# SOLUBILIZATION AND RECONSTITUTION OF THE L-GLUTAMIC ACID TRANSPORTER FROM RAT BRAIN

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## 1. Introduction

High affinity $Na^+$-dependent uptake systems for a variety of established and putative neurotransmitters have been detected in brain preparations such as synaptosomes [1–4]. These systems have been implicated in the termination of transmitter action on postsynaptic receptors [1], as well as in maintaining constant levels of transmitters in the neurons [5]. Various observations have led to the proposal that ion gradients, generated primarily by devices such as the $(Na^+ + K^+)$-ATPase are the immediate driving force for neurotransmitter accumulation [6–8]. Recently this idea has been supported more directly using membrane vesicles [9,10]. Surprisingly, these studies also have revealed that in addition to $Na^+$ dependence, other ions are required. Thus, $\gamma$-aminobutyric acid (GABA) translocation is dependent on the presence of external small monovalent anions of which $Cl^-$ is the most effective [9], while translocation of L-glutamic acid requires the presence of internal $K^+$ [10]. Moreover, the gradients of these additional ions, $Cl^-$ (out > in) and $K^+$ (in > out) serve as driving forces, for GABA or L-glutamic acid accumulation, respectively, in addition to the $Na^+$ gradient (out > in) [9,10]. The dependence on these gradients cannot be explained by the mere electrogenicity of the processes [9,10].

Direct flux experiments are required to determine if these ions in fact are translocated coupled obligatorily to the translocation of the neurotransmitters. These experiments will probably be successful only in reconstituted proteoliposomes, containing the highly purified transporters, which are expected to have a low ion permeability.

Recently the solubilisation of the GABA transporter with the non-ionic detergent Triton X-100 and its functional incorporation into liposomes has been described [11]. GABA transport in this crude reconstituted system displays all the features observed in the native system [11]. However, the solubilisation and reconstitution of the L-glutamic acid transporter by this method was unsuccessful (B.I.K., I.S., unpublished experiments). This report describes the solubilisation of the L-glutamic acid transporter with the ionic detergent cholate and its functional incorporation into liposomes. The transport of L-glutamic acid catalysed by these proteoliposomes is very similar in its ion dependency and energisation to that observed with the native membrane vesicles.

## 2. Materials and methods

Cholic acid from Schwarz/Mann was recrystalised from 70% ethanol [12] and soybean phospholipids (asolectin, Associated Concentrates) were partially purified [12]. L-[G-$^3$H]glutamic acid was from Amersham. Valinomycin was from Sigma. Nigericin was a generous gift from Dr R. J. Hosley, Eli Lilly.

Isolated membrane vesicles were prepared by osmotic lysis of synaptosomes, derived from rat brains [9]. Aliquots were stored in liquid air.

For solubilisation of the L-glutamic acid carrier, these stored membrane vesicles were used and all subsequent steps were performed at 0–4°C. Membrane vesicles (15–20 mg protein) were incubated at 12–15 mg/ml with 0.26 M sucrose, 8 mM Tris–sulfate (pH 7.4), 0.4 mM EDTA, 0.8 mM $MgSO_4$,

1 mM dithiothreitol, 10% saturated ammonium sulfate and 2% cholic acid neutralised with NaOH (last addition). The mixture then was centrifuged in a Beckman 50 Ti rotor for 60 min at 46 000 rev./min. The supernatant was carefully removed with a pasteur pipette and was used for reconstitution experiments.

Reconstitution was done using the cholate dialysis procedure [12]. Soybean phospholipids were dried under a stream of nitrogen and suspended, using a bath-type sonicator, at 30 μmol/ml in dialysis buffer (see below). This phospholipid suspension, 18–20 μmol/ml was incubated with the solubilised protein, 1–2 mg/ml, and with 0.8% cholic acid neutralised with NaOH (last addition). This mixture was dialysed for 18–20 h against 200–300 vol. dialysis buffer containing either 120 mM potassium phosphate, pH 6.8 (unless indicated otherwise, see fig.4 legend), 0.32 M sucrose, 10 mM Tris–sulfate (pH 7.4), 0.5 mM EDTA, 1 mM $MgSO_4$, 1 mM dithiothreitol and 1% glycerol. The proteoliposomes were used for the transport experiments within 8 h of dialysis termination.

Transport of L-glutamic acid was measured by adding 30–40 μl proteoliposomes to 0.36 ml incubation mixture containing (unless indicated otherwise in the figure legends) 0.15 M NaCl, 1% (v/v) glycerol, 2 mM $MgSO_4$, 0.1 μM L-[G-$^3$H]glutamic acid at 28 Ci/mmol, and the additions indicated in the figure legends. The reactions were carried out at room temperature (21–24°C). After termination, filtration and washing [11], the retained radioactivity was determined using liquid scintillation spectrometry. Experimental values were up to 10–20-fold the 0-time values. Unless indicated otherwise, inhibition of transport by nigericin was always more than 90%. Protein was assayed by the Lowry method [13].

## 3. Results and discussion

A time-dependent L-glutamic acid uptake is observed when proteoliposomes, formed in a potassium phosphate solution, are diluted into 0.15 M NaCl in the presence of valinomycin (fig.1). Under these conditions artificial gradients of ions such as $Na^+$ (out > in) and $K^+$ (in > out) are generated. The uptake of L-glutamic acid, both with regard to initial rate and extent, is somewhat lower, but in the same order of magnitude (15–35%) of that under similar conditions in the native system [10]. The optimal reconstitution of the L-glutamic acid system requires both exogeneously-added phospholipids as well as the solubilised protein fraction. No significant uptake was detected with either of these two components alone (fig.1).

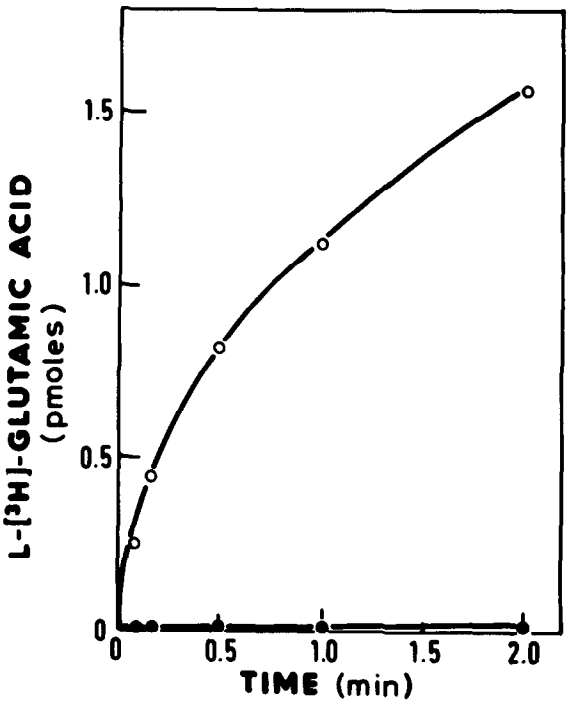


Fig.1. Dependence of reconstituted L-glutamic acid transport activity on phospholipids and solubilised protein. Reconstitution was performed as in section 2 using 18.6 μmol soybean phospholipids/ml and 1.25 mg protein/ml (o–o) or either component alone (•–•). For transport assays, done in the presence of 2.5 M valinomycin, 30-μl aliquots of the dialysed mixtures were used.

The reconstituted L-glutamic acid uptake is also similar to the native system with respect to stereospecificity. The uptake of L-glutamic acid radioactivity is inhibited by addition of an excess of L-glutamic acid but not by the addition of the D-isomer (fig.2). This indicates that L-glutamic acid is the preferential substrate in the reconstituted system.

When the cation gradients artificially imposed across the proteoliposomes are abolished using the ionophore nigericin, an almost complete inhibition of L-glutamic

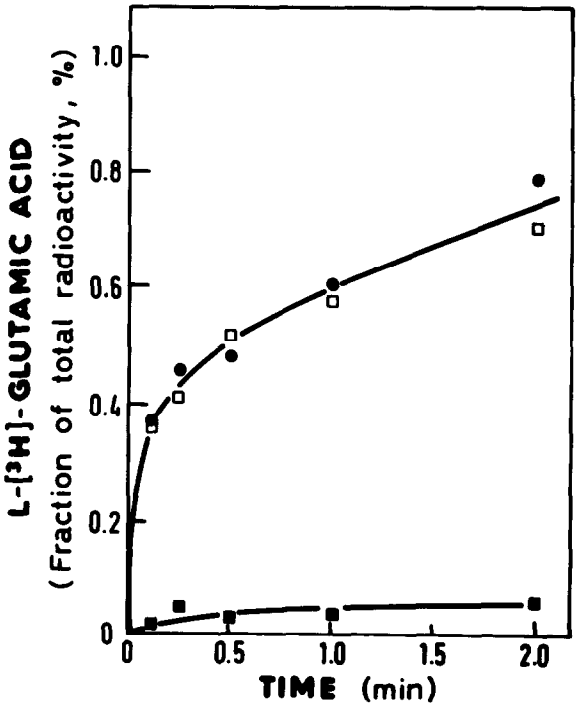


Fig.2. Stereospecificity of reconstituted glutamic acid transport. Reconstituted vesicles, 42.3 μg protein, were assayed for transport as in section 2 except that the reaction contained 3.5 μM L-[G-$^3$H]glutamic acid, 1.58 Ci/mmol. Additions of unlabelled glutamic acid: none (□–□); 50 μM L-glutamic acid (■–■); 50 μM D-glutamic acid (●–●).

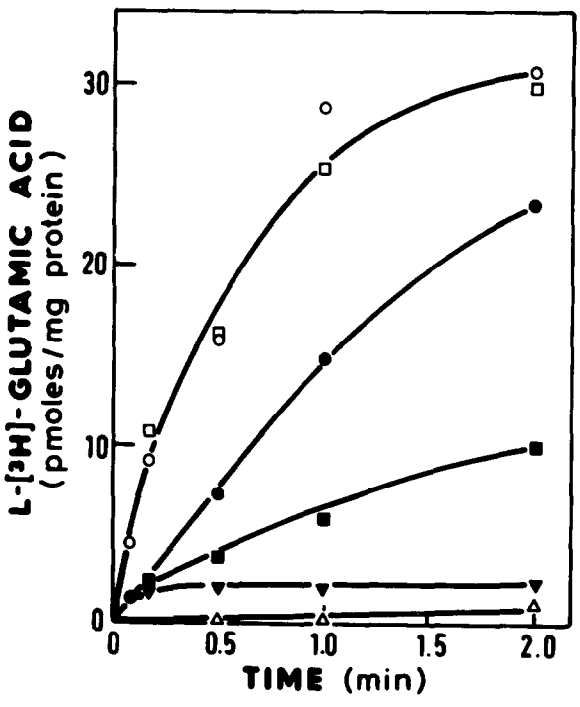


Fig.3. The effect of ionophores and external ions on reconstituted L-glutamic acid transport activity. Reconstituted vesicles, 42.3 μg protein, were assayed for transport as in section 2. The reaction medium contained 1% (v/v) glycerol, 2 mM $MgSO_4$, 0.1 μM L-[G-$^3$H]glutamic acid and the following additions: 150 mM NaCl + 2.5 μM valinomycin (○–○); 150 mM NaCl (●–●); 150 mM NaCl + 2.5 μM valinomycin + 5 μM nigericin (△–△); 100 mM sodium phosphate (pH 6.8) + 50 mM sucrose + 2.5 μM valinomycin (□–□); 100 mM sodium phosphate (pH 6.8) + 50 mM sucrose (■–■); 150 mM LiCl (▼–▼).

acid transport is observed (fig.3). Moreover, the addition of the ionophore valinomycin stimulates the initial rate of transport about 4-fold. Thus, the electrogenicity of L-glutamic acid transport [10] is preserved in the reconstituted system. Although the effects of nigericin and valinomycin are similar to those observed in the native system [10] the stimulation by the latter ionophore is considerably larger in the reconstituted system. This indicates that the passive permeability for potassium ions is lower in this system. It is likely that this is due to the excess of phospholipids added. The inhibition of L-glutamic acid transport by nigericin in NaCl containing media which is also observed without valinomycin (data not shown) is usually $> 95\%$. However, upon standing of the proteoliposomes for several days, an apparent increase in transport activity was observed, but this increase was found to be nigericin resistant. Therefore the activity of the proteoliposomes was always assayed within 8 h of dialysis termination.

The remarkable ion specificity of the L-glutamic acid transport system [10] is also preserved upon solubilisation and reconstitution. With regard to external ions, the system is absolutely dependent on $Na^+$; when external NaCl is replaced by LiCl no L-glutamic acid transport is detected (fig.3). The low level of radioactivity observed in the LiCl medium was resistant to nigericin. With external KCl results were obtained similar to those with LiCl (data not shown). The external anion is not crucial for L-glutamic acid transport in native [10] and reconstituted systems, since in the presence of external sodium

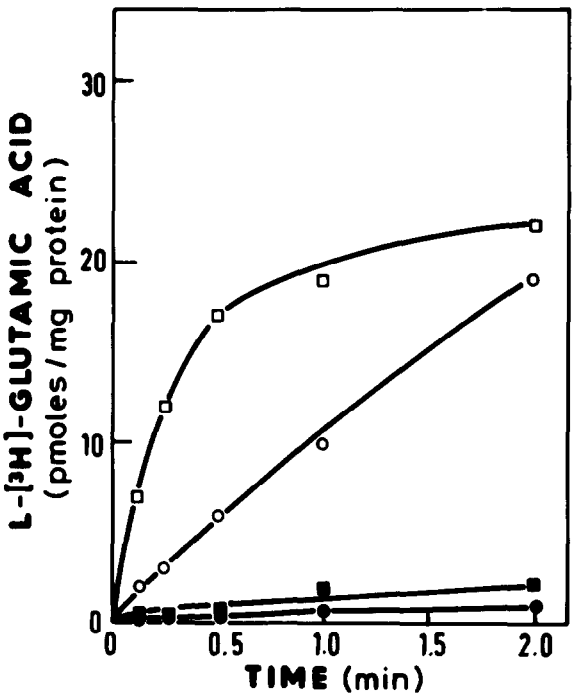


Fig.4. The effect of internal $K^+$ on reconstituted L-glutamic acid transport activity. Proteoliposomes were reconstituted as in section 2 (K-loaded vesicles) or with 120 mM Tris–phosphate in the dialysis medium (pH 6.8), instead of potassium phoshate (Tris-loaded vesicles) and assayed for transport (37.5 μg protein each) in either 150 mM NaCl or 150 mM NaSCN containing media as indicated. K-loaded vesicles: 150 mM NaCl (○–○); 150 mM NaSCN (□–□). Tris-loaded vesicles: 150 mM NaCl (●–●); 150 mM NaSCN (■–■).

phosphate still considerable transport is observed (fig.3), in contrast to the GABA system [9,11]. The lower initial rates in the phosphate containing medium apparently reflect the lower membrane permeability of this anion than that of $Cl^-$. The relative stimulation by valinomycin in the phosphate containing medium is larger than in the $Cl^-$ medium (fig.3), suggesting that in the absence of valinomycin glutamic acid transport is limited by the permeability of the external anion. The faster initial rate in the presence of the highly permeable thiocyanate ion than in the presence of $Cl^-$ (fig.4) supports this contention. With regard to internal ions, the absolute dependency of L-glutamic acid transport on potassium persists upon reconstitution. With internal Tris–phosphate no L-glutamic acid transport at all was detected, even in the presence of external sodium thiocyanate (fig.4). Thus it is highly unlikely (as well as in the native system) that the dependency on internal $K^+$ is merely due to satisfy the requirement for a membrane potential (interior negative).

The reconstituted L-glutamic acid system described here thus appears to be similar to that observed in isolated membrane vesicles [10] with respect to ion specificity, electrogenicity and stereospecificity. This reconstitution assay will be useful as a functional assay for the L-glutamic acid carrier during its purification. Similarly a functional assay has been developed for the GABA system [11]. The use of detergents during the purification of some neurotransmitter transport systems, however, has to be carefully examined, since Triton X-100 inactivates the L-glutamic acid system, but not the GABA system.

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