Sodium-gradient-stimulated transport of L-alanine by plasma-membrane vesicles isolated from liver parenchymal cells of fed and starved rats

Crucial role of the adrenal glucocorticoids

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The ability of liver efficiently to take up amino acids, particularly L-alanine, during starvation was studied in a cell-free system by isolating plasma-membrane vesicles in a transport-competent state from rat liver parenchymal cells. These membrane vesicles have the capacity to accumulate L-alanine against an apparent concentration gradient when exposed to an artificial and transient transmembrane Na^+ gradient (extravesicular Na^+ concentration greater than inside). The rate of accumulation of L-alanine is dependent on the plasma-membrane vesicle concentration, and the steady-state concentration attained is inversely related to the osmolarity of the medium. The Na^+ -mediated stimulation is not exhibited if the membrane vesicles are pre-equilibrated with NaCl, if K^+ or Li^+ are substituted for Na^+ , or if SO_4^{2-} replaces Cl^- as the counterion. The apparent active transport of L-alanine into the membrane vesicles appears to occur by an electrogenic mechanism: (1) the use of NaSCN significantly heightens the early concentrative phase of transport when compared with the effect of NaCl; (2) an enhanced active transport is also observed when a valinomycin-induced K^+ efflux occurs concomitant with Na^+ and L-alanine influx. Plasma-membrane vesicles isolated from liver parenchymal cells of a 24 h-starved rat exhibit an initial L-alanine transport rate that is 3–4 times that for membrane vesicles derived from a fed animal. The increased rate of L-alanine transport by plasma-membrane vesicles from starved animals can be obliterated by adrenalectomy and restored by administration of glucocorticoid. These results establish that stimulation of the gluconeogenic pathway by starvation involves a plasma-membrane-localized change affecting L-alanine transport which is regulated in part by the glucocorticoid hormones.

The liver is the main site of gluconeogenesis, and amino acids, particularly L-alanine, serve as major substrates for this biosynthetic pathway (Exton, 1972). The alanine used for glucose synthesis by the liver comes from muscle, primarily as a result of synthesis *de novo* (Odessey *et al.*, 1974; Garber *et al.*, 1976). The uptake of amino acids by the liver has been shown to be a key event in regulating amino acid-based hepatic gluconeogenesis (Mallette *et al.*, 1969a,b). The hepatocyte plasma membrane contains several amino acid transport systems, which vary in their substrate specificity (Kilberg *et al.*, 1980) and the means by which they are regulated. The 'A'-system, which is largely responsible for the transport of L-alanine, is known to be controlled by the concentrations of intracellular amino acids (Kelley & Potter, 1978, 1979; Fehlman

et al., 1979a), and by several hormones (LeCam & Freychet, 1976, 1978; Fehlman *et al.*, 1979b; Kelley *et al.*, 1980a,b; Kilberg *et al.*, 1980).

The regulation of hepatic amino acid transport has been studied by using intact liver, liver slices and isolated hepatocytes. Although the use of isolated hepatocytes in culture provides distinct advantages over the use of intact animals, studies on active amino acid transport in these cultured cells are complicated by subsequent metabolic conversions and intracellular compartmentalization. Additional factors affecting amino acid transport are exchange diffusion, trans-stimulation and trans-inhibition (Christensen, 1975). Some of these problems have been overcome by using non-metabolizable analogues of the natural amino acids or by using metabolic inhibitors. However, these modifications

create non-physiological conditions, and the significance of such findings is subject to interpretation.

The above problems encountered with the use of intact cells can be overcome by studying amino acid transport in isolated plasma-membrane vesicles. This approach has been used successfully with a variety of cell types as membrane sources (Hopfer *et al.*, 1973; Colombini & Johnstone, 1974; Hamilton & Nilsen-Hamilton, 1976; Quinlan & Hochstadt, 1976; Lever, 1977; Hochstadt *et al.*, 1979; Pilch *et al.*, 1980). Although even total rat liver cell-derived plasma-membrane vesicles have been used for amino acid transport studies (Van Amelsvoort *et al.*, 1978; Sips *et al.*, 1980a), much less information is available on the alanine transport mechanism present in plasma-membrane vesicles isolated from purified rat liver parenchymal cells (Sips *et al.*, 1980b), and essentially no information is available on the use of this cell-free transport system to study hormonal regulation of alanine transmembrane movement in rat liver parenchymal cells.

In the study reported here we describe the isolation of plasma-membrane vesicles from rat liver parenchymal cells which are competent to carry out the active transport of L-alanine in the presence of an artificial transmembrane Na^+ gradient in a manner consistent with an electrogenic mechanism. In addition, we also present data dealing with the L-alanine-transport activity of plasma-membrane vesicles prepared from liver parenchymal cells of fed and starved rats. It was found that membrane vesicles from starved rats exhibit an elevated rate of Na^+ -gradient-stimulated L-alanine transport compared with those from fed rats, and that adrenalectomy abolished the increase in transport owing to starvation.

Experimental

Materials

L-[2,3- ^3H]Alanine and 3-O-methyl[1- ^3H]glucose were purchased from Amersham Corp. Valinomycin and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. Nitrocellulose filters were from Millipore Corp. All other chemicals were either reagent or ultra-pure grade.

Methods

Isolation and characterization of plasma-membrane vesicles. Liver parenchymal cells were isolated from adult rats (150–300 g each) by the collagenase-perfusion technique of Bonney *et al.* (1974). The cells were homogenized in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH/5 mM-MgCl₂ (pH 7.4) in a Potter-Elvehjem homogenizer until over 90% of the cells

were broken. The particulate material was collected by centrifugation for 20 min at 200 000 g_{max} , and resuspended to 63% (w/v) sucrose in the Hepes/KOH/MgCl₂. The plasma membrane was floated away from other subcellular fractions by a modification of the isopycnic flotation technique of Perdue *et al.* (1971). A 40–55% linear sucrose gradient was layered on top of the 63%-sucrose-containing homogenate and centrifuged for 60 min at 90 000 g_{max} in a SW-27 rotor. On completion of the centrifugation step there were four visible bands (fraction 1 pooled from the 40%- and 45%-sucrose zones, fraction 2 from the 48%-sucrose zone, fraction 3 from the 53%-sucrose zone), and a pellet at the 63%-sucrose zone (fraction 4). Each fraction was removed from the gradient and analysed for marker-enzyme activities and DNA content (Table 1). The plasma-membrane material was identified by monitoring two marker-enzyme activities, 5'-AMPase (EC 3.1.3.5) (Bischoff *et al.*, 1975) and adenylate cyclase (EC 4.6.1.1) (Brown *et al.*, 1971; Butcher *et al.*, 1975). To check for contamination of the putative plasma-membrane material by other cellular organelles, additional marker enzymes were monitored: cytochrome *c* reductase (EC 1.6.99.3) for microsomal fractions (Mackler & Green, 1956) and succinate dehydrogenase for mitochondria (King, 1968). In addition, DNA content was measured (Burton, 1956) as a marker for nuclei. Also, the original cell homogenate was monitored for marker-enzyme distribution. The two plasma-membrane marker enzymes selected, adenylate cyclase and 5'-AMPase, were located predominantly in fractions 1 and 2. Although fraction 2 had 3–4-fold greater total activity for these markers, the degree of contamination by microsomal fractions (cytochrome *c* reductase), mitochondria (succinate dehydrogenase) and nuclei (DNA content) was significantly less in fraction 1. In addition, the enrichment for each plasma-membrane marker enzyme was greater in fraction 1 than in fraction 2 when based on enzyme activity in the original cell homogenate. Finally, the Na^+ -gradient-stimulated transport activity for L-alanine in fraction 1 was 6 times that for fraction 2 (results not shown). Therefore only fraction-1 material was subsequently used in transport experiments. This material was removed from the gradients with a Pasteur pipette, pooled, and centrifuged free of sucrose at 200 000 g_{max} for 20 min. The resultant plasma-membrane pellet was resuspended in 20 mM-Hepes/KOH/5 mM-MgCl₂/250 mM-sucrose (pH 7.4) at 3–10 mg of protein/ml, and used in transport assays either fresh or stored at -85°C in 10% (v/v) glycerol (freezing and thawing once allows at least 80% retention of transport activity).

Amino acid transport by plasma-membrane vesicles. A modification of the method of Quinlan *et*

Table 1. *Distribution of subcellular fractions after sucrose-gradient centrifugation of cell homogenates from fed animals*

The marker-enzyme analysis for the subcellular fractions from liver of starved animals was not significantly different from that for fed animals. The positions of fractions 1–4 in the centrifuged sucrose gradient, and the methods used to monitor each marker, are described in the Experimental section. Specific activity (sp.act.) units: adenylate cyclase (pmol of cyclic AMP/10 min per mg of protein); 5'-AMPase (μ mol of P_i /10 min per mg of protein); cytochrome *c* reductase and succinate dehydrogenase (nmol of dichlorophenolindophenol reduced/min per mg of protein); DNA (μ g/mg of protein). Specific-activity values represent means $\pm$ S.E.M. for at least seven different animals, except for 5'-AMPase, which represents the mean of two separate preparations; % values represent total activity relative to homogenate (Homog.). Total percentage recovery represents the summation of %-total values for fractions 1–4.

Organelle marker	Cell fractions tested									
	Homog. sp.act.	Fraction 1		Fraction 2		Fraction 3		Fraction 4		Total percentage recovery
Adenylate cyclase	0.04 $\pm$ 0.01	0.16 $\pm$ 0.04	20.5	0.05 $\pm$ 0.02	76.7	0.03 $\pm$ 0.03	3.4	0	0	100.6
5'-AMPase	0.2	2.4	26.4	0.6	73.0	0.3	5.5	0	0	104.9
Cytochrome <i>c</i> reductase	83.7 $\pm$ 22.7	48.1 $\pm$ 1.2	6.0	161.8 $\pm$ 10	67.3	86.3 $\pm$ 13.1	10.0	15.6 $\pm$ 8.6	5.9	89.2
Succinate dehydrogenase	45.8 $\pm$ 8.1	10.9 $\pm$ 1.6	2.5	44.9 $\pm$ 6.3	29.4	63.9 $\pm$ 4.6	47.0	15.2 $\pm$ 6.5	3.3	82.2
DNA	19.3 $\pm$ 10.2	0	0	3.1 $\pm$ 1.8	0.8	1.1 $\pm$ 1.3	1.7	86.2 $\pm$ 23	85.6	88.1

al. (1976) was used to measure transport activity. In the standard assay, approx. 50 μ g of plasma-membrane protein was incubated in 9.0 mM-Hepes/KOH (pH 7.5)/240 mM-sucrose/0.2 mM-CaCl₂/4.8 mM-MgCl₂ (total reaction volume 50 μ l in separate test tubes). These membrane suspensions were pre-incubated at 34°C for 5 min, after which time NaCl (final concn. 100 mM) and L-[2,3-³H]alanine (final concn. 100 μ M; sp. radioactivity 1.8 Ci/mmol) were added together to initiate transport. At various times thereafter samples were diluted 1:20 with 4°C wash buffer [9.0 mM-Hepes/KOH (pH 7.5)/240 mM-sucrose/0.2 mM-CaCl₂/100 mM-NaCl]. These samples were immediately collected on nitrocellulose filters (0.3 μ m pore size) under vacuum and washed with 2 $\times$ 1 ml of 4°C wash buffer. The filters were air-dried and monitored for radioactivity content by liquid-scintillation spectrometry with a toluene-based scintillant.

An alternative protocol which gives qualitatively similar results is as follows. Rather than using separate test tubes for each assay point, a single reaction volume of 200 μ l containing all reaction components is used, with 20 μ l portions being removed and added directly to the filter at the appropriate time. This adsorbed membrane-vesicle material is then washed with 2 $\times$ 2.0 ml of 4°C wash buffer. All subsequent steps are as described above.

The intravesicular volume of the vesicle populations was determined by measuring the accumulation of 1.0 mM-3-O-methyl[³H]glucose by the membrane-filter assay. An average value of 1.45 $\pm$ 0.30 μ l intravesicular space/mg of protein was obtained. All subsequent transport measurements with [³H]alanine were expressed as concentration-in-

(C_i)/concentration-out (C_o), or as pmol/ μ l of intravesicular space.

In certain experiments the above standard transport assay was varied as follows: (1) pre-equilibrating the membrane vesicles in 100 mM-NaCl for 15 min; (2) substituting different salts for NaCl during co-addition with L-[³H]alanine; (3) varying the membrane-vesicle protein concentration, as well as extravesicular osmolarity, in the reaction medium. In addition, the membrane vesicles were hyperpolarized by the use of valinomycin and intravesicular KCl.

Staging animals for cell isolation. Rats (Sprague-Dawley) were maintained on a 12 h-light/12 h-dark schedule with standard chow (Purina) given *ad lib*. Liver parenchymal cells were isolated 3–5 h into the light schedule. Food had been withdrawn from the starved animals 24 h before the isolation of the cells. Bilateral adrenalectomy was performed on animals 5 days before isolation of cells. Adrenalectomized animals were maintained on 0.9% NaCl and were subjected to the same light/dark schedule as described above with food present *ad lib*.

Protein determination. Protein content was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Na⁺-stimulated transport of L-alanine by isolated plasma-membrane vesicles

The time-dependent ability of plasma-membrane vesicles derived from liver parenchymal cells of fed rats to transport L-alanine is depicted in Fig. 1. In the absence of extravesicular Na⁺, intravesicular

Fig. 1. Transport of L-alanine by plasma-membrane vesicles from fed-rat liver parenchymal cells

The time-dependent accumulation (plotted as C_i/C_o) of L-alanine was measured in membrane vesicles added to reaction mixtures of the following composition: L-[^3H]alanine alone (O, no NaCl present); L-[^3H]alanine and 100 mM-NaCl present together (●). In addition, membrane vesicles were also preincubated with 100 mM-NaCl for 15 min and then added to a NaCl-free reaction mixture containing L-[^3H]alanine (▲). L-[^3H]Alanine concentration and assay protocols are as indicated in the Experimental section. Each point represents the mean of two separate determinations, and the pattern reflects the results obtained from six different membrane preparations.

accumulation of L-alanine is linear with time for 2–3 min, after which a steady-state value is maintained for at least 15 min. When 100 mM-NaCl is co-added along with the L-[^3H]alanine, a marked 'overshoot' is measurable, which reaches a peak at about 3 min, with a C_i/C_o value of about 2.5. This active or concentrative accumulation of L-alanine is dissipated beyond 3 min, with the steady-state value attained by approx. 10 min. The asymmetric effect of Na^+ on L-alanine transport into the vesicles is evident from the observation (Fig. 1) that only a transient Na^+ gradient across the vesicle membrane (Na^+ concentration out > in) permits concentrative uptake. Pre-equilibration of the membrane vesicles with 100 mM-NaCl (15 min preincubation) before addition of L-[^3H]alanine results in an accumulation pattern nearly identical with that measured in the absence of Na^+ . Preliminary experiments determined that L-alanine accumulation was directly proportional to membrane-vesicle protein content, and inversely proportional to the reaction mixture osmolarity (results not shown).

Cation and anion effects on L-alanine transport

As indicated in Fig. 1, a Na^+ gradient established across the vesicle membrane permits an apparent concentrative or active transport of L-alanine. The specificity of this Na^+ effect was examined, with the results presented in Table 2. Neither K^+ nor Li^+ gradients across the vesicle membrane (extra-vesicular concentration > intravesicular) stimulate L-alanine transport to the extent produced by a Na^+ gradient, the latter resulting in a C_i/C_o value of 3.3. The transport of L-alanine supported by the Li^+ gradient is greater than that supported by the K^+ gradient. A gradient of choline chloride also failed to enhance L-alanine accumulation by the plasma-membrane vesicles (results not shown). It must be noted, however, that the influence of a Na^+ gradient on transport is markedly dependent on the type of anion used as the Na^+ counterion. For example, the stimulatory effects of a Na^+ gradient are enhanced greater than 2-fold when Cl^- is replaced by SCN^- . In contrast with this, the stimulatory effects are eliminated when SO_4^{2-} is substituted for Cl^- . Since the membrane-permeant nature of these anions is $\text{SCN}^- > \text{Cl}^- > \text{SO}_4^{2-}$, these results are consistent with the Na^+ -stimulated transport of L-alanine being an electrogenic process.

Valinomycin can enhance the stimulatory effects of a transmembrane Na^+ gradient

The electrogenic nature of the Na^+ -gradient-stimulated transport of L-alanine can be further substantiated by the use of valinomycin, an ionophore for K^+ . As the results from the experiment depicted in Fig. 2 show, membrane vesicles preloaded with K^+ and then added to a valinomycin + Na^+ reaction medium exhibit an increased ability to concentrate L-alanine when compared with

Table 2. Effect of cations and anions on gradient-stimulated transport of L-alanine

Plasma-membrane vesicles were used in the standard transport assay as described in the Experimental section, except that various salts other than NaCl were also co-added with the L-[^3H]alanine. The total length of time of the transport assay was 1 min. C_i/C_o was determined after 1 min of transport; values are expressed as means $\pm$ S.E.M. from three separate experiments.

Salt co-added with [^3H]alanine	Apparent concentration gradient, C_i/C_o
100 mM-NaCl	3.29 ± 0.38
100 mM-KCl	0.92 ± 0.22
100 mM-NaSCN	7.09 ± 0.67
100 mM-KSCN	0.69 ± 0.11
100 mM-LiCl	1.28 ± 0.23
50 mM- Na_2SO_4	1.04 ± 0.16

Fig. 2. Transport of L-alanine is influenced by an induced K^+ -permeation event brought about by valinomycin. Plasma-membrane vesicles were preincubated for 20 min at 34°C in a reaction medium containing 50 mM-KCl with (●) or without (○) valinomycin ($2\mu\text{g}$ in 2% ethanol/mg of membrane protein). The membrane vesicles were then diluted 8-fold into a transport reaction medium containing 100 mM-NaCl and $100\mu\text{M}$ -L-[^3H]alanine. As a function of time thereafter, transport was terminated and samples were processed as described in the Experimental section. The membrane vesicles not preincubated with valinomycin were in a reaction medium that contained 2% ethanol.

membrane vesicles not pre-loaded with K^+ . That is, an apparent induced K^+ permeation from inside to outside results in about a 2-fold greater ability of the Na^+ gradient (external concentration > internal) to concentrate L-alanine inside the vesicle interior. This type of response by the putative Na^+/L -alanine transport complex to a greater intravesicular electro-negativity is again consistent with an electrogenic transport mechanism.

L-Alanine transport by plasma-membrane vesicles isolated from liver parenchymal cells of fed and starved rats

Earlier studies with perfused rat liver *in vitro* have shown that L-alanine transport is a crossover point in the utilization of this amino acid for gluconeogenesis when hepatic gluconeogenesis is stimulated by starvation of rats (Mallette *et al.*, 1969a,b). Furthermore, studies using the non-metabolizable amino acid α -aminoisobutyric acid have also shown that amino acid transport is hormonally regulated (Kletzien *et al.*, 1975, 1976; Pariza *et al.*, 1977;

Fig. 3. Comparative L-alanine accumulation (plotted as C_i/C_o) by plasma-membrane vesicles isolated from liver parenchymal cells of fed and starved rats

Plasma-membrane vesicles were isolated from each type of rat as described in the Experimental section. A portion of the membrane vesicles was preincubated in 100 mM-NaCl, before initiation of the transport reaction, by adding membrane vesicles (○, fed rat; ●, starved rat) to a NaCl-free medium. Other membrane-vesicle populations (△, fed rat; ▲, starved rat) were added directly to 100 mM-NaCl (no preincubation) and $100\mu\text{M}$ -L-[^3H]alanine to initiate transport of the amino acid. At various times thereafter transport was terminated and samples were processed as described in the Experimental section. The inset represents the initial time-course of accumulation for those membrane vesicles preincubated with NaCl before exposure to L-[^3H]alanine. Each point represents the mean of three separate determinations $\pm$ S.E.M. (represented as bars). During the course of this study, we observed that the absolute magnitude of the transport rates varied between different membrane preparations, but a 4–5-fold difference in rate between vesicle preparations from fed and starved animals was observed in each case. Therefore the pattern depicted reflects the results obtained in one experiment, but the difference in transport rates was observed in five different membrane vesicle preparations from fed and starved animals.

Fehlman *et al.*, 1979a,b; Kelley *et al.*, 1980a,b). From these above observations, one can hypothesize that liver parenchymal-cell plasma-membrane vesicles from starved rats will transport

Fig. 4. Adrenalectomy blocks the starvation-induced increase in initial transport activity for L-alanine by rat liver parenchymal-cell-derived plasma-membrane vesicles

Bilateral adrenalectomy was followed by a 5-day recovery period during which time animals were fed. Over the next 24h animals were either fed (○), starved (●), or starved plus dexamethasone-replete (▲). Animals were made replete with a total of 100µg of dexamethasone/100g body wt. over the 24h starvation period with five individual doses of intramuscularly injected hormone. All transport assays on isolated plasma-membrane vesicles were performed in the presence of a 100mM transmembrane Na⁺ gradient. Each point represents the mean of three separate determinations ± S.E.M. (represented by bars). The data reflect the results observed in two different membrane-vesicle preparations from the fed, starved and starved/replete animals.

L-alanine at a rate greater than membrane vesicles from liver parenchymal cells of fed rats. The data presented in Fig. 3 support this hypothesis for both Na⁺-gradient-stimulated, and possibly non-gradient-stimulated, transport of this neutral amino acid. Within the first 60s of transport, the gradient-stimulated concentrative accumulation of L-alanine by membrane vesicles from liver parenchymal cells of starved rats is about 3–5 times that exhibited by membrane vesicles from fed rats.

L-Alanine transport by plasma-membrane vesicles from liver parenchymal cells of fed and starved adrenalectomized rats

Although the starvation-induced increase in L-alanine transport activity exhibited by liver parenchymal cells is at least partly mediated *in vivo* by increased plasma glucagon, the glucocorticoids may

also play an important 'permissive' role, on the basis of their effects on amino acid transport in cultured hepatocytes (Kletzien *et al.*, 1975; Pariza *et al.*, 1977; Kelley *et al.*, 1980b). In order to determine whether the putative glucagon–glucocorticoid interaction *in vivo* results in an enhanced L-alanine transport capability by parenchymal-cell-derived plasma-membrane vesicles, rats were subjected to bilateral adrenalectomy before starvation. These adrenalectomized rats were then subjected to one of three treatments: (1) feeding, (2) starvation, or (3) starvation along with dexamethasone administration (details are given in the Experimental section and in the legend to Fig. 4). After these treatments plasma-membrane vesicles are isolated as before and monitored for Na⁺-gradient-stimulated L-alanine transport activity. As opposed to the effects of starvation on uptake observed without adrenalectomy (Fig. 3), the results presented in Fig. 4 demonstrate that adrenal-gland removal essentially eliminates this response. That is, L-alanine transport after starvation is at the same level as after feeding. However, liver parenchymal-cell plasma-membrane vesicles isolated from adrenalectomized rats also given dexamethasone exhibited a 3–4-fold greater Na⁺-gradient-stimulated L-alanine transport rate. Thus glucocorticoid administration permits the enhanced uptake activity brought about by starvation as seen in non-adrenalectomized animals.

A summary of the data depicted in Figs. 3 and 4 is presented in Table 3, with transport activity expressed as pmol of L-alanine accumulated/µl intravesicular space. Most important is the 4–5-fold difference in initial (45 s) transport rates for L-

Table 3. Specific L-alanine transport activities of liver parenchymal-cell plasma-membrane vesicles isolated from fed, starved and adrenalectomized rats

Rats were fed or starved as described in the Experimental section; some rats were subjected to bilateral adrenalectomy (Adx) with or without glucocorticoid (dexamethasone) repletion as described for Fig. 4. Transport activity values are expressed as pmol of L-alanine accumulated per µl intravesicular space (1.45 ± 0.03 µl/mg of protein); transport assays were carried out in the presence of a transmembrane Na⁺ gradient. Transport values represent means of duplicate determinations, ± S.E.M.

Rat treatment	Specific transport activity	
	45 s	10 min
Fed	155 ± 33	129 ± 26
Starved	672 ± 158	198 ± 51
Adx/fed	252 ± 79	141 ± 22
Adx/starved	148 ± 35	131 ± 38
Adx + dexamethasone/starved	758 ± 127	202 ± 34

alanine by liver parenchymal-cell plasma-membrane vesicles from fed versus starved rats and from adrenalectomized/starved versus dexamethasone-repleted animals, as opposed to the very similar steady-state accumulation values (10 min).

Discussion

Much progress has been made in understanding cellular transport processes by utilizing experimental systems in which homogeneous populations of cells are used rather than cells in the intact organism or in tissue slices. A further extension of this step-down in degree of complexity involves the use of transport-competent plasma-membrane vesicles instead of intact cells. Membrane-vesicle transport systems allow the investigator to monitor the uptake of natural substrates while avoiding metabolic conversion or trapping as well as the complications of intracellular compartmentalization. Although these advantages are certainly important, the critical factor in determining the fidelity of such a cell-free transport system involves the demonstration that plasma-membrane-vesicle transport properties mirror those observed in the intact cells from which the membranes have been isolated.

Our results demonstrate that plasma-membrane vesicles prepared from rat liver parenchymal cells transport L-alanine in a manner similar to the intact liver and liver parenchymal cells. At an extracellular L-alanine concentration of 1 mM, the intact hepatocyte transports about 85% of this amino acid through the Na⁺-dependent A- and ASC-systems, and about 15% through the Na⁺-independent L-system. About 30% of the Na⁺-dependent transport of L-alanine can be sustained by replacing extracellular Na⁺ with Li⁺, with this representing transport through the ASC-system (Edmonson *et al.*, 1979). Our results, with 100 mM extravesicular NaCl, KCl or LiCl, indicate that at a 100 μ M extravesicular L-alanine concentration Na⁺-dependent transport is about 72% of the total, of which about 84% is through the A- and the balance (about 16%) through the ASC-system (Table 2). These values are in reasonable agreement with those obtained by using intact hepatocytes.

The Na⁺-gradient-supported transport of L-alanine by these rat liver parenchymal-cell-derived plasma-membrane vesicles is concentrative and/or active (Fig. 1), as well as dependent on the nature of the Na⁺ counterion used (Table 2). The pattern of anion stimulation (SCN⁻ > Cl⁻ > SO₄²⁻) indicates that more lipophilic anions result in an enhanced initial transport rate for L-alanine. The stimulation of L-alanine transport by extravesicular Na⁺ could be augmented by a valinomycin-induced efflux of pre-loaded K⁺ from the membrane vesicles. These findings together suggest that an electrogenic mechanism

energizes the active accumulation of L-alanine in these membrane vesicles, as it does in the intact cell (Kristensen, 1980). The steady-state accumulation of L-alanine by the membrane vesicles is inversely proportional to the extravesicular osmolarity, suggesting that L-alanine is accumulated within the intravesicular space rather than simply being adsorbed to the vesicle surface.

The isolation of plasma-membrane vesicles from rat liver, and the characterization of active L-alanine transport by these vesicles, has been accomplished by others (Van Amelsvoort *et al.*, 1978; Sips *et al.*, 1980a). It is difficult to compare our data with those of Sips *et al.* (1980a,b) and Van Amelsvoort *et al.* (1978), since they have used whole rat liver as a membrane source for most of their experimental results, and because they use the non-physiological salt, NaSCN, to generate a transmembrane gradient. Owing to the use of NaSCN, rather than NaCl, the time-course of L-alanine accumulation measured by those investigators exhibits an earlier and narrower overshoot when compared with our results (Fig. 1). Although Sips *et al.* (1980a) demonstrate the L-alanine transport mechanism exhibited by their membrane vesicles to be electrogenic, the difference between NaCl-stimulated and NaSCN-stimulated uptake is only about 10%. Our results (Table 2) indicate a 215% difference. This dissimilarity could be explained, in part, by the use of whole rat liver cells by Sips *et al.* (1980a) as a membrane source rather than only liver parenchymal cells, or by their use of a different membrane isolation procedure.

Amino acid transport into intact cells is markedly stimulated by the depletion of amino acids from primary cultures of hepatocytes (Kelley & Potter, 1978, 1979) or by starving the animals before hepatocyte isolation (Fehlman *et al.*, 1979a). This pattern of amino acid uptake regulation is reflected in the plasma-membrane vesicles isolated from rat liver parenchymal cells (Fig. 3). That is, membrane vesicles from starved rats exhibit a 3–4-fold greater initial rate of L-alanine transport than do vesicles from fed rats. Starvation is accompanied by an increase in the circulating concentrations of glucagon and the glucocorticoids (Exton, 1972). It is well established that these hormones regulate the hepatic gluconeogenic pathway (Exton, 1972) and that amino acid transport is a rate-determining step for the pathway (Mallette *et al.*, 1969a,b). The effects of glucagon on the pathway are multifocal, and the glucocorticoids, although exerting little effect by themselves, amplify the effects of glucagon (Exton, 1972). In studies on primary cultures of liver parenchymal cells, we have shown that glucocorticoids only slightly modify amino acid transport activity, but they greatly amplify glucagon stimulation of the 'A'-system (Kletzien *et al.*, 1975; Kelley *et al.*, 1980b). The results presented here show that

adrenalectomy abolishes the increased transport activity that is observed in membrane vesicles from starved rats, whereas glucocorticoid administration restores the increase. Thus the adrenal glucocorticoids play a vital role in the regulation of hepatic amino acid transport. Since amino acid depletion in primary cultures of hepatocytes also induces the 'A'-system for amino acid transport (Kelley & Potter, 1978), it is possible that longer-term starvation, which results in lowered plasma amino acid concentrations, could induce the 'A'-system through a hormone-independent mechanism.

Our results establish that the increased accumulation of L-alanine by rat liver *in vivo* and *in vitro* involves a change at the level of the cell's surface membrane. Although these results indicate that the transport rate differences measured in intact hepatocytes can be observed in their isolated surface membrane, they do not eliminate the possibility of a membrane-potential change (Villereal & Cook, 1978) or an alteration in the Na^+/K^+ pump (Spector *et al.*, 1980, 1981) contributing to this regulation phenomenon *in vivo*. In addition, at the level of the intact hepatocyte, the data presented here do not allow us to distinguish (for starved rats) between activation of a pre-existing transporter, increased synthesis and/or insertion of transporters, or changes in surface membrane fluidity (Carter-Su & Czech, 1980; Pilch *et al.*, 1980).

The demonstration that the physiological regulation of amino acid transport exhibited by the intact hepatocyte is also reflected in isolated plasma-membrane vesicles makes this cell-free transport system an ideal one in which to study the regulation of amino acid uptake by various hormones. The transport activity of the amino acid 'A'-system is enhanced by several hormones, though the specific role of plasma-membrane components in this process is currently not known. Transport studies using membrane vesicles prepared from hormone-treated hepatocytes could provide such information. Since the vesicular transport studies can be carried out with L-alanine, the major substrate in gluconeogenesis as well as an important negative allosteric effector of glycolysis (Foster & Blair, 1978), these studies may contribute to an understanding of overall glucose homeostasis.

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