# Astrocytic glucose-6-phosphatase and the permeability of brain microsomes to glucose 6-phosphate

Robert J. FORSYTH,*‡ Kim BARTLETT,* Ann BURCHELL,† Hazel M. SCOTT† and Janet A. EYRE*

*Department of Child Health, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, and †Department of Obstetrics and Gynaecology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, U.K.

Cells from primary rat astrocyte cultures express a 36.5 kDa protein that cross-reacts with polyclonal antibodies to the catalytic subunit of rat hepatic glucose-6-phosphatase on Western blotting. Glucose-6-phosphate-hydrolysing activity of the order of 10 nmol/min per mg of total cellular protein can be demonstrated in cell homogenates. This activity shows latency, and is localized to the microsomal fraction. Kinetic analysis shows a $K_m$ of 15 mM and a $V_{max.}$ of 30 nmol/min per mg of microsomal protein in disrupted microsomes. Approx. 40% of the total phosphohydrolase activity is specific glucose-6-phosphatase, as judged by sensitivity to exposure to pH 5 at 37 °C. Previous reports that the brain microsomal glucose-6-phosphatase system does not distinguish glucose 6-phosphate and mannose 6-phosphate are confirmed in astrocyte microsomes. However, we demonstrate significant phosphomannose isomerase activity in brain microsomes, allowing for ready interconversion between mannose 6-phosphate and glucose 6-phosphate ($V_{max.}$ 15 nmol/min per mg of microsomal protein; apparent $K_m$ < 1 mM; pH optimum 5–6 for the two-step conversion). This finding invalidates the past inference from the failure of brain microsomes to distinguish mannose 6-phosphate and glucose 6-phosphate that the cerebral glucose-6-phosphatase system lacks a 'glucose 6-phosphate translocase' [Fishman and Karnovsky (1986) J. Neurochem. **46**, 371–378]. Furthermore, light-scattering experiments confirm that a proportion of whole brain microsomes is readily permeable to glucose 6-phosphate.

## INTRODUCTION

Astrocytes comprise approx. 25% of the cells of the central nervous system [1] and have several neuronal support functions, including spatial buffering of $K^+$ ions, anapleurotic neurotransmitter recycling [2,3] and release of neurotrophic factors [4]. They also contain the large majority of the cerebral stores of glycogen [5], of the order of 50 nmol/mg of protein in size, although there is considerable regional variation [6]. These stores exhibit high turnover. Stored glycogen can also be demonstrated in cultured astrocytes [7].

Glycogenolysis occurs *in vitro* in both cultured astrocytes and brain slices on exposure to noradrenaline, $K^+$ and vasoactive intestinal peptide (VIP) [8–11]; however, the fate of the glycogen remains unclear.

The demonstration of significant glucose-6-phosphatase (G6Pase; EC 3.1.3.9) activity in a glycogen-containing cell has implications for the likely fate of glucosyl units after glycogenolysis. Although various workers have reported histochemical studies of G6Pase expression within brain [12,13], the non-specific nature of the histochemical techniques used has resulted in a confused picture of its extent and localization. However, recent immunohistochemical studies in human brain using specific polyclonal antibodies to the catalytic subunit of hepatic G6Pase confirm expression of this protein, and indicate an exclusively astrocytic localization, although not all astrocytes express G6Pase [14].

The present paper is a study of the immunological and biochemical properties of G6Pase in cultured astrocytes and brain microsomes.

## EXPERIMENTAL

### Materials

Animals used were Wistar rat newborns and adults from the University of Newcastle upon Tyne breeding colony. All components of the culture medium were obtained from GIBCO (Paisley, U.K.). Culture flasks were from Nunc (Roskilde, Denmark). Histone (type II-AS), glucose 6-phosphate (G6P; monosodium and dipotassium salts) and mannose 6-phosphate (M6P; monosodium salt) were supplied by Sigma (Poole, Dorset, U.K.). [U-$^{14}$C]G6P, [U-$^{14}$C]glucose and [U-$^{14}$C]mannose were from Amersham International (Amersham, Bucks., U.K.). Yeast hexokinase and G6P dehydrogenase were from Boehringer Mannheim (Lewes, Sussex, U.K.). All other reagents were of analytical grade.

### Astrocyte culture

Primary cultures of rat brain homogenates were prepared by the method of McCarthy and de Vellis [15], with a culture medium comprising Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 10% (v/v) foetal-calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin, Eagle's MEM non-essential amino acids and vitamins. All experiments were performed using confluent unpassaged cultures (approx. 7–10 days age). Characterization of mature cultures by fluorescence-activated cell sorting analysis of glial fibrillary acidic protein expression (GFAP; a specific astrocytic cytoskeletal protein [16,17]) confirmed that virtually all cells were GFAP-expressing astrocytes, in confirmation of previous reports [17].

Abbreviations used: ER, endoplasmic reticulum; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase (EC 3.1.3.9); M6P, mannose 6-phosphate; PGI, phosphoglucose isomerase (EC 5.3.1.9); PMI, phosphomannose isomerase (EC 5.3.1.8); VIP, vasoactive intestinal peptide; [$^3$H]DIDS, 4,4′-di-isothiocyano-1,2-diphenyl[$^3$H]ethane-2,2′-disulphonic acid.

‡ To whom correspondence should be addressed.

### Microsome preparation from cultured astrocytes

All procedures were performed at 4 °C. Medium was decanted from confluent astrocyte cultures (total six 80 $cm^2$ flasks) and the cells were washed in ice-cold medium A (0.25 M sucrose/5 mM Hepes, pH 6.5). Cells were harvested with a mechanical scraper, resuspended in 0.5 ml of medium A and homogenized with 10 strokes of a tight-fitting Dounce tissue homogenizer. The homogenate was subjected to a low-speed centrifugation, and the pellet rehomogenized to increase yield. Homogenates were pooled and centrifuged at 10000 *g* in a Beckmann Optima TLX refrigerated centrifuge (TLA 120.2 rotor, $r_{av.}$ 31.8 mm) for 6 min. The supernatant was centrifuged at 200000 *g* for 6 min.

Transmission electron microscopy of a pellet showed a uniform population of membrane vesicles and ribosomes. No mitochondrial contamination was observed.

### Microsome preparation from whole brain

Adult male Wistar rats (180–230 g) were killed by cervical dislocation, and the brains were rapidly dissected and homogenized in 5 vol. (v/w) of ice-cold medium A by using five passes of a Potter–Elvehjem Teflon/glass homogenizer. In the light-scattering experiments (see below), medium A was replaced by a homogenization medium of 0.32 M sucrose/20 mM Hepes, pH 7.2. The homogenate was centrifuged at 10000 *g* in a Sorvall RC5B centrifuge (SS34 rotor, $r_{av.}$ 82.6 mm) for 15 min and the supernatant was centrifuged at 100000 *g* for 70 min in a Sorvall OTD 55B centrifuge (TFT 80-13 rotor; $r_{av.}$ 64.2 mm).

### Microsome disruption

Histone was used to disrupt microsomes functionally without the inhibition of G6Pase reported in liver if high concentrations of detergents are used [18]. In preliminary experiments, addition of histone to a final concentration of 0.7 mg/ml revealed an additional latent G6Pase activity of approx. 100% that of untreated astrocytic microsomes. Pretreatment of astrocytic microsomes with sodium deoxycholate concentrations of 0.015–0.3% (w/v) (0.225–4.5 $\mu$g of deoxycholate/$\mu$g of microsomal protein) before exposure to 0.7 mg/ml histone resulted in progressive inhibition of the histone effect, decreasing the magnitude of the latent activity revealed to 46% or less of the untreated microsomal activity over this range of deoxycholate concentrations (results not shown).

### [U-$^{14}$C]M6P and [U-$^{14}$C]G6P synthesis

[U-$^{14}$C]M6P (monosodium salt) was synthesized from [U-$^{14}$C]-mannose. For this, 2 GBq of [U-$^{14}$C]mannose (200 nmol; 10 MBq/nmol) was incubated in 5 ml of 100 mM glycylglycine buffer, pH 8.0, containing 2 $\mu$mol of ATP, 2 $\mu$mol of $MgCl_2$ and 20 units of hexokinase for 60 min at room temperature. The reaction mixture was applied to a Dowex 1 (formate) column, the column was eluted with a formic acid gradient (0.3–1 M) and fractions were collected. Some 5% of the original radioactivity was eluted in the earliest fractions (unconverted [U-$^{14}$C]-mannose), the remainder being eluted as a well-defined peak of [U-$^{14}$C]M6P in the middle fractions. Any $P_i$ present was eluted immediately after the M6P, and thus contamination with $P_i$ could be minimized. The appropriate fractions were pooled, freeze-dried, and redissolved in 240 mM unlabelled M6P, pH 6.8, to a final specific radioactivity of 5 Bq/nmol.

Initially [U-$^{14}$C]G6P was available from Amersham. In later experiments, [U-$^{14}$C]G6P was synthesized in an identical manner from [U-$^{14}$C]glucose.

### G6Pase assay

A radiochemical assay was used. The assay system comprised (final concns.): 10 mM sodium cacodylate buffer [recrystallized from 90% (v/v) ethanol], 2 mM EDTA, pH 6.8, and [U-$^{14}$C]-G6P or -M6P (monosodium salts; working sp. radioactivity 5 Bq/nmol; pH 6.8) at substrate concentrations of 2, 5, 10, 20 and 80 mM. When appropriate, histone was added to a final concentration of 0.7 mg/ml.

Incubations were started by the addition of 20 $\mu$l of microsomal suspension (approx. 2 mg of microsomal protein/ml) to 80 $\mu$l of assay mixture in a 1.5 ml conical plastic tube pre-equilibrated to 30 °C in a water bath, and stopped by addition of 50 $\mu$l of 25% (v/v) $HClO_4$, followed by neutralization with 100 $\mu$l of 2 M $KHCO_3$. Samples incubated at 30 °C with $HClO_4$ added before the microsomal sample were used as blanks.

After neutralization, samples were centrifuged and 200 $\mu$l samples of the supernatant were applied to Dowex formate columns (3 cm × 0.5 cm diameter). The columns were eluted with 2 ml of water, and the eluates were collected in scintillation vials. Radioactivity was determined by liquid-scintillation counting using the external-standard channels-ratio method.

The reaction was linear to 30 min at a substrate concentration of 1 mM, but linear to 60 min at 30 mM. Assay times were accordingly limited to 15 min. The assay was linear with respect to protein (by the Lowry method [19],with BSA as standard) to at least 6 mg of microsomal protein/ml.

### pH 5 exposure

The effect of pre-exposure to pH 5 at 37 °C was used as a measure of specific G6Pase activity [20]. Astrocytic microsomal pellets were resuspended in 10 mM sodium acetate buffer at pH values from 4.8 to 5.2 and incubated at 37 °C for 10 min. The pH was then returned to pH 6.5 by addition of 0.4 M K-Hepes, and samples were assayed for G6Pase activity at a substrate concentration of 20 mM G6P in the presence of histone.

### Phosphomannose isomerase (PMI)/phosphoglucose isomerase (PGI) assay

The ability of whole brain microsomes to catalyse the two-step process

$$\text{M6P} \xrightarrow{\text{PMI}} \text{fructose 6-phosphate (F6P)} \xrightarrow{\text{PGI}} \text{G6P}$$

was assessed by incubating 10 $\mu$l samples of whole brain microsome suspension (approx. 5 mg of protein/ml) in 40 $\mu$l of 60 mM sodium acetate buffer, pH 5.5, containing M6P at appropriate concentrations. For pH-optimum determinations, the sodium acetate buffer was replaced with 60 mM sodium citrate/HCl (pH 4.5), imidazole/HCl (pH 6.5), triethanolamine/HCl (pH 7.5) and Tris/HCl (pH 8.5).

Incubations at 30 °C were terminated with $HClO_4$ and $KHCO_3$ as above, and supernatants were assayed for the presence of G6P by using an automated G6P dehydrogenase-based assay monitoring the production of NADPH fluorimetrically on a Cobas Bio centrifugal analyser [21].

### Immunoblot analysis

Polyclonal sheep anti-(rat G6Pase) antibodies were prepared as described by Countaway et al. [22]. IgG was further purified by

$(NH_4)_2SO_4$ fractionation [23]. SDS/PAGE was carried out in 7–16%-acrylamide gels [24] and proteins separated on SDS/polyacrylamide gels were transferred electrophoretically to nitrocellulose [25]. The Western blot was immunostained with sheep anti-(rat G6Pase) IgG, and immunoreactive polypeptides were detected by using a biotin–streptavidin–peroxidase-linked detection system with 4-chloro-1-naphthol as substrate [26].

Rat lung and muscle homogenates were used as negative controls on immunoblots, as they have previously been shown not to contain the G6Pase enzyme [20].

### Light-scattering experiments

The scattering of light by dilute suspensions of membrane vesicles increases as average vesicle size falls [27], and this property has been used to study the permeability of sarcoplasmic-reticulum and endoplasmic-reticulum (ER) vesicles to various solutes [28]. A dilute suspension of vesicles is allowed to equilibrate in a hypo-osmotic buffer while the degree of light-scattering is monitored. A concentrated solution of the solute of interest is added, and the osmotically induced loss of water from the vesicles results in shrinkage, causing a rapid rise in the light-scattering signal. If the vesicles are permeable to the solute, solute and water then re-enter the vesicles, which re-expand, and the amount of light scattered slowly falls again. The rate of this decline gives a measure of the vesicles' permeability to the solute in question.

The method of Fulceri et al. [29] to assess liver microsomal permeability to G6P was adapted to whole brain microsomes. Brains were homogenized in 0.32 M sucrose/20 mM Hepes, pH 7.2, and microsomes were prepared as above. The pellet was resuspended (50 mg of protein/ml) in a cytosol-like buffer of composition KCl 100 mM, NaCl 20 mM, $MgCl_2$ 1 mM, Mops 20 mM, pH 7.2, and then diluted 600-fold into 5 mM K-Pipes, pH 7.0, at 25 °C. Microsomal preparations were used within 3 h. A 3 ml portion of this dilute suspension was placed in a temperature-controlled (25 °C) cuvette in a Perkin–Elmer LS-3 fluorimeter, and stirred continuously. Light-scattering at 90° to the incident light path was monitored on a chart recorder at a wavelength of 400 nm: pieces of fine metal mesh were placed in the incident light path to attenuate the incident light and prevent signal overload. Once a stable baseline had been obtained, 0.3 ml samples of the test solutes (0.5 M KCl, 0.75 M sucrose and 0.75 M G6P dipotassium salt, all in 5 mM K-Pipes and adjusted to pH 7.0) were injected through the compartment lid under continuous stirring.

Chart-recorder output was scanned on a flat-bed scanner and the trace converted into a text file of absolute $x,y$ co-ordinates, given the known horizontal and vertical scales, by using the DataThief program [30].

### Numerical methods

Estimates of kinetic parameters ($K_m$ and $V_{max.}$) were obtained by fitting a Michaelis–Menten hyperbola directly to untransformed kinetic data by using unweighted iterative non-linear regression (Marquardt–Levenberg algorithm [31]) implemented on a Macintosh computer (Kaleidagraph, Abelbeck software).

The same algorithm was used to fit exponential decay curves of the form $y = a + be^{-ct}$ ($a$, $b$ and $c$ to be determined) to the data derived from the light-scattering traces and allow estimates of the half-life ($= \log_e 2/c$) of light-scattering decays.

All data are expressed as mean ± S.D. unless otherwise stated. All tests of statistical significance were performed on untransformed data by using the unpaired two-tailed Student's $t$ test.

## RESULTS

### Protein expression

Cultured astrocytes express a protein of the same molecular mass (36.5 kDa) as the catalytic subunit of hepatic G6Pase. Figure 1 shows a Western blot of cultured astrocytes, and positive (liver) and negative (muscle, lung) controls.

### Kinetic characterization and subcellular localization

The results of a typical kinetic study with G6P as substrate are shown in Figure 2. Maximal rates are of the order of 30 nmol/min per mg of microsomal protein. The data also demonstrate that the activity exhibits latency, with an increase in activity in histone-treated microsomes of approx. 40% above untreated microsomes over the substrate concentration range 5–20 mM.

Estimates for $K_m$ and $V_{max.}$ for both treated and untreated microsomes with either M6P or G6P as substrate are shown in Table 1. The $K_m$ values show a significant fall with histone treatment for both substrates (G6P, $P < 0.02$; M6P, $P < 0.002$). However, there is no evidence of an ability of either untreated or disrupted microsomes to distinguish between G6P and M6P as substrates, as judged by a lack of significant differences between the estimated $K_m$ and $V_{max.}$ values ($P > 0.2$ in all instances).

The subcellular localization of the activity was determined by assaying a whole-cell homogenate of cultured astrocytes and preparing microsomes from a sample as detailed above, with the exception that the final 200000 $g$ centrifugation was prolonged

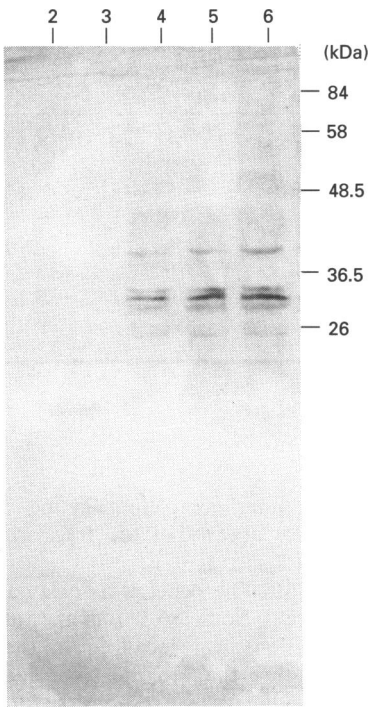


**Figure 1 Immunoblot analysis of G6Pase catalytic-subunit expression in astrocytes**

Tissue samples were electrophoresed on a 7–16%-polyacrylamide gel before electrophoretic transfer to nitrocellulose, which was immunoblotted with an antibody to the catalytic subunit of hepatic G6Pase. Lanes: 2, control rat muscle homogenate (40 $\mu$g); 3, rat lung homogenate (40 $\mu$g); 4, rat liver microsomes (3 $\mu$g); 5 and 6, cultured astrocyte microsomes (28 and 32 $\mu$g respectively). Prestained molecular-mass standards (Sigma) were of 26, 36, 48, 58, 84, 116 and 180 kDa.

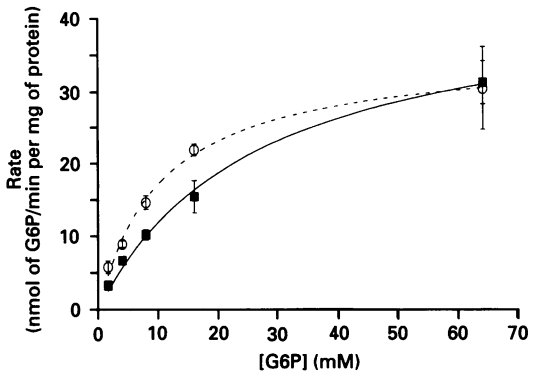


**Figure 2 Kinetic properties of G6Pase expression in cultured astrocytes**

The Figure shows the G6Pase activity of untreated (■) and histone-disrupted (○) microsomes. Points are means ± range of duplicate assays minus duplicate blanks at each point. Curves represent best-fit Michaelis–Menten equations fitted to each data set (see the text).

**Table 1 Summary of estimates of kinetic parameters ($K_m$ and $V_{max.}$) for the handling of G6P and M6P by untreated or histone-disrupted astrocytic microsomes**

Data are expressed as means ± S.D., with numbers of measurements in parentheses. Note that in some microsome preparations the lack of a reliable estimate of protein content meant that a $K_m$, but not a $V_{max.}$, could be calculated.

| Substrate | Microsomes | $K_m$ (mM) | $V_{max.}$ (nmol/min per mg of protein) |
|---|---|---|---|
| G6P | Untreated | 27.7 ± 6.9 (5) | 30 ± 6 (4) |
| | Disrupted | 15.4 ± 4.8 (4) | 27 ± 6 (4) |
| M6P | Untreated | 25.9 ± 6.0 (4) | 33 ± 7 (3) |
| | Disrupted | 10.6 ± 3.1 (5) | 34 ± 12 (3) |

to 20 min to ensure more complete pelleting of membrane fragments. The G6Pase activity of the fractions under $V_{max.}$ conditions is shown in Table 2.

The activity in the 10000 *g* pellet is associated with incompletely homogenized cells. Some 86.5% of the total activity of the post-10000 *g* fractions was localized to the microsomal membrane fraction rather than to the soluble post-200000 *g* fraction, suggesting that the enzyme responsible for the measured activity is membrane-bound rather than cytosolic.

### Effects of pH 5 exposure

Acid preincubation of astrocytic microsomes as described in the Experimental section was associated with a fall in G6Pase activity to 60% of control values when a threshold of pH 5 was crossed (Figure 3). In comparison, incubation of rat liver microsomes at

**Table 2 Distribution of G6Pase activity within subcellular fractions**

Fractions were prepared from a sample of the whole cell homogenate as described in the Experimental section, except that the second-stage 200000 *g* centrifugation was prolonged for 20 min. Pellets were resuspended in known volumes of homogenization medium and assayed for activity at a substrate concentration of 80 mM over a period of 10 min. *Specific activities of fractions are given as means ± S.D. of quadruplicate estimations. Activities were expressed as a percentage of the measured activity of the whole cell homogenate (note that totals in final column do not equal 100%).

| Fraction | Specific activity (nmol/min per mg of protein) | Proportion of total activity (%) |
|---|---|---|
| Whole cell homogenate | 7.9 ± 0.3 | 100* |
| 10000 *g* pellet | 7.3 ± 0.3 | 42 |
| 200000 *g* pellet | 10.7 ± 0.5 | 44 |
| Post-200000 *g* supernatant | 1.5 ± 0.5 | 7 |

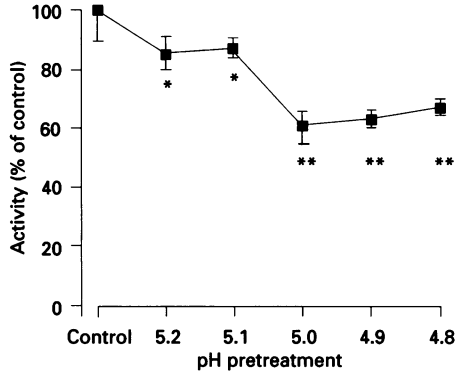


**Figure 3 Effects of pH 5 exposure**

Effect of pre-exposure of astrocyte microsomes to 10 mM sodium acetate buffer at pH 4.8–5.2 for 10 min at 37 °C, expressed as a percentage of control G6Pase activity at a substrate concentration of 20 mM G6P in the presence of histone. Points are means ± S.D. for quadruplicate estimations at each point. Significance of difference from control activity as shown by asterisks: $*P < 0.1$; $**P < 0.001$.

pH 5 for 10 min at 37 °C resulted in a fall in activity to 7.3% of control values. Sensitivity to pH 5 exposure is used to distinguish specific microsomal G6Pase from non-specific phosphatase activity (see Burchell and Waddell [20] for a recent review). By this criterion, 40% of the total measured phosphatase activity in cultured astrocytes is due to specific G6Pase.

### PMI/PGI activity

Whole brain microsomes can convert M6P into G6P, presumably as a two-step process via F6P by the sequential actions of PMI and PGI. This two-step conversion has an apparent $K_m$ of < 1 mM M6P and a $V_{max.}$ of the order of 15 nmol/min per mg of microsomal protein (Figure 4a) and a broad pH maximum at pH 5.5–6.5 (Figure 4b).

### Permeability of brain microsomes to G6P

Independent assurance of the intactness of a microsomal preparation is necessary before meaningful conclusions about vesicle uptake functions can be made. Because of our demonstration of significant PMI activity in brain, the latency of the low-affinity

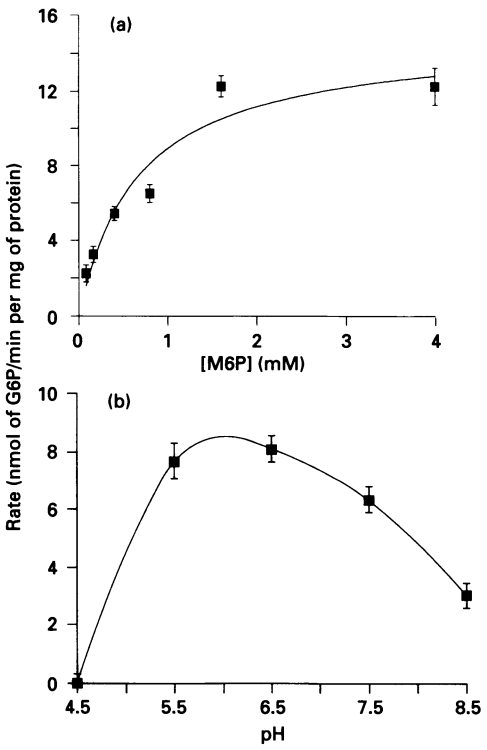


**Figure 4 Conversion of M6P into G6P by brain microsomes**

(**a**) Dependence of the overall activity of the two-step conversion of M6P to G6P via F6P on substrate concentration at pH 5.5. Points are means ± S.D. of eight measurements at each substrate concentration in a single experiment. The curve represents the best-fit Michaelis–Menten curve: apparent $K_m$ = 0.7 mM; apparent $V_{max.}$ = 15 nmol/min per mg of protein. The experiment was repeated once with similar results. (**b**) Effect of pH on rate at a substrate concentration of 4 mM. Points are means ± S.D. of 7–8 measurements at each point in a single experiment.

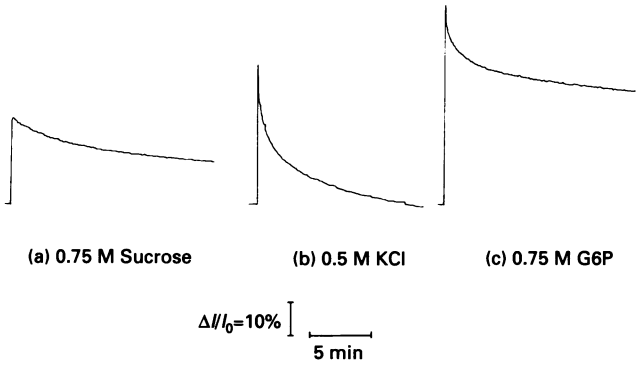


**Figure 5 Light-scattering by whole brain microsomes**

The degree of light-scattering demonstrated by whole brain microsomes reflecting osmotically induced changes in vesicle size on addition of various solutes. All traces are to the same scale: the horizontal and vertical scale bars represent 5 min and 10% of baseline signal, respectively. The concentrations shown adjacent to each panel represent the concentration of the solute added: the final concentration of solute in the cuvette is 0.1 times this value (e.g. 50 mM KCl). Typical traces from one microsome preparation shown. This was repeated in four experiments with similar results.

G6Pase activity towards M6P recommended by Arion et al. [32] as an index of microsomal intactness in liver is unusable. Measurements of [$^3$H]sucrose efflux by rapid filtration have been used as indices of brain microsomal intactness [33], since brain (and liver) microsomes are relatively impermeable to sucrose, but these methods are inconvenient for routine use. Sucrose entry as assessed by light-scattering (Figure 5a) gives similar results to those seen in liver [29], and was used in the present study as an index of intactness.

The effects of the three solutes, KCl (50 mM), dipotassium G6P (75 mM) and sucrose (75 mM) (final concns.) on the light-scattering properties of whole brain microsomes were studied. Regular assessments of sucrose permeability were made during an experiment. Typically, sucrose permeability began to rise after about 3 h, indicating decreasing intactness, at which point the preparation was discarded.

Representative traces are shown in Figure 5. The light-scattering response to KCl (Figure 5b) is characterized by a rapid rise and a fast exponential decay approximately to the initial baseline with a half-life of 2.3 ± 0.1 min ($n$ = 3 in one experiment). Conversely, sucrose addition is accompanied by a rapid rise and then slow decay with half-life 4.9 ± 0.8 min ($n$ = 5 in one experiment) (Figure 5a).

Addition of G6P to a final concentration of 75 mM was accompanied by a distinct response (Figure 5c): a decay with a half-life (2.0 ± 0.5 min; $n$ = 5) not significantly different from that with KCl ($P$ = 0.3), to a new baseline of 67 ± 4% ($n$ = 5) of the maximum initial deflection. The half-life for the light-scattering signal decay for 75 mM G6P was significantly less than that for sucrose ($P < 0.0001$).

The rapid return approximately to original baseline for KCl (Figure 5b), and comparably rapid fall to a consistently higher baseline for G6P (Figure 5c), may reflect the high permeability of most brain microsomes to KCl, with a comparable permeability in a smaller proportion of brain microsomes to G6P. Given the heterogeneous nature of a brain 'microsome' preparation, and the demonstration that G6Pase is confined to astrocytes [14], it would be surprising if all brain microsomes were capable of G6P uptake. Other interpretations may be possible, however: published results in liver [29] show decay in the light-scattering signal for KCl that falls markedly below the original baseline. This has been attributed to changes in vesicle aggregation on adding solute [28].

## DISCUSSION

Classical microsomal G6Pase is strongly expressed by liver, kidney and β-cells of the pancreatic islets, and at lower levels by small intestine [34] and gall bladder [35]. It is a multicomponent system, comprising specific transporters for delivery of substrate into, and glucose and $P_i$ products out of, the lumen of the ER, where the catalytic subunit itself is situated [36]. The present study provides evidence for the presence of the catalytic subunit of G6Pase in astrocytes. The subunit in astrocytes is immunologically very similar to that of liver, kidney, β-cells, gall bladder and intestine, and has a molecular mass of 36.5 kDa in all these tissues as judged by SDS/PAGE.

Furthermore, this study provides evidence for the first time of G6Pase activity in astrocytes *in vitro*, showing several of the properties of hepatic G6Pase, including latency, sensitivity to exposure to pH 5 and evidence of association with membranes. Reports that G6P does not have ready access to brain G6Pase, and the implication that therefore the enzyme must be of limited physiological significance [37] (and see below), are contradicted by the results of light-scattering experiments demonstrating that a proportion of brain microsomes is permeable to G6P. The expression of G6Pase in cultured astrocytes, and the demonstration of characteristic activity *in vitro* and of accessibility of

substrate to the G6Pase catalytic subunit, support recent reports of the expression of the protein in astrocytes *in vivo* in humans [14]. Together they provide evidence that the enzyme may play an important role in mammalian brain.

The specific activity of the G6Pase in cultured astrocytic microsomes is considerably lower than the typical values in liver of 300 nmol/min per mg of microsomal protein [38], but it is in the same range as previously reported for human gall bladder [35] and intestinal mucosa [39]. The $K_m$ of the astrocytic enzyme is higher than its hepatic counterpart, although this may be an effect of culture. Indeed, in this regard it is interesting that the G6Pase activity of cultured hepatocytes declines rapidly with time even in a strong counter-regulatory environment [40]. In contrast, the astrocytic activity appears relatively stable and persists after several passages *in vitro* (R. J. Forsyth, unpublished work).

The isolation of 'cerebral glucose-6-phosphatase' was reported by Anchors and Karnovsky in 1975 [41]. The purification method was surprisingly simple, i.e. 1% (w/v) deoxycholate extraction of a brain fraction, followed by Sephadex G-100 and DEAE Sephadex chromatography, and resulted in a 146% yield of 'glucose 6-phosphatase' activity [41]. However, the properties of the purified protein were very different from those of the hepatic G6Pase enzyme. For example, the specific activity of 7.9 $\mu$mol/min per mg of enzyme protein is at least 50 times lower than can be calculated for the liver enzyme [22]. The liver enzyme is very unstable during purification [35,42]; in contrast, the brain enzyme apparently gained activity during the purification [41]. Furthermore, the brain enzyme had a molecular mass of 28 kDa, whereas the catalytic subunit of hepatic G6Pase has a molecular mass of 36.5 kDa. The demonstration of a protein of the same molecular mass as hepatic G6Pase in astrocytes *in vitro* (the present paper) and *in vivo* [14] suggests that the protein purified by Anchors and Karnovsky [41] was misidentified.

To be fully functional in a tissue, all the elements of the multicomponent G6Pase system must be expressed, including T1 (the specific transporter of G6P into the lumen of the ER), T2 (for $P_i$ efflux) and T3 (for glucose efflux) [20]. In 1982, Karnovsky's group reported the purification of a 4,4′-diisothiocyano-1,2-diphenyl[$^3$H]ethane-2,2′-disulphonic acid ([$^3$H]-DIDS)-binding protein in liver and suggested that it must be T1, since DIDS inhibits G6P transport into the lumen of the ER [43]. They subsequently questioned the expression of T1 in brain [37] on the grounds that (i) they were unable to identify a [$^3$H]DIDS-binding protein in brain and (ii) brain microsomes did not demonstrate the preferential hydrolysis of G6P rather than M6P seen in intact hepatic microsomes that is taken as evidence of the presence in liver of the G6P-specific transporter T1.

[$^3$H]DIDS has since been shown to label specifically the $Na^+$-dependent bile acid transport protein in hepatocyte plasma membranes [44], as well as non-specifically binding with other microsomal proteins. Thus liver T1 has yet to be isolated, and a failure to demonstrate a [$^3$H]DIDS-binding protein in brain does not imply absence of T1 [22,45,46].

Although in this paper we confirm the inability of astrocytic microsomes to distinguish between M6P and G6P [37], the demonstration of significant PMI activity in brain homogenates [47] and in whole brain microsomes (the present paper) provides an explanation for this inability. Whereas the physiological role of PMI in brain is unclear, the activity is sufficient to permit the support of brain metabolism in isolated brain preparations by mannose alone [48]. The assay conditions used by Fishman and Karnovsky [37] under which they could not distinguish M6P and G6P (pH 5.5; substrate concentrations of M6P up to 80 mM) are optimal for M6P-to-G6P interconversion, given the pH maximum (Figure 4b) and low $K_m$ (Figure 4a) of the combined PMI/PGI activity of brain microsomes.

Light-scattering experiments provide direct independent support for the hypothesis that at least a proportion of brain microsomes is permeable to G6P, and that therefore the G6Pase catalytic subunit in the lumen of the ER is accessible by G6P. Direct evidence for G6P uptake into microsomes and the multicomponent model for G6Pase has been difficult to obtain until recently, even in liver (compare, e.g., Fulceri et al. [29] with Berteloot et al. [49]).

There has been considerable debate regarding the expression of G6Pase in brain, primarily because of a need to validate the assumption underlying the 2-deoxyglucose method [50] for the estimation of local cerebral metabolic rates, that once transported into brain and phosphorylated by hexokinase, the product 2-deoxyglucose 6-phosphate is metabolically stable [51–54]. A demonstration of an apparent rapid decline in the $^3H/^{14}C$ ratio in the cerebral glucose pool of conscious rats in the few minutes following bolus administration of [2-$^3$H,U-$^{14}$C]glucose [55,56] was interpreted as providing evidence for significant substrate cycling in brain between glucose and G6P. Subsequent studies have shown this to be an artefact, and have failed to demonstrate any significant decline in the $^3H/^{14}C$ ratio of the glucose pool under these conditions [54,57,58]. Nevertheless, significant G6Pase activity may not be excluded by these results: in liver, relative detritiation studies have been reported to underestimate the true magnitude of the glucose/G6P cycle by up to 50% as a result of isotope effects [59,60]. Furthermore, locus coeruleus activation as an 'arousal' response [61] in the restrained conscious rats used in the studies by Huang and Veech [55,56] and Nelson and colleagues [54,57,58] could result in cerebral glycogenolysis ([62], and see below) for a significant fraction of the short study period, and dilution of the G6P pool with unlabelled G6P from glycogen.

The vulnerability of the 2-deoxyglucose method to demonstrations of G6Pase in brain has long been realized. Fishman and Karnovsky's report [37] of the apparent absence of the G6P translocase formed the basis of the 'time-lag hypothesis' [63], that absence of a translocase gave a time window after injection of 2-deoxyglucose before loss of 2-deoxyglucose 6-phosphate by G6Pase activity became significant. Although no evidence of such a time lag could be detected [64], the 2-deoxyglucose method nevertheless gives estimates for regional and whole brain metabolic rates for glucose that are in reasonable agreement with those obtained by other methods [50]. We hypothesize that G6Pase-involving processes in cortex (see below) may operate on a smaller temporal and spatial scale than previously appreciated, and below the resolution of the 2-deoxyglucose and related [2-$^{18}$F]fluoro-2-deoxyglucose positron-emission-tomography methods for local cerebral metabolic rate determination, and that this may underlie the apparent tolerance of these methods to significant G6Pase activity in brain.

Neurons are largely isolated from direct contact with cerebral capillaries, which are almost completely enveloped by astrocytic endfeet. There is evidence for important roles for astrocytes in the uptake of glucose into the brain [65], with strong expression of GLUT3, the brain-specific facilitative plasma-membrane glucose transporter, around cerebral capillaries, probably in astrocytic endfeet [66]. Thus it appears likely that the majority of the glucose entering the brain initially enters astrocytes, and that the glucose passing from cerebral capillary to neuron traverses an astrocytic compartment. Moreover, a 'nutritional' role for glia has been demonstrated in invertebrate systems, with glucose uptake solely into glia before transfer of an as yet unidentified metabolite from glia to neuronal cells [67].

Astrocytic glycogenolysis occurs *in vitro* on exposure to noradrenaline, $K^+$ and VIP [8–10]. *In vivo*, these substances are products of neuronal activity, and their levels reflect such activity, since the brain is protected from systemic sources of these compounds by the blood/brain barrier [68]. This has led to suggestions that local energy-homoeostatic mechanisms may exist in mammalian brain, with astrocytic glycogenolysis occurring in response to increased neural metabolic rates [62]. Inverse relationships between neuronal activity and astrocytic glycogen stores [69] and between neuronal activity and extracellular concentrations of glucose [70] have been seen *in vivo*. Although the factors controlling astrocytic glycogenolysis are well characterized, the metabolic fate of the glycogen remains unclear. Since astrocytes have a number of neuronal support roles, it would be expected that their own metabolic requirements will rise at times of increased neuronal activity. Nevertheless, the presence of G6Pase activity in a glycogen-containing cell suggests that at least some glucosyl units may be exported to neurons as glucose at times of increased neuronal metabolic demand, thus modulating the total glucose flux to neurons, and serving local glucose homoeostasis in cerebral cortex.

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