport was linear over a 10 min time interval. Their vesicles were

Fig. 4. Amino acid uptake by vesicles reconstituted from liver plasma membranes fractionated on concanavalin A-agarose

Liver plasma-membrane vesicles were solubilized in MEGA-10 and subjected to concanavalin A-agarose affinity chromatography as described in the Materials and methods section. The protein fraction bound to the column was eluted with 0.15 M-D-mannose/20 mM-Tris/HCl/0.5% MEGA-10, pH 7.4. After concentration to approx. 1 mg/ml, the protein fraction was reconstituted with phospholipids and the uptake of alanine (0.1 mM) or glutamine (0.1 mM) was measured. (a) Alanine; (b) glutamine; $\blacktriangle$, in the presence of NaCNS; $\triangle$, in the presence of KCNS. The apparent binding at zero time was subtracted as in Fig. 3. The data shown are representative of results obtained in five such fractionation experiments.

Fig. 5. Separation of liver plasma-membrane proteins by lectin-affinity chromatography

Portions (0.1 mg) of solubilized liver plasma-membrane vesicles and of the protein fraction obtained by concanavalin A-affinity chromatography in Fig. 4 were subjected to SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). After staining with Coomassie Blue, the gel was scanned. Molecular-mass markers (Sigma) were run on a parallel track. (a) Liver plasma-membrane vesicles; (b) protein fraction that contains alanine-transport activity eluted from the concanavalin A column with mannose.

presumably much larger than those obtained by the present method, since the extent of amino acid uptake was considerably greater. Only AIB transport was measured by Bracy *et al.* (1987), and it is therefore not known whether their method achieved the reconstitution of transport systems other than System A. The procedure described here is more rapid and can be completed in 3–4 h.

The reconstitution method presented here employs the detergent MEGA-10. This detergent has a number of particular advantages for membrane-protein fractionation studies (see also Hildreth, 1982). It is uncharged, thus facilitating protein separation based on ion-exchange chromatography, it does not absorb light at 280 nm and it is readily removed from proteins by gel filtration. The ease and rapidity of this procedure makes it particularly suitable as an assay system for amino acid transport proteins.

As an example of the potential application of this method, it was shown that alanine-transport activity, but not glutamine-transport activity, could be reconstituted from the protein fraction absorbed on concanavalin A affinity columns. This finding provides the first direct evidence that System A is a glycoprotein, and is consistent with the earlier finding of Barber *et al.* (1983) that induction of AIB transport in cultured hepatocytes is inhibited by tunicamycin, an inhibitor of asparagine-linked glycoprotein synthesis. In contrast, System N activity does not bind to concanavalin A, providing further evidence that the transport activities termed System A and System N are catalysed by different proteins. The finding that System A activity binds to concanavalin A should open the way to the eventual identification of this transport protein and to the elucidation of the mechanism of its long-term regulation.

In summary, the reconstitution procedure described here is a useful method which is complementary to that of Bracy *et al.* (1987) and should be of particular application in approaches to carrier-protein identification based on protein fractionation.

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