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TRANS-STIMULATION AND DRIVING FORCES FOR GSH TRANSPORT IN SINUSOIDAL MEMBRANE VESICLES FROM RAT LIVER

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SUMMARY: Sinusoidal membrane vesicles from rat liver were employed to study the characteristics of GSH transport. Saturable concentration dependent uptake was best described by the sum of a high and low K_m transport. Preloading with GSH markedly stimulated the initial uptake of GSH. GSH transport was electrogenic; uptake was enhanced by an inwardly directed K^+ gradient which could be blocked by the K^+-channel blocker, Ba^{2+}. The other cations such as Na^+, Li^+ were poor substitutes for K^+. These results therefore show that net GSH transport involves movement of K^+.

INTRODUCTION: The release of GSH from the liver, mainly into plasma, can account for essentially all of the turnover of hepatocellular GSH (1). Moreover, plasma GSH is nearly all derived from the liver and is cleared by other organs (2). Our laboratory has used the isolated perfused rat liver (3) and freshly isolated hepatocytes (4) while Inoue et al have used sinusoidal membrane vesicles (SMV) (5) to characterize the kinetics of GSH efflux. With all three models a saturable transport of GSH has been observed consistent with facilitative carrier-mediated transport. However, saturability of transport may be due to other factors and therefore does not prove carrier-mediation. The demonstration of trans-stimulation would provide more definitive evidence for carrier-mediated transport. With the SMV preparation used in the studies of Inoue et al (5), the high ionic permeability precluded determination of trans-stimulation or the driving forces for transport. Since GSH is a charged substance, a number of possible types of carriers may be involved in transport, such as an electrogenic or electroneutral uniport, a symport or an antiport. Therefore, in the current study, we have employed a recently described technique for isolation of SMV to characterize these important aspects of GSH transport.

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MATERIALS AND METHODS: Fed male Sprague-Dawley rats (200-250g) were used. All chemicals were from routine commercial sources. [Glycine-2-^{3}H]GSH (35.6 Ci/mmol) and [^{14}C] L-alanine (8.5 Ci/mmol) were obtained from new England Nuclear, Boston, MA.

Sinusoidal membrane vesicle preparation. SMV were prepared according to the method of Blitzer and Donovan using a self-generating Percoll gradient (6). The final enrichment of Na^+-K^+ ATPase specific activity ranged between 20-40-fold and that of Mg^{2+} ATPase, alkaline phosphatase and glucose 6-phosphatase between 2-4-fold. Preparations were de-enriched in succinate dehydrogenase and acid phosphatase. A minimum of 20-fold enrichment of Na^+-K^+ ATPase was used as a criterion for using the preparation. In addition, each SMV preparation was checked for leakiness; only preparations showing a minimum of 2-3-fold overshoot in labelled alanine uptake (100 uM) and an osmotic dependence on equilibrium uptake of GSH were used. Equilibrium space for alanine averaged 0.5 ul/mg vesicle protein, and binding at equilibrium represented 50% of the vesicle associated radioactivity as determined by the y-intercept of uptake (ordinate) versus 1/osmolality (abscissa) using varying concentrations of raffinose. However, during the first 10 sec of uptake, from which initial rates were determined, binding was negligible. Using the HPLC method of Reed et al (7), >95% of the label associated with vesicles at 10 sec was in the form of GSH.

Transport assay. Vesicles were stored at -70° in 2 ml aliquot quantities for <4 weeks in 10 mM Hepes-sucrose, pH 7.4, without loss of transport activities. Prior to assay, vesicles were thawed rapidly at 37° in the same buffer or with substitution of sucrose isosmotically with various ions. Because of a greater propensity for leakiness of SMV to ions to occur at 37°, all assays were performed at 25°. The incubation mixture contained in a final volume of 30 ul the following: SMV, 2.0 mg/ml; GSH, 0-10 mM, and Hepes-sucrose 300 mM, pH 7.4. The salts, whenever present, isosmotically replaced Hepes-sucrose. Uptake of GSH was initiated by addition of GSH and stopped by addition of 1 ml ice-cold Hepes-sucrose. The mixture was filtered onto 0.45 um filters and the membranes washed 2x successively with 3 ml buffer. For GSH preloading experiments, vesicles were preincubated in Hepes-sucrose alone or in the presence of 3 mM GSH at 25° for 3h. Uptake was initiated by addition of ^{3}H-GSH in 300 mM Hepes-sucrose or in buffer in which 50 mM KCl isosmotically substituted for sucrose. GSH uptake was nearly linear for the first 10-15 sec; thus, initial uptake rates were routinely determined using 10 sec times.

RESULTS: Kinetics of GSH uptake. Using 5 individual cell preparations, saturable uptake of GSH (10 sec time points) was observed (Fig. 1). The data for each individual SMV prep were fitted best by the sum of two Michaelis-Menten functions with the following mean parameter values for all 5 preps: K_{m1} = 0.05 mM, V_{max1} = 0.33 nmol/mg/min and K_{m2} = 3.93 mM, V_{max2} = 12.3 nmol/mg/min. The value for the low K_m transport was lower than that reported by Inoue et al (5) but otherwise the results are in reasonable agreement with these investigators.

Trans-stimulation. Vesicles were preincubated for 3h at room temperature with 3 mM GSH or Hepes-sucrose without GSH and then resuspended in 3 mM GSH containing radiolabelled GSH in the presence or absence of 50 mM KCl. Uptake

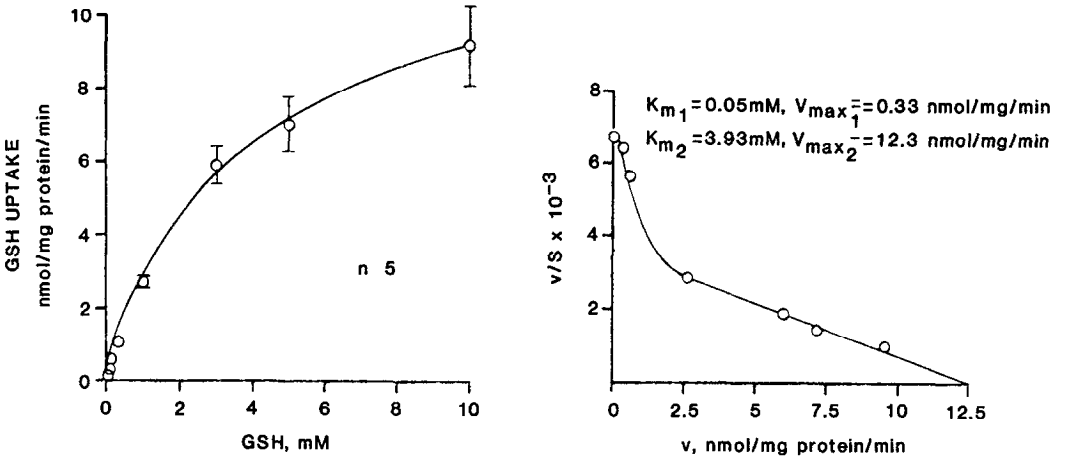

Fig. 1. Kinetics of GSH uptake by sinusoidal membrane vesicles. Varying GSH was added to SMV at room temperature and rapidly filtered onto 0.45 um filters after 10 sec incubation. Medium and vesicles contained 300 mM sucrose, 10 mM Hepes, pH 7.4. Filters were washed with cold stop solution and intravesicular radioactive GSH was counted. Data from five individual SMV preparations were fitted by nonlinear least squares to the sum of two Michaelis-Menten functions shown as the solid line on the left. The transformed fit is shown on the right.

of label was determined at 10 sec and 30 min. Preloaded vesicles showed enhanced initial uptake of the label with Hepes-sucrose and with a 50 mM inwardly directed KCl gradient (Fig. 2). A similar trans-stimulation of GSH uptake was seen in the absence of a membrane potential, i.e., under voltage-clamped conditions with 50 mM KCl in and out plus 10 ug/ml valinomycin (not shown). Preloading had no effect on the equilibrium uptake of radiolabel GSH (not shown), indicating that preloading did not influence vesicle volume.

Effect of cation and anion substitutions. An inwardly directed KCl gradient enhanced GSH uptake compared to control (Hepes-sucrose) whereas NaCl had no effect (Fig. 3). In addition, inwardly directed LiCl or choline Cl gradients also did not enhance GSH uptake (not shown). This effect of K^+ on GSH uptake was further examined in detail by stimulating K^+ conductance with valinomycin or inhibiting with $BaCl_2$. The presence of valinomycin did not enhance GSH uptake by an inwardly directed KCl gradient which indicates that the vesicles have a very high conductance for K^+ so that no further enhancement was seen with valinomycin. Further work is required to define this phenomenon. In contrast, addition of 10 mM $BaCl_2$ significantly inhibited

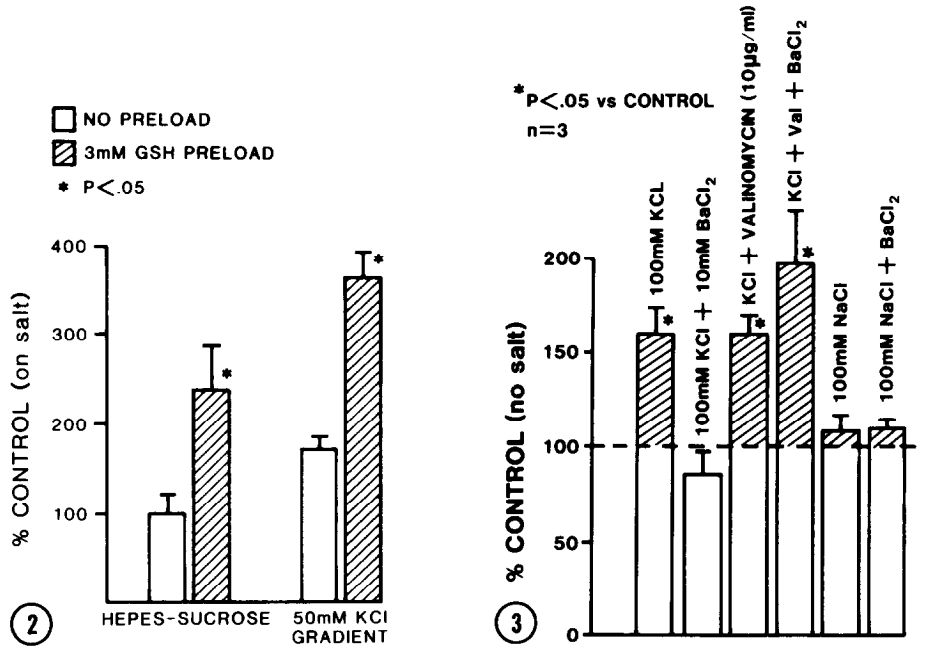

Fig. 2. Trans-stimulation of GSH uptake. Vesicles were preincubated in Hepes-sucrose alone (control) or containing 3 mM GSH for 3 h at room temperature. Uptake of 3 mM GSH at 10 sec is shown in 300 mM Hepes-sucrose or in buffer in which KCl was isosmotically substituted for sucrose. Control uptake was 0.44 ± 0.04 nmol/mg/10 sec in sucrose-Hepes and 0.75 ± 0.10 nmol/mg/10 sec in the presence of 50 mM KCl (n=3 preparations). Statistical comparison used the paired t-test.

Fig. 3. Effect of K^+ and Na^+ gradients on GSH uptake. GSH uptake at 10 sec was determined using 3 mM GSH in the medium. Control (dotted line) is uptake in Hepes-sucrose (no salt) and was 0.40 ± 0.01 nmol/mg/10 sec (n=3 preparations). Other conditions are listed in the figure. Valinomycin (10 ug/ml) was preincubated with SMV for 10 min prior to the uptake studies. Statistical comparison used the paired t-test.

the enhanced uptake of GSH by the inwardly directed KCl gradient (Fig. 3), suggesting that GSH transport is related to movement of K^+. The inhibition by Ba^{2+} was overcome by incubation with valinomycin which provided an alternate route for K^+ movement.

Anion substitutions were assessed in the presence of an inwardly directed 50 mM K^+ gradient. Maximum initial uptake rates were seen with the relatively impermeant anion, SO_4^{2-}, and the order of uptake rates was K_2SO_4 > KCl > KSCN > KNO_3 in the following ratios; 2.7: 1.6: 1.2: 1.0 (n=3). All the rates were significantly different from each other ($p < 0.05$) except KSCN vs KNO_3. The possibility that GSH transport is mediated by an anion antiport was studied by examining GSH uptake in voltage-clamped conditions (50 mM KCl in and out and

10 ug/ml valinomycin) in the presence of 10-fold outwardly directed Cl^- or HCO_3^- gradients (50 mM Cl^- and HCO_3^-, respectively). No difference was observed compared to Hepes-sucrose controls (not shown).

DISCUSSION: GSH efflux in the perfused liver and isolated hepatocyte is saturable with sigmoidal kinetics consistent with three transport sites. Using SMV, we found saturable uptake by vesicles similar to the studies by Inoue et al (5), with the data best fitted by the sum of two Michaelis-Menten functions. The reason for the difference in kinetics between intact cells and vesicles is uncertain but may be due to other physiologic factors modulating transport in cells, the mixed orientation of the vesicle preparations, or the effects of different isolation procedures.

The uptake of GSH by the SMV preparation was enhanced by preloading the vesicles with GSH. This indicates that we are not simply measuring binding of GSH and that GSH is not moving through a simple channel or pore. Rather, this finding strongly suggests a facilitative carrier-mediated transport process which would be expected to show trans-stimulation.

The driving forces and possible ion requirements for sinusoidal GSH transport have not been previously defined. These driving forces may be of critical importance in the regulation of GSH efflux from the liver in vivo and therefore, may modulate hepatic GSH turnover and its interorgan homeostasis. Because GSH has a net negative charge at physiologic pH, its transport may be affected by the electrical potential across the membrane and/or specific inorganic ions. An inwardly directed KCl gradient was found to enhance GSH transport whereas NaCl, LiCl and choline Cl were without effect. Using inwardly directed K^+ gradients, transport was affected by anions; specifically, uptake was enhanced with impermeable SO_4^{2-} and decreased with NO_3^- or SCN^- compared to Cl^-. These data are consistent with an electrogenic, potential-driven transport for GSH.

The possibility of a K^+-GSH symport versus K^+ conductance creating a membrane potential (inside positive) to drive GSH uptake was assessed using

the K^+ channel blocker, Ba^{2+}. Barium inhibited the K^+ gradient stimulated uptake of GSH but inhibition was overcome by valinomycin, a K^+ ionophore which provided an alternate path of K^+ diffusion not inhibited by Ba^{2+}. The findings thus excluded a K^+-GSH symport; in addition, a Cl^- or OH^-/HCO_3^- antiport was also excluded. The results therefore favor the existence of an electrogenic uniport for GSH transport.

Based on studies of the perfused organ, isolated hepatocytes and membrane vesicles, hepatic GSH release into sinusoidal blood can be viewed as being mediated by a membrane carrier which exhibits saturability (in all models) and trans-stimulation (in the vesicle model). The reason for lack of trans-stimulation noted previously in the intact cell is uncertain but may be due to the steep outwardly directed electrochemical gradient which makes entry of GSH into the hepatocyte highly unfavorable.

GSH release from the liver has been noted to increase under conditions of exhaustive exercise or fasting *in vivo* and under the influence of hormones in the perfused liver (8). Since we have defined membrane potential as the major driving force for GSH transport, hormonal effects on membrane potential or K^+ conductance may mediate the changes on GSH efflux which have been observed by others experimentally.

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