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Permeability of rat liver microsomal membrane to glucose 6-phosphate

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Light-scattering measurements of osmotically induced changes in the size of rat liver microsomal vesicles pre-equilibrated in a low-osmolality buffer revealed the following. (1) The increase in extravesicular osmolality by addition of glucose 6-phosphate or mannose 6-phosphate (25 mM each) caused a rapid shrinking of microsomal vesicles. After shrinkage, a rapid swelling phase ($t_{\frac{1}{2}}$ approx. 22 s) was present with glucose 6-phosphate but absent with mannose 6-phosphate, indicating that the former had entered microsomal vesicles, but the latter had not. (2) Almost identical results were obtained in the absence of any glucose 6-phosphate hydrolysis, i.e. with microsomes pre-treated with 100 μM-vanadate. (3) The anion-channel blocker 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS) suppressed the glucose 6-phosphate-induced swelling phase. (4) The swelling phase was more prolonged as the glucose 6-phosphate concentration increased ($t_{\frac{1}{2}} = 16 \pm 3$, 22 ± 3 and 35 ± 4 s with 25 mM, 37.5 mM- and 50 mM-glucose 6-phosphate respectively). The behaviour of glucose-6-phosphatase activity of intact and disrupted microsomes measured in the presence of high concentrations (< 30 mM) of substrate also indicated the saturation of the glucose 6-phosphate permeation system by extravesicular concentrations of glucose 6-phosphate higher than 20–30 mM. Additional experiments showed that vanadate-treated microsomes pre-equilibrated with 0.1 mM- and 1.0 mM-glucose 6-phosphate (and [1-^{14}C]glucose 6-phosphate as a tracer) rapidly ($t_{\frac{1}{2}} < 20$ s) released [1-^{14}C]glucose 6-phosphate when diluted in a glucose 6-phosphate-free medium. The efflux of [1-^{14}C]glucose 6-phosphate was largely prevented by DIDS, allowing an evaluation of the intravesicular space of glucose 6-phosphate of approx. 1.0 μl/mg of microsomal protein.

INTRODUCTION

Liver glucose-6-phosphatase (G-6-Pase; EC 3.1.3.9) catalyses the terminal reaction of gluconeogenesis and glycogenolysis and plays a major role in the maintenance of blood glucose homoeostasis [1]. Recognized since the 1950s [2], the enzyme is intimately associated with the endoplasmic reticulum (ER) and its activity is within the ER lumen. P_i hydrolysed from G-6-P by G-6-Pase accumulates within the lumen of the intact ER or of ER-derived vesicles (microsomes) when it is trapped by cations such as Pb^{2+} [3,4] or Ca^{2+} [5,6]. A main feature of liver G-6-Pase is its latency. The enzyme is more active *in vitro* in disrupted microsomal vesicles than in intact microsomes (for reviews see [1,7,8]).

To explain the intrareticular compartmentation of G-6-Pase activity, two major hypotheses have been proposed. The first was the conformational model [9–12], which postulates that the enzyme, embedded in the microsomal membrane, binds G-6-P at the external face and releases the products (P_i and glucose) into the microsomal lumen by changing its molecular conformation. According to this model, permeation of the microsomal-vesicle membrane by G-6-P is not required. In contrast, in the second hypothesis, called the substrate-transport model [13,14], G-6-Pase exists as a multifunctional system comprising (i) a G-6-P-specific translocase and (ii) a non-specific phosphatase which has its active site at the lumenal surface of the (reticular) microsomal membrane. According to this hypothesis, the substrate-specificity is due to a selective permeation of G-6-P into the microsomal lumenal space via a translocase termed T_1.

Direct experimental proofs for an actual translocation of G-6-P into microsomal vesicle space would be crucial to support the substrate-transport or the conformational model. In this respect, however, previous results are conflicting and far from conclusive. Rapid-centrifugation experiments showed that ^{14}C or ^{32}P from labelled G-6-P [and mannose 6-phosphate (M-6-P)] became associated with rat liver microsomal vesicles [15]. In rapid-filtration experiments, ^{14}C or ^{32}P from labelled G-6-P (but not from M-6-P) have been found associated with human [16] and rat [17] liver microsomes incubated with the labelled hexose phosphates. However, on the basis of radioisotope-flux measurements, the liver microsomal membrane appears to be relatively impermeable to G-6-P and related monophosphates [18]. Recently, studies using a fast-sampling rapid-filtration technique and rat liver microsomes [19] failed to supply direct evidence for an intravesicular translocation of external [^{14}C]G-6-P. In the present study, direct evidence for the entry of G-6-P into rat liver microsomal vesicles is given.

EXPERIMENTAL

Materials

G-6-P (dipotassium salt), M-6-P (monosodium salt), G-1-P (monosodium salt) and 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS) were from Sigma, St. Louis, MO, U.S.A. Na_3VO_4 was from Fisher Science Co., Pittsburgh, PA, U.S.A. [1-^{14}C]G-6-P (49 mCi/mmol) and [^{3}H]inulin (500 mCi/g) were from DuPont–New England Nuclear, Dreieich, Germany. Cellulose nitrate filter membranes were from Sartorius,

Abbreviations used: G-6-P, glucose 6-phosphate; G-6-Pase, glucose-6-phosphatase; M-6-P, mannose 6-phosphate; G-1-P, glucose 1-phosphate; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid; ER, endoplasmic reticulum.

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Gottingen, Germany. All other chemicals were of analytical grade.

Preparation of liver microsomes

Male Sprague–Dawley rats (180–230 g) were used. Microsomes were isolated from liver homogenates (20%, w/v, in 0.3 M-sucrose/20 mM-Hepes buffer, pH 7.2) as described previously [20]. Microsomal fractions were resuspended (approx. 80 mg of protein/ml) in a medium of the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 1; Mops, 20, pH 7.2. Suspensions were maintained at 0–4 °C and used within 4 h. Intactness of the microsomal membrane was ascertained by measuring the latency of mannose-6-phosphatase activity [21]. This activity of microsomes was less than 15% of that of fully disrupted microsomal vesicles in all the preparations employed.

Light-scattering measurements

Osmotically induced changes in microsomal vesicle size and shape were monitored at 400 nm at right angles to the incoming light beam (whose intensity was decreased by 80% with the aid of a copper grid) by using a fluorimeter (Perkin–Elmer model LS-3B) equipped with a recorder, a temperature-controlled cuvette holder (22 °C) and magnetic stirrer as described elsewhere [22]. Microsomal suspensions (80 mg of protein/ml) were diluted 1000-fold in a low-osmolality buffer (5 mM-K-Pipes, pH 7.0). Diluted microsomal suspension (2 ml) was placed in the fluorimeter cuvette, and vesicles were equilibrated and stirred until a stable baseline was obtained. Osmotically induced changes in the vesicles were initiated by increasing the osmolality of the vesicle medium. To this end, 0.2 ml portions of concentrated solutions of the different solutes (0.75–0.25 M in 5 mM-K-Pipes, adjusted to pH 7.0 with KOH or HCl) were added to the vesicle suspension through a hole in the sample compartment cover by using a microlitre syringe.

Measurement of [^{14}C]G-6-P isotope space and efflux rate

Liver microsomes were washed (80000 *g*, 45 min) and resuspended (15 mg of protein/ml) in a cytosol-like medium with the following composition (mM): KCl, 100; NaCl, 20; potassium phosphate buffer, pH 7.2, 1; $MgCl_2$, 1; Mops, 20, pH 7.2. Vanadate (Na_3VO_4, 100 μM) was also present to suppress G-6-P hydrolysis. The microsomal suspension was incubated in the presence of 0.1 mM- or 1.0 mM-G-6-P plus [14C]G-6-P (8–9 μCi/ml) for 5 min at 22 °C and subsequently diluted (100-fold) with the incubation medium including no G-6-P. At the indicated time intervals, samples of the diluted suspension (containing 75 μg of protein each) were taken to measure ^{14}C associated with microsomes by rapid filtration [5].

Other analytical procedures

G-6-Pase activity was measured as reported in detail elsewhere [23]. Protein was determined by the Lowry method [24], with BSA as standard. The intravesicular water space was measured by using ^{3}H-labelled inulin [25].

RESULTS AND DISCUSSION

In a first set of experiments, the permeability of liver microsomal vesicles to G-6-P was assessed by determining their osmotic behaviour, as measured by light-scattering. The rationale of this method is the following. After an increase in the osmolality of the medium, microsomal vesicles rapidly shrink as water leaves the vesicles, thereby increasing their light-scattering properties. Subsequently, as the osmotically active molecule and/or ions and water enter the vesicles, vesicles swell and the signal returns to that of the control. The amplitude of the signal and the rate of swelling are essentially a function of the permeability of the vesicles to the solutes (see [22,26] for details). Preliminary experiments to assess the osmotic behaviour of our microsomal preparation were consistent with the rationale of the method. KCl (Fig. 1, trace *a*) causes a transient increase in microsomal light-scattering, followed by a rapid decrease in the signal as expected, since Cl^- and K^+ easily cross the microsomal membrane (see, e.g., [18]). Moreover, the complete permeabilization of vesicles to K^+ by valinomycin (1 μM) further accelerated the decrease in the signal (results not shown), indicating that Cl^- possesses the highest conductance, whereas K^+, despite possessing a high conductance, is the rate-limiting ion of the pair [18,22]. Almost identical patterns of increase/decrease in light-scattering were observed by replacing KCl with NaCl (results not shown), as expected, since also Na^+ easily crosses the liver microsomal membrane [18]. In contrast, sucrose (Fig. 1, trace *b*) causes a rapid but sustained increase in the light-scattering intensity, owing to the poor permeability of the microsomal membrane to the sugar (e.g. [18]). As a control, the decrease in light-scattering due merely to the dilution of the microsomal suspension by the addition of solvent alone is shown in Fig. 1 (trace *c*).

Fig. 1 (trace *d*) shows that the increase in extravesicular osmolality upon addition of G-6-P (25 mM final concn.) causes a transient increase in light-scattering, followed by a relatively rapid decrease in the signal, indicating that G-6-P had entered the vesicles. Qualitatively similar results were obtained with other G-6-P concentrations ranging from 12.5 to 75 mM (see also Fig. 2). By contrast, the stereoisomer M-6-P (25 mM final concn.) causes a rapid and sustained increase in light scattering (Fig. 1, trace *e*), which was almost unchanged for prolonged incubation times (10–15 min; results not shown), indicating that M-6-P does not enter the microsomal vesicles. A sustained increase in the light-scattering intensity was also evident with higher concentrations of M-6-P (up to 75 mM; results not shown). The lack of entry of M-6-P into the microsomal vesicles is consistent with the fact that M-6-P is a good substrate for the G-6-Pase enzyme in disrupted vesicles, but not a substrate in intact vesicles [14].

The possibility that the swelling phase observed with G-6-P is due to the intravesicular generation of P_i and glucose by G-6-Pase activity, rather than to the entry of G-6-P itself, was ruled out by inhibiting the microsomal G-6-Pase activity with vanadate [27]. For this, microsomes were treated with 100 μM-vanadate for 15 min before measurement of G-6-P-induced light-scattering changes. The rate of G-6-P hydrolysis (μmol of P_i/60 min per mg of protein; mean ± S.E.M., $n = 3$) was 2.55 ± 0.30 and 0.15 ± 0.05 in control and vanadate-treated microsomes respectively, when measured under the experimental conditions for the light-scattering experiments (25 mM-G-6-P, 22 °C). In vanadate-treated microsomes the patterns of shrinking/swelling by G-6-P (Fig. 1, trace *g*) and M-6-P (Fig. 1, trace *e*) are essentially unmodified as compared with those of control microsomes (Fig. 1, traces *d* and *f*). The basal microsomal light-scattering intensity and its changes effected by KCl or sucrose additions were also unmodified in vanadate-treated microsomes (results not shown). Similarly to M-6-P, the isomer G-1-P causes a rapid and sustained microsomal shrinking (Fig. 1, trace *h*), which was essentially maintained up to 10 min of incubation (results not shown). Other phosphoesters tested (i.e. ATP, ADP, AMP and fructose, 1,6-bisphosphate, 25 mM each; results not shown) caused a rapid and prolonged shrinking of vanadate-treated liver microsomes. The pattern of increase/decrease in light-scattering by G-6-P (as K^+ salt) was unaffected by valinomycin (results not shown), indicating that the membrane permeability to K^+ does not limit the rate of G-6-P entry into vesicles.

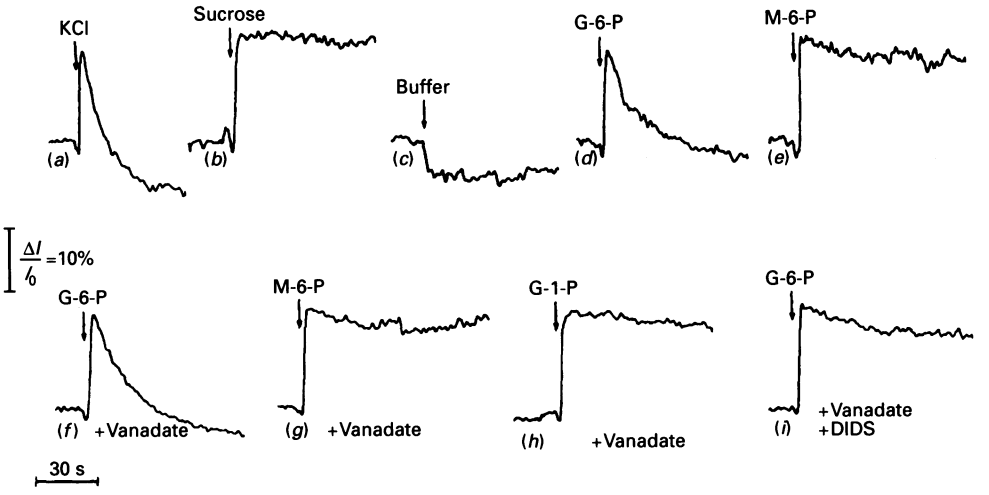

Fig. 1. Osmotically induced changes in light-scattering intensity of rat liver microsomal vesicles caused by various solutes

Rat liver microsomes (80 mg of protein/ml) were diluted 1000-fold with a low-osmolality buffer (5 mM-K-Pipes, pH 7.0) in a fluorimeter cuvette, and vesicles were equilibrated and stirred at 22 °C until a stable baseline was obtained. Where indicated, diluted microsomes were pre-treated with 100 μM-vanadate (15 min) and 100 μM-DIDS (2 min). Concentrated solutions of the different solutes (0.75–0.25 M, in 5 mM-K-Pipes adjusted to pH 7.0 with KOH or HCl) were added ($\frac{1}{10}$ of the suspension volume) to the microsomal suspension when indicated by arrows. The resulting final concentrations (mM) were 25 for G-6-P, M-6-P and G-1-P, 50 for KCl and 75 for sucrose. Light-scattering changes were monitored at right angles to the incoming beam at 400 nm (see the Experimental section for details). A typical set of experiments out of three to five is reported.

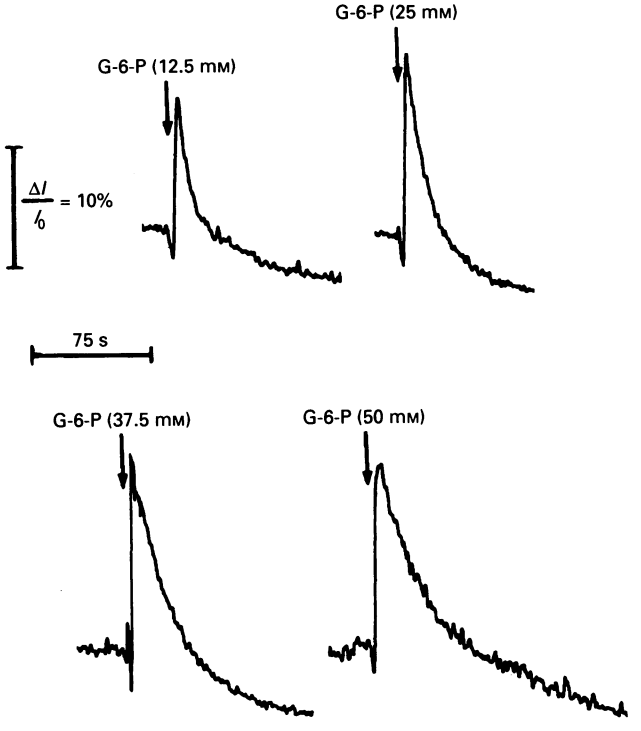

Fig. 2. Osmotically induced changes in light-scattering intensity of rat liver microsomal vesicles caused by different concentrations of G-6-P

Rat liver microsomes (80 mg of protein/ml) were diluted 1000-fold with a low-osmolality buffer (5 mM-K-Pipes, pH 7.0) in a fluorimeter cuvette, and vesicles were equilibrated and stirred at 22 °C until a stable baseline was obtained. Diluted microsomes were pre-treated with 100 μM-vanadate (15 min) before adding G-6-P. Concentrated solutions of G-6-P (0.5–0.125 M, in 5 mM-K-Pipes adjusted to pH 7.0 with HCl) were added ($\frac{1}{10}$ of the suspension volume) to the microsomal suspension when indicated by arrows to give the desired final concentrations of G-6-P. Light-scattering changes were monitored at right angles to the incoming beam at 400 nm (see the Experimental section for details). A typical set of experiments out of three is reported.

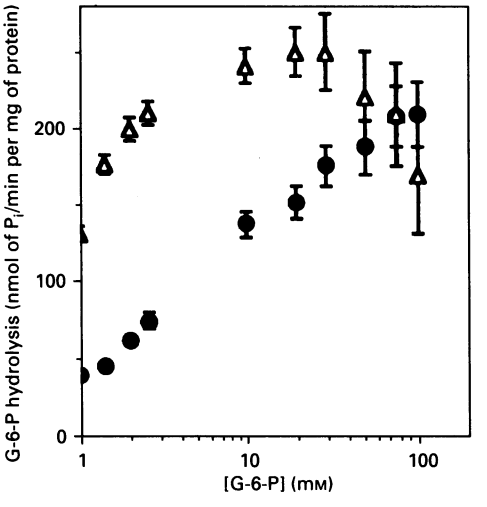

Fig. 3. Rate of hydrolysis of G-6-P by intact and disrupted rat liver microsomes incubated in the presence of different concentrations of G-6-P

The rate of hydrolysis of G-6-P by intact (●) or histone 2A-disrupted (▲) rat liver microsomes were measured as described elsewhere [23] at the indicated G-6-P concentrations. Data are means ± S.E.M. of four or five experiments.

It was previously reported [28,29] that DIDS inhibits the G-6-Pase activity of intact liver microsomes. The inhibition was counteracted by G-6-P. It was therefore suggested [28,29] that DIDS binds and inhibits a putative microsomal translocase for G-6-P. In line with these reports, DIDS suppresses the rapid swelling phase which follows G-6-P-induced microsomal shrinking (Fig. 1, trace *i*). However, at later times after G-6-P addition a decrease in light-scattering is present ($t_{\frac{1}{2}} \leqslant 15$ min, $n = 3$; 25 mM-G-6-P). The rate of swelling after KCl-induced shrinking is also decreased in DIDS-treated microsomes as compared with control ones ($t_{\frac{1}{2}}$ 20 ± 4 and 9 ± 3 s respectively; mean ± S.E.M., $n = 3$). This indicates that DIDS also acts as an

unspecific anion-channel blocker, as can be predicted on the basis of various reports (e.g. see [30]).

In further experiments the rate of G-6-P entry into vesicles has been evaluated at different concentrations of extravesicular G-6-P in vanadate-treated microsomes. As shown in Fig. 2, the addition of G-6-P concentrations ranging from 25 to 50 mM to the microsomal suspension causes similar increases in the light-scattering intensity, whereas at lower concentrations (12.5 mM) the increase is somewhat minor. Thus G-6-P concentrations $\leqslant$ 25 mM appears to cause a maximal shrinking of vesicles. On the other hand, the swelling phase is more prolonged as the G-6-P concentration increases (Fig. 2). The $t_{\frac{1}{2}}$ values for the swelling phase (means $\pm$ S.E.M., $n = 3$, or mean, $n = 2$) are the following: 16 ± 3 s, 22 ± 3 s, 35 ± 4 s and 61 s with G-6-P concentrations of 25, 37.5, 50 and 75 mM respectively. These results suggest that the microsomal permeation system for G-6-P is limited and saturated, at least at G-6-P concentrations higher than 25 mM. Unambiguous data were not provided by the light-scattering method with G-6-P concentrations lower than 12.5 mM, since the osmotically induced shrinkage of vesicles was minor. In line with these findings was the behaviour of G-6-Pase activity of intact and disrupted microsomes measured in the presence of high concentrations (<30 mM) of substrate. In disrupted microsomes the rate of G-6-P hydrolysis decreases at G-6-P concentrations higher than 20–30 mM (Fig. 3), indicating some inhibition of the phosphohydrolase activity by high concentrations of its own substrate. On the other hand, this decrease in G-6-Pase activity is absent from the intact microsomal vesicles (Fig. 3). A logical interpretation of these data is that the access of G-6-P to the intralumenal phosphohydrolase may be limited by the saturation of the G-6-P permeation system by extravesicular concentrations of G-6-P higher than 20–30 mM.

The osmotic behaviour of liver microsomes, as assessed by light-scattering, strongly indicates the entry of G-6-P itself into the microsomal space. However, quantitative information on intravesicular G-6-P space cannot be clearly obtained by this method, as discussed extensively elsewhere [22]. In addition, only high concentrations of G-6-P ($\leqslant$ 15 mM) can be usefully investigated. Therefore, in additional experiments the flux rate and the intravesicular space of G-6-P were investigated radioisotopically by rapid filtration. To this end, the time-dependent loss of the microsome-associated radioactivity was evaluated in vanadate-treated microsomes pre-equilibrated with G-6-P (0.1 and 1.0 mM plus [^{14}C]G-6-P as a tracer) and diluted in a G-6-P-free medium. It is unlikely that ^{14}C associated with microsomes might be accounted for by [^{14}C]glucose already present in the [^{14}C]G-6-P solution, or derived from some hydrolysis of [^{14}C]G-6-P, because [^{14}C]glucose in the incubation medium never exceeded 3% of total radioactivity (as measured at the beginning and at the end of the experiments after the removal of [^{14}C]G-6-P by barium precipitation [31]; result not shown).

Figs. 4(*a*) and 4(*b*) (● symbols) show that after diluting [^{14}C]G-6-P-challenged microsomes in a G-6-P-free medium some microsome-associated [^{14}C]G-6-P (approx. 0.6 nmol/mg of protein) is present at the shortest time interval of the ultrafiltration technique (30 s). The amount of [^{14}C]G-6-P associated with microsomes decreases within 2–2.5 min (Figs. 4*a* and 4*b*; ●). However, two major problems exist with rapid-filtration assays: (i) rapid-permeating compounds can be lost during filtration (and the washing procedure if used); (ii) radioactivity associated with vesicles might reflect both the entry into or binding to vesicles. Since DIDS greatly decreases the permeability of the microsomal membrane to G-6-P (see above; Fig. 1), it was employed to overcome these problems. Treatment with DIDS of microsomes already equilibrated with [^{14}C]G-6-P results in an increase in the initial microsome-associated [^{14}C]G-6-P which

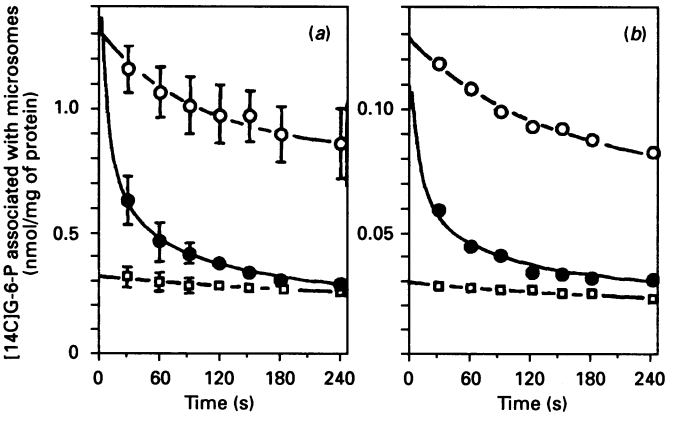

Fig. 4. Efflux of [^{14}C]G-6-P from [^{14}C]G-6-P-pre-equilibrated liver microsomes after dilution in a G-6-P-free medium

Liver microsomes wre pre-treated with 100 μM-vanadate (15 min at 22 °C), incubated in the presence of 1.0 mM- (*a*) or 0.1 mM (*b*) G-6-P plus [^{14}C]G-6-P (8–9 μCi/ml) for 5 min at 22 °C and subsequently diluted (zero time) with a G-6-P-free medium (●). In parallel samples 500 μM-DIDS was also added to the microsomal suspension 2 min before pre-equilibration with G-6-P (□) or after the 5 min pre-equilibration time but 1 min before dilution (○); in both instances G-6-P-free medium also included 100 μM-DIDS. At the indicated time intervals after dilution, samples were taken to measure [^{14}C]G-6-P associated with microsomes by rapid filtration. Data are means $\pm$ S.E.M. of three experiments (*a*) or means of two experiments (*b*).

decreases slowly after dilution in the G-6-P-free medium (Figs. 4*a* and 4*b*, ○ symbols). In contrast, microsomes treated with DIDS before (and during) [^{14}C]G-6-P challenge contained relatively low amounts of [^{14}C]G-6-P (Figs. 4*a* and 4*b*, □ symbols); these amounts probably represent the binding of [^{14}C]G-6-P to microsomal membrane. Taking into account the binding of G-6-P to microsomes, a net efflux rate of G-6-P from microsomal vesicles with $t_{\frac{1}{2}} \leqslant 20$ s can be calculated from the data reported in Fig. 4. This value is in agreement with those calculated for the influx of G-6-P in the light-scattering experiments (see above). The intravesicular water space was calculated on the basis of the amount of [^{14}C]G-6-P retained by microsomes pre-equilibrated with [^{14}C]G-6-P and subsequently treated with DIDS to minimize the efflux of G-6-P. In microsomes not treated with DIDS the efflux rate of G-6-P was too fast to allow reliable measurements. By extrapolating the initial rate of G-6-P efflux to the time point of dilution (zero time; Fig. 4, ●) and after the subtraction of the G-6-P bound to the membrane (Fig. 4, □), the calculated values of intravesicular G-6-P were 1.05 and 0.106 nmol/mg of protein, after pre-equilibration with 1.0 mM- and 0.1 mM-G-6-P respectively. These values would correspond to a vesicular space for G-6-P of approx. 1 μl/mg of microsomal protein in both instances. Pre-equilibration times longer than that used in the experiments shown in Fig. 4 (5 min) did not result in higher values of G-6-P vesicular space. Intravesicular water content was also measured in the microsomal fraction employed as [^{3}H]inulin-impermeable space. The value is 4.7 ± 0.5 μl/mg of microsomal protein (mean $\pm$ S.E.M., $n = 4$). It appears therefore that the vesicular space for G-6-P accounts for only a portion of the intravesicular water space (approx. $\frac{1}{4}$). This difference might be due to the presence in the microsomal fraction of vesicles with different permeability to G-6-P, or to a limited steady-state equilibrium under the experimental conditions employed. These conditions were suitable to mimic, at best, the conditions in the intact liver cell, as a 'cytosol-like' medium (for ion composition and pH; see the Experimental section) and physiological concentrations of

G-6-P were used. A similar value for the steady-state intravesicular space of G-6-P itself can be calculated from previous data [16,32] obtained in human liver microsomes from control patients and patients with type 1b or 1c glycogen-storage disease who are genetically lacking T_1, the 'putative' G-6-P translocase, or T_2, the phosphate translocase.

Concluding remarks

Definitive proof for the permeability to G-6-P of liver microsomal membranes has not previously been obtained by radioisotopic techniques, as the results obtained could have been due to either entry into and/or binding to microsomal vesicles (e.g. [15,16]). We have provided compelling evidence that G-6-P is translocated into the lumen of liver microsomal vesicles, whereas other similar compounds, e.g. M-6-P and G-1-P, are not. The fact that G-6-P is indeed translocated into the lumen of microsomes means that the conformational model of G-6-Pase is an unnecessary hypothesis. This is consistent with recent studies of the type 1 glycogen-storage diseases, which provide compelling evidence that for normal G-6-Pase activity *in vivo* at least six different polypeptides are required, including ER translocase systems for G-6-P, glucose and P_i [33–36]. Recently, 'transport' proteins termed T_2 and T_3, which respectively translocate glucose and Pi (the products of G-6-Pase) across the reticular membrane, have been identified and purified [36,37]. Now it has been demonstrated that the 'putative' microsomal T_1 translocase for G-6-P exists, further work must be done to identify unequivocally and purify and clone T_1, as DNA probes for the diagnosis of type Ib glycogen-disease are urgently needed.

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