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Glycine transport in mouse eggs and preimplantation conceptuses

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At least two Na^+-dependent systems for glycine transport became detectable, while another became undetectable during preimplantation development of mouse conceptuses. Glycine was taken up by a process in eggs and cleavage-stage conceptuses which closely resembles system Gly. Mediated transport at these stages was more rapid at higher Cl^- concentrations, sigmoidally related to the exogenous Na^+ concentration, and strongly inhibited by sarcosine but not by amino acids with larger side chains. Moreover, neither Li^+ nor choline could substitute for Na^+ in stimulating glycine transport. System Gly was the only mediated process detected for glycine uptake in unfertilized and fertilized eggs and two-cell conceptuses, but two, less conspicuous, sarcosine-resistant, Na^+-dependent components of transport also appeared to be present in eight-cell conceptuses. One of the latter components seemed to remain relatively inconspicuous when conceptuses formed blastocysts, while system Gly became undetectable. In contrast, the other less conspicuous component in eight-cell conceptuses appeared to become the most conspicuous transport process in blastocysts. The latter process, previously designated system $B^{0,+}$, was shown here also to interact strongly with a broad scope of zwitterionic and cationic amino acid structures. Moreover, transport of glycine via system $B^{0,+}$ was more rapid at higher Cl^- concentrations, and this Na^+-dependent process as well as Na^+-independent leucine uptake were inhibited by choline. Furthermore, Na^+-dependent amino acid transport in two-cell conceptuses and blastocysts was inhibited by 1.0 or 10 mM ouabain, but the inhibition was incomplete at both concentrations. Since Na^+/K^+-ATPase has not been detected in two-cell conceptuses, inhibition of amino acid transport by ouabain may not have been due solely to an effect on this enzyme. The level of system Gly activity decreased during the development of eight-cell conceptuses from eggs, and this decrease could contribute to an associated decline in intracellular glycine. Since other amino acids begin to compete strongly with glycine for transport when system $B^{0,+}$ replaces system Gly in conceptuses, this qualitative change in transport activity may help account for a further decrease in the glycine content of conceptuses, reported elsewhere to occur after they form blastocysts.

Introduction

The results of prior studies have been used to support the theory that amino acid transport system Gly decreases, whereas system A increases in activity during preimplantation development of mouse conceptuses [1–5]. Uptake of amino acids via system Gly is restricted mainly to glycine and

Abbreviations: BCO, 3-amino-*endo*bicyclo[3.2.1]octane-3-carboxylic acid; MeAIB, 2-(methylamino)isobutyrate; p.c., post coitum.

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its *N*-methylated derivatives [1] and, therefore, the observation that saturable glycine uptake by cleavage-stage conceptuses is not inhibited by L-leucine, L-alanine, or 2-(methylamino)isobutyrate (MeAIB) is consistent with the possibility that system Gly is present at these developmental stages [3]. Moreover, like system Gly [6], the manner in which glycine uptake by two-cell conceptuses is affected by variations in the Na^+ concentration is consistent with the possibility that two sodium ions are transported with each glycine molecule [4], although the stoichiometry of the associated fluxes of Na^+ and glycine have not been measured in preimplantation conceptuses. In contrast, leucine and alanine strongly inhibit Na^+-dependent glycine transport in preimplantation conceptuses after they form blastocysts [3], and these data are consistent with the possibility that system A mediates glycine transport at the latter stage of development [3,4]. System A accepts a much broader scope of amino acids as substrates than does system Gly [1], so Na^+-dependent transport of several other zwitterionic amino acids in blastocysts also has been used to support the view that system A is present in these conceptuses [2,5].

Although these prior studies are provocative, the substrate selectivities and other characteristics of the transport activities in preimplantation conceptuses may not have been studied well enough to conclude that they are systems Gly and A. For example, it has not been determined whether *N*-methylglycine (sarcosine) inhibits glycine uptake by cleavage-stage conceptuses, but such inhibition should be strong if transport is via system Gly [1]. Moreover, MeAIB is usually regarded as a system A-specific substrate [1], but its inability to inhibit glycine transport in blastocysts has been left unexplained [3]. Similarly, kinetic evidence for the involvement of two sodium ions with each glycine molecule transported by blastocysts [4] is inconsistent with similar kinetic studies of system A [6,7]. In fact, the data discussed in the preceding paragraph may be insufficient support even for the less than definitive conclusion that different systems mediate glycine transport in cleavage-stage conceptuses and in blastocysts. The substrate selectivity of a transport system apparently can change somewhat when one type of cell develops into another, as would the system's susceptibility to competitive and possibly noncompetitive inhibition [1,8,9]. Much of the argument in favor of the possibility that one system is replaced by another during development of preimplantation conceptuses depends, however, on the greater susceptibility of glycine uptake to inhibition by leucine and alanine when conceptuses form blastocysts [3,4]. For all of these reasons, a more thorough investigation of the characteristics of glycine transport in preimplantation mouse conceptuses seems warranted. When transport of substrates other than glycine has been studied according to established criteria [1,6,8,10,11], the results have supported the conclusion that the most conspicuous transport processes in mouse blastocysts [12–14] are unlike any of the well-characterized mammalian and avian processes such as systems Gly and A [1,8,10].

Some investigators may not have detected novel transport processes in preimplantation mouse conceptuses because they may have designed their experiments to determine if certain well-known transport systems were present there. Such approaches have proved limiting, however, and hence interactions among a wide variety of amino acid structures for transport ought to be investigated in cell types not yet examined in this manner [1,8]. Analog inhibition studies and 'ABC' testing are usually needed to detect heterogeneity of transport processes and to determine the range of substrate structures accepted by apparently homogeneous transport activities [1,8,10,11,15]. As part of such investigations in our laboratory [1,12–14], we have studied further the characteristics of glycine transport during preimplantation development of mouse conceptuses. A preliminary report of some of these findings has appeared previously [16].

Materials and Methods

The methods for obtaining eggs and conceptuses and measuring their ability to take up amino acids have been described recently [12,13]. Briefly, groups of Swiss ICR mice (Harlan Sprague Dawley Inc.) were induced to ovulate and mate at about the same time utilizing gonadotropins [17]. Unfertilized eggs were removed from the ampulla of the oviduct in warm Brinster's medium [18] about 15 h after administration of human chorionic

gonadotropin (about 3 h after ovulation). Eggs were freed from cumulus cells by briefly (less than 5 min) exposing them to 145 IU of hyaluronidase (Sigma Chemical Company) in 1.0 ml of Brinster's medium. One-, two-, or eight-cell conceptuses were obtained from the oviduct about 3, 28 and 54 h post coitum (p.c.), respectively. Blastocysts were flushed from the uterine lumen with a stream of culture medium about 82 h p.c. The zonae pellucidae were removed from some conceptuses by placing them in an isotonic solution at pH 2.5 for a few seconds [19]. All eggs and conceptuses were washed and stored briefly (less than 6 h) in Brinster's medium in a humidified incubator containing an atmosphere of 5% CO_2/95% air at 37°C (pH 7.4). Transport activity was not observed to change in these eggs or conceptuses during storage in this manner (data not shown), although transport activity has been observed to change rapidly during incubation of blastocysts obtained nearer the time of implantation [13]. In some cases, blastocysts were incubated with 5 μg of cytochalasin B (Sigma) per ml of Brinster's medium for 1 or 2 h to collapse them, and some conceptuses were treated with 1.0 or 10 mM ouabain (Sigma) for 1 or 2 h. Stock solutions of cytochalasin B were prepared in dimethylsulfoxide and diluted 200-fold in medium.

Eggs or conceptuses were incubated with [^{3}H]glycine, L-[^{3}H]leucine, L-[^{3}H]lysine or L-[^{3}H]alanine (20–60 Ci/mmol; ICN Pharmaceuticals) and various concentrations of nonradioactive amino acids as indicated in the figures and tables. Amino acids were dissolved in Brinster's medium or a modification of this medium ($NaHCO_3$ replaced with $KHCO_3$ and sodium salts of pyruvate and lactate replaced with NaCl) when the labeling period was 15 or 20 min. Amino acids were usually dissolved in phosphate-buffered NaCl (57 mM Na_2HPO_4, 18 mM KH_2PO_4, 46 mM NaCl and 1.0 mg/ml bovine serum albumin; pH 7.1) when the labeling period was 5 min. For one series of experiments, a more dilute concentration of phosphate was used to buffer the isotonic solutions in which transport was measured at pH 7, as described in the legend of Table III. Each of the phosphate-buffered media made it possible to maintain the desired H^+ concentration while conceptuses were kept in this medium on a slide warmer for 5 min at 37°C during labeling. These methods permitted us to perform more rapid and reproducible analog inhibition and kinetic studies than when conceptuses were kept in bicarbonate-buffered medium in a CO_2 incubator during a 5 min labeling period. On the other hand, an environment more like those known to promote development of conceptuses in vitro (e.g., Brinster's medium in an atmosphere of 5% CO_2 at 37°C) was considered preferable when a longer labeling period of 15 or 20 min was utilized. Uptake of glycine from a 100 μM solution in modified Brinster's medium increased linearly with time for more than 20 min (data not shown). In some cases, Cl^- in the medium was replaced with acetate. In other cases, Na^+ was replaced with choline or Li^+ during labeling. Two-cell conceptuses did not appear to be irreversibly altered by Na^+-free medium, since they exhibited a normal level of Na^+-dependent amino acid transport after incubation in Na^+-free Brinster's medium for 1 h [20]. Eggs or conceptuses were processed as described previously [12,13] to determine the amount of a labeled substrate they had taken up. Commonly used statistical methods were used to analyze the data [21,22] as indicated in the legends of the figures and tables.

The concentrations of amino acids in the medium are unlikely to have changed during experiments in ways that would have significantly affected the results. Eggs and conceptuses do not contain enough free amino acids [23] to have increased the total concentration of these substances in the medium by more than a total of 0.5 μM, and the concentrations of radioactivity in the medium did not change throughout the experiments. Nonradioactive amino acids were purchased from Sigma Chemical Company (MeAIB, L-glutamate, L-serine, L-lysine, glycine, L-pipecolate, L-alanine, L-leucine, L-proline and sarcosine) or were a gift from Professor Carmen Avendaño (3-amino-*endo*bicyclo[3.2.1]octane-3-carboxylic acid, BCO; Ref. 24).

Results

Effects of ions on transport

In order to study the effect of the Na^+ concentration on amino acid transport it is necessary

TABLE I

EFFECT OF ADDITION OF CHOLINE AND OTHER SUBSTANCES TO BRINSTER'S MEDIUM ON UPTAKE OF GLYCINE BY PREIMPLANTATION CONCEPTUSES

Transport was measured by incubating conceptuses with 1.0 μM [^{3}H]glycine for 20 min in Brinster's medium (140 mM Na^+) to which the indicated substance had been added. The means ± S.E. per conceptus of four to 16 replicate determinations (number shown in parentheses) are reported for data obtained in two to eight independent experiments at each stage of development (five conceptuses/determination). Groups that could be distinguished statistically are marked with different superscripts ($P < 0.05$; analysis of variance). Transport rates at different stages of development were not compared statistically.

Substance added to medium	Percent of glycine uptake relative to 'none' group ± S.E. (n)			
	one-cell	two-cell	eight-cell	blastocyst
None	100.0 ± 9.2 (4)	100.1 ± 3.2 [a] (16)	100.1 ± 5.0 (9)	99.9 ± 3.4 [x] (12)
70 mM choline chloride	91.0 ± 8.1 (4)	79.1 ± 6.3 [b] (16)	77.5 ± 6.5 (9)	43.3 ± 3.6 [y] (12)
70 mM lithium chloride	–	88.0 ± 7.0 [a,b] (10)	–	74.4 ± 5.2 [x] (4)
70 mM sodium acetate	–	101.0 ± 7.1 [a] (10)	–	115.4 ± 12.3 [x] (4)
140 mM mannitol	87.2 ± 16.1 (4)	80.6 ± 5.0 [b] (16)	90.7 ± 8.4 (7)	86.6 ± 7.7 [x] (10)
70 mM sodium chloride	140.2 ± 18.2 (4)	111.3 ± 7.6 [a] (15)	113.3 ± 14.5 (9)	105.5 ± 6.7 [x] (12)

to replace it with another cation. Such substitutes for Na^+ should not stimulate or inhibit the transport processes to be examined. When a cation with unknown effects on transport is merely substituted for Na^+, however, similar results are expected for both a lack of stimulation and for inhibition. Therefore, it may be necessary to add substances to medium (Tables I–III), as well as substitute them for Na^+ (Figs. 1 and 2), in order to ascertain their actual effects on transport. We found that glycine uptake was predominantly Na^+-dependent in unfertilized and fertilized eggs, cleavage-stage conceptuses and blastocysts, and neither Li^+ nor choline substituted appreciably for Na^+ in stimulating glycine transport (Fig. 1). On the other hand, when choline and Li^+ were added to Brinster's medium in addition to the salts it already contained, choline, but not Li^+, inhibited uptake of glycine in blastocysts (Tables I and II), but not in cleavage-stage conceptuses (Table I). Although added choline may have slowed glycine uptake in two-cell conceptuses somewhat, this effect could not be attributed specifically to choline and may have resulted, instead, from a negative effect of increased osmolality on transport in these conceptuses (Table I). Na^+-independent leucine uptake by blastocysts also appeared to be inhibited by addition of choline to an isotonic solu-

TABLE II

EFFECT OF ADDITION OF CHOLINE AND OTHER SUBSTANCES TO BRINSTER'S MEDIUM ON UPTAKE OF L-ALANINE, GLYCINE, L-LYSINE AND L-LEUCINE BY BLASTOCYST

Transport was measured by incubating blastocysts with 1 μM ^{3}H-labeled substrate for 5 min in Brinster's medium (140 mM Na^+) to which the indicated substance had been added. The means (±S.E.) of four replicate determinations are reported (approx. five blastocysts/determination). Statistically significant inhibition is indicated by double asterisks ($P < 0.01$; analysis of variance).

Substance added to medium	Percent of uptake relative to 'none' group ± S.E.			
	L-alanine	glycine	L-lysine	L-leucine
None	100.0 ± 8.8	100.0 ± 12.9	100.0 ± 6.2	100.0 ± 6.1
70 mM choline chloride	54.2 ± 3.4 **	41.3 ± 6.1 **	59.5 ± 4.3 **	47.5 ± 8.2 **
70 mM lithium chloride	104.3 ± 7.0	104.8 ± 6.9	107.7 ± 9.7	117.7 ± 4.5
70 mM sodium acetate	99.3 ± 10.2	129.5 ± 10.8	76.4 ± 3.7	98.4 ± 7.3
140 mM mannitol	92.0 ± 7.8	97.1 ± 2.0	103.4 ± 11.7	127.0 ± 12.9
10 mM sarcosine	93.2 ± 9.3	84.3 ± 13.2	96.5 ± 3.2	87.6 ± 8.8

TABLE III

EFFECT OF ADDITION OF CHOLINE AND OTHER SUBSTANCES TO AN ISOTONIC MEDIUM ON Na^+-INDEPENDENT L-LEUCINE UPTAKE BY BLASTOCYSTS

Transport was measured by incubating blastocysts with 0.4 μM [^{3}H]leucine for 5 min in 150 mM LiCl or choline chloride each of which was buffered with 0.75 mM potassium phosphate (pH 7) and to which the indicated substance had been added. The mean (±S.E.) of four replicate determinations (approx. six blastocysts/determination) are reported for data obtained in two independent experiments. Groups that could be distinguished statistically are marked with different superscripts ($P < 0.05$; analysis of variance). Moreover, although it is not indicated in the table, transport in 150 mM choline chloride was approx. 44% slower than that in 150 mM LiCl ($P < 0.01$).

Substance added to medium	Percent of uptake relative to 'none' group ± S.E.	
	in 150 mM lithium chloride	in 150 mM choline chloride
None	100.0 ± 9.5 ^{b}	100.0 ± 5.0 ^{x}
70 mM lithium chloride	85.9 ± 4.6 ^{b}	97.1 ± 4.1 ^{x}
70 mM choline chloride	57.3 ± 2.6 ^{c}	78.6 ± 4.5 ^{y,z}
70 mM potassium chloride	61.1 ± 5.2 ^{c}	71.8 ± 3.6 ^{z}
70 mM sodium chloride	281.1 ± 26.7 ^{a}	274.1 ± 23.4 ^{w}
140 mM mannitol	81.6 ± 2.2 ^{b}	81.6 ± 2.4 ^{y,z}
140 mM sucrose	80.0 ± 3.5 ^{b}	89.3 ± 3.9 ^{x,y}

tion of LiCl (Table III). An almost linear relationship between glycine transport in blastocysts and the Na^+ concentration was observed when Li^+ was substituted for various amounts of Na^+ (Fig. 2A). In contrast, the shape of the curve seemed to be a function of both Na^+ stimulation and choline inhibition when the concentrations of choline and Na^+ were varied inversely (Fig. 2A). There was a sigmoidal relationship between glycine transport in two-cell conceptuses and the Na^+ concentration (Fig. 2A). Nevertheless, leaving the numerical value of the Na^+ concentration at its first power resulted in a good fit of the latter data to a linear transformation (correlation coefficient = 0.995), whereas raising it to higher powers produced curved lines (Fig. 2B). Glycine flux was also more rapid in blastocysts and two-cell conceptuses at higher Cl^- concentrations (Fig. 3), and a 64% reduction in glycine uptake by eight-cell conceptuses occurred when the Cl^- concentration was reduced from 46 to 0 mM in phosphate-buffered medium (data not shown). We attribute slower transport to lower Cl^- concentrations, instead of inhibition by acetate, because addition of acetate to Brinster's medium did not inhibit amino acid uptake (Tables I and II).

Tests for the presence of more than one glycine transport process in eggs and conceptuses

Uptake of glycine from a 1.0 μM solution decreased during cleavage and then increased again when blastocysts formed (Fig. 1). During cleavage, only glycine and sarcosine inhibited most of the Na^+-dependent uptake of radiolabeled substrate. In contrast, several amino acids inhibited almost all of the glycine transport in blastocysts (Fig. 1), whereas no effect of sarcosine was detected. Similar results were obtained after the zona pellucida was removed from the two-cell conceptus or blastocyst, suggesting that this structure does not greatly alter the characteristics of glycine transport (data not shown). MeAIB, BCO, pipecolate (structurally similar to proline), alanine and leucine emerged as either weak or partial inhibitors of glycine uptake by conceptuses during cleavage (Fig. 1). Therefore, most of these substances were used in analog inhibition studies to test for possible heterogeneity of mediated transport processes.

The results of experiments with various concentrations of alanine, leucine and MeAIB supported the conclusion that these amino acids weakly inhibit the most conspicuous component of glycine transport in unfertilized eggs (Fig. 4) and eight-cell conceptuses (Fig. 5). Proline was a stronger inhibitor than alanine, leucine or MeAIB, and sarcosine appeared to inhibit mediated glycine uptake strongly and completely (Fig. 4) or nearly completely (Fig. 5) in unfertilized eggs and eight-cell conceptuses, respectively. Lysine did not inhibit the most conspicuous component of glycine transport at the latter stages of development. In blastocysts, in contrast, sarcosine was a weak inhibitor of glycine flux, inhibition by proline was somewhat stronger and lysine inhibited almost all of the glycine uptake strongly and completely (Fig. 6). Similar results were obtained when blastocysts were collapsed with cytochalasin B prior to measuring glycine transport (data not shown).

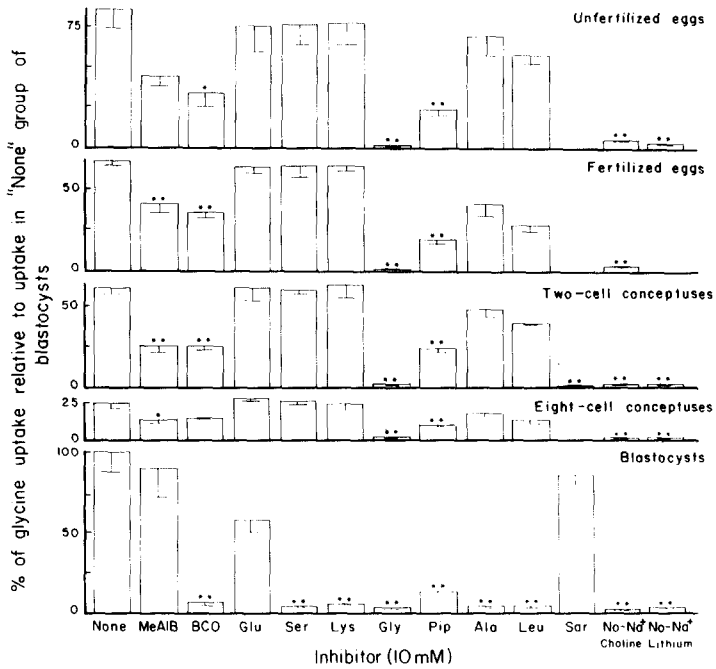

Fig. 1. Effect of various amino acids or lack of Na^+ on glycine uptake by eggs and conceptuses. Eggs or conceptuses were incubated with 1.0 μM [^{3}H]glycine for 20 min in Brinster's medium, modified Brinster's medium or these media in which Li^+ or choline was substituted for Na^+. Each column represents the mean ± S.E. uptake per egg or conceptus of four replicate determinations (five eggs or conceptuses/determination) obtained in two independent experiments (two determinations for effect of alanine and leucine on uptake in one-cell, two-cell and eight-cell conceptuses). Significant inhibition, relative to the 'none' group at each stage of development, is indicated by single ($P < 0.05$) or double ($P < 0.01$) asterisks as determined with analysis of variance (means of only two determinations were not included in the analyses). Uptake decreased significantly between the two-cell and eight-cell stages ($P < 0.05$) and then increased again when blastocysts formed ($P < 0.01$). Pip, L-pipecolate; Sar, sarcosine.

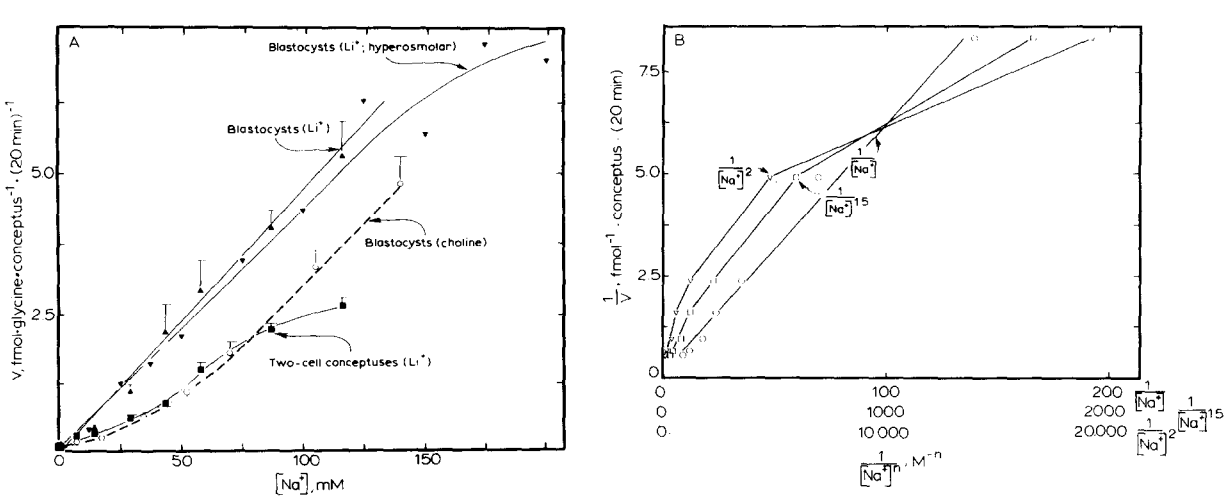

Fig. 2. Relationship between the Na^+ concentration and glycine uptake by two-cell conceptuses and blastocysts. (A) Conceptuses were incubated with 1.0 μM [^{3}H]glycine for 20 min in Brinster's medium (open symbols), modified Brinster's medium (filled symbols) or these media in which some or all of the Na^+ was replaced with Li^+ or choline. Each point represents the mean ± S.E. uptake per conceptus of four replicate determinations (five conceptuses/determination) obtained in two independent experiments (two determinations in hyperosmolar medium). The total concentration of Na^+ plus Li^+ was made to equal 200 mM in the hyperosmolar medium by adding the appropriate chloride salts (total of 84 mM) to modified Brinster's medium. Nonsaturable uptake has not been subtracted from the data presented. (B) Double-reciprocal relationship between net Na^+-dependent glycine uptake by two-cell conceptuses and the numerical value of the Na^+ concentration left at its first power (circles), or raised to its second (triangles) or 1.5th (squares) power. Net uptake was calculated by deducting glycine taken up in the absence of Na^+. The correlation coefficients are 0.995, 0.974 and 0.948 for the first, 1.5th and second powers of the value of the Na^+ concentration, respectively.

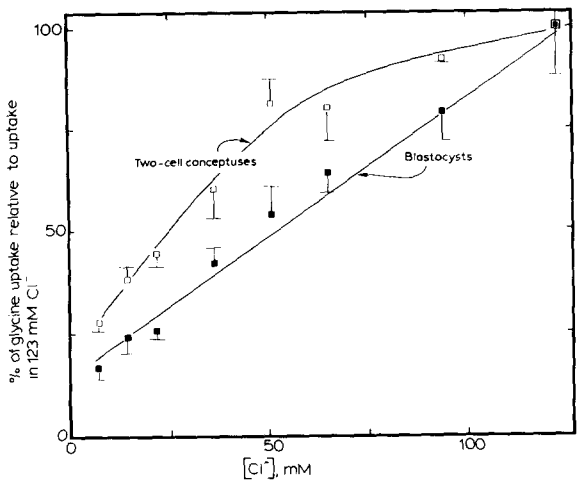

Fig. 3. Relationship between the Cl^- concentration and glycine uptake in two-cell conceptuses and blastocysts. Conceptuses were incubated with 1.0 μM [^{3}H]glycine for 20 min in modified Brinster's medium or this medium in which the Cl^- concentration was varied by replacing different amounts of it with acetate. Each point represent the mean $\pm$ S.E. uptake per conceptus of four replicate determinations (five conceptuses/determination) obtained in two independent experiments. Nonsaturable uptake has not been subtracted from the data presented.

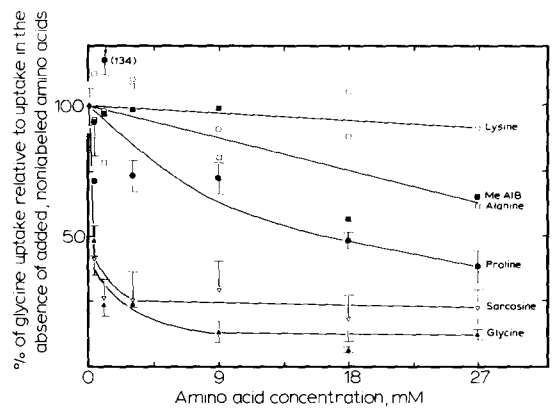

Fig. 5. Effect of various concentrations of sarcosine, L-proline, MeAIB, L-alanine and L-lysine on uptake of glycine by eight-cell conceptuses. Conceptuses were incubated with 2.0 μM [^{3}H]glycine and the indicated concentration of a nonradioactive amino acid for 5 min in phosphate-buffered NaCl. Each point represents the mean $\pm$ S.E. uptake per conceptus of five replicate determinations (approx. five conceptuses/determination) obtained in three independent experiments (four determinations at each concentration for alanine, sarcosine and MeAIB). Where the S.E. is not shown it is approx. 13% of the value reported.

An apparently small, lysine-resistant component of Na^+-dependent glycine transport was also detected in blastocysts (Fig. 6), but this component too was not inhibited by sarcosine (Fig. 7). Likewise, a less conspicuous, sarcosine-resistant component of Na^+-dependent glycine uptake was detected in eight-cell conceptuses, and this component appeared to be more completely inhibited by

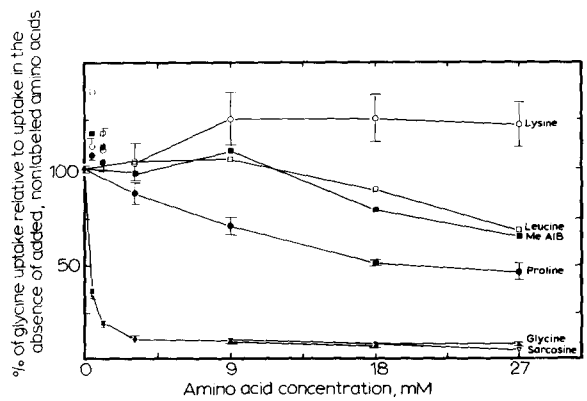

Fig. 4. Effect of various concentrations of sarcosine, L-proline, MeAIB, L-leucine and L-lysine on uptake of glycine by unfertilized eggs. Eggs were incubated with 2.0 μM [^{3}H]glycine and the indicated concentration of a nonradioactive amino acid for 5 min in phosphate-buffered NaCl. Each point represents the mean $\pm$ S.E. uptake per egg of four replicate determinations (nine or ten eggs/determination) obtained in two independent experiments (two determinations at each concentration for leucine, sarcosine and MeAIB).

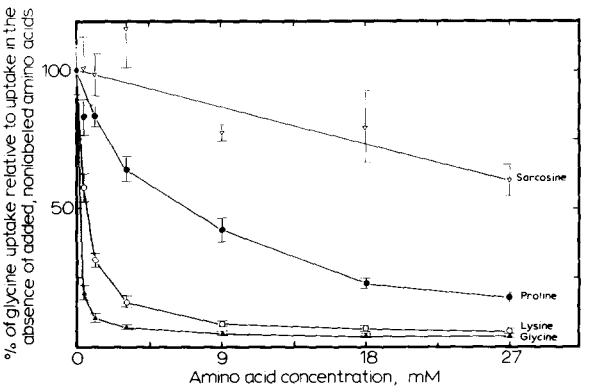

Fig. 6. Effect of various concentrations of sarcosine, L-proline and L-lysine on uptake of glycine by blastocysts. Blastocysts were incubated with 2.0 μM [^{3}H]glycine and the indicated concentration of a nonradioactive amino acid for 5 min in phosphate-buffered NaCl. Each point represents the mean $\pm$ S.E. uptake per blastocyst of four or five replicate determinations (approx. five blastocysts/determination) obtained in two independent experiments.

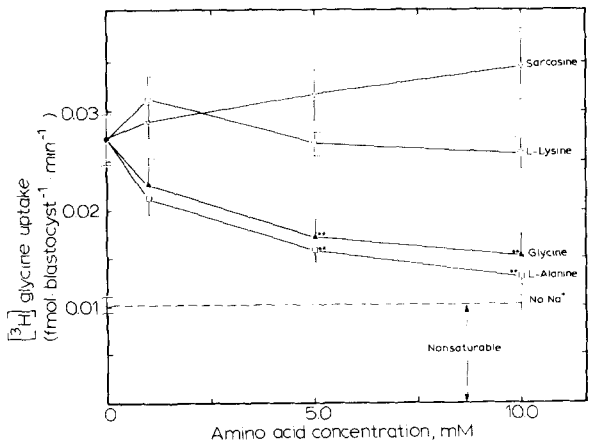

Fig. 7. Effect of various concentrations of L-alanine and sarcosine on L-lysine-resistant glycine uptake by blastocysts. Blastocysts were incubated with 2.5 μM [^{3}H]glycine and 20 mM L-lysine for 5 min in phosphate-buffered NaCl, or this medium in which Na^+ was replaced by Li^+ or choline to determine inhibition of Na^+-dependent transport by the indicated amino acids in addition to that provided by 20 mM lysine. Data for uptake in phosphate-buffered choline and lithium chloride were indistinguishable and were pooled. Each point represents the mean ± S.E. uptake of eight replicate determinations (approx. eleven blastocysts/determination) obtained in four independent experiments. Statistically significant inhibition beyond that provided by 20 mM lysine is indicated by double asterisks ($P < 0.01$) as determined with analysis of variance. Virtually all of the Na^+-independent glycine uptake detected (dashed line; mean ± S.E. uptake in Na^+-depleted media) appeared to occur via a nonsaturable route (i.e., most of the [^{3}H]glycine taken up in the presence of 10 mM glycine and 20 mM lysine).

alanine than by lysine (Fig. 8). In contrast, no sarcosine-resistant component of Na^+-dependent glycine transport was detected in unfertilized (Fig. 4) or fertilized (Fig. 8) eggs or two-cell conceptuses (data not shown). Virtually all of the lysine- or sarcosine-resistant Na^+-independent glycine uptake detected in blastocysts or cleavage-stage conceptuses (dashed lines in Figs. 7 and 8), respectively, appeared to occur via a nonsaturable route. Because of their apparently small contributions to total transport and the extra cost of performing experiments in which the minor components of Na^+-dependent glycine transport are quantified separately, only Na^+-independent or nonsaturable uptake was subtracted from total uptake to produce the data analyzed kinetically and shown later in Figs. 10, 11 and 12. First-order kinetics were assumed instead of more complex formulations (e.g., Ref. 25) to calculate the contribution of nonsaturable glycine uptake to total net uptake. These more complex descriptions may be necessary when significant exodus of the radiolabeled substrate being taken up occurs via a nonsaturable route while its net uptake is being measured [25], but glycine exodus and exchange

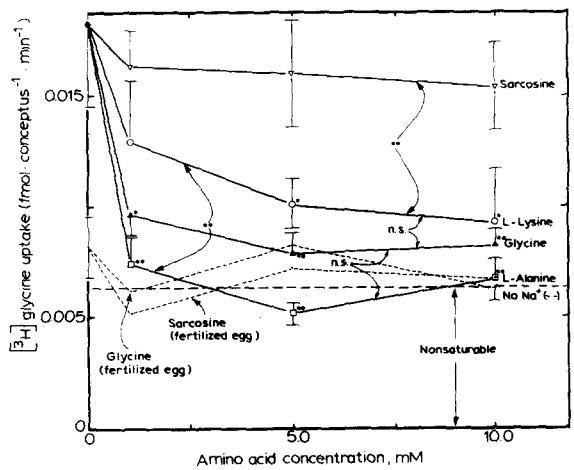

Fig. 8. Effect of various concentration of L-alanine and L-lysine on sarcosine-resistant glycine uptake by eight-cell conceptuses. Conceptuses were incubated with 2.5 μM [^{3}H]glycine and 20 mM sarcosine for 5 min in phosphate-buffered NaCl, or this medium in which Na^+ was replaced by Li^+ or choline to determine inhibition of Na^+-dependent glycine transport by the indicated amino acids in addition to that provided by 20 mM sarcosine. Data for uptake in phosphate-buffered choline and lithium chloride were indistinguishable and were pooled. Each point represents the mean ± S.E. uptake of eight replicate determinations (approx. nine conceptuses/determination) obtained in four independent experiments. Statistically significant inhibition beyond that provided by 20 mM sarcosine is indicated by single ($P < 0.05$) or double ($P < 0.01$) asterisks as determined with analysis of variance. In addition, statistically significant (**) and insignificant (n.s.) differences between the effects of various amino acids are indicated with arrows pointing to the lines for the pairs of amino acids compared. Virtually all of the Na^+-independent glycine uptake detected (dashed line; mean ± S.E. uptake in Na^+-depleted media) appeared to occur via a nonsaturable route (i.e., most of the [^{3}H]glycine taken up in the presence of 10 mM glycine and 20 mM sarcosine). In similar experiments, no sarcosine-resistant glycine transport was detected in fertilized eggs. The dotted lines represent uptake by fertilized eggs in the presence of various concentrations of glycine or sarcosine in addition to 20 mM sarcosine and were statistically indistinguishable. In the latter cases, each point represents the mean of four replicate determinations and the S.E. was about 13% of each mean.

are not detectable in preimplantation mouse conceptuses during 10-min incubation periods [3].

Effects of cytochalasin B and ouabain

The surface of the preimplantation mouse blastocyst is composed of a single layer of cells known as the trophoblast. This structure defines the fluid-filled blastocyst cavity and contains the inner cell mass. Na^+/K^+-ATPase appears to be located in the membrane on the inside surface of the trophoblast next to the blastocyst cavity [26–28]. Therefore, ouabain presumably can be brought into contact with this enzyme in blastocysts and the effect of this treatment on Na^+-de-

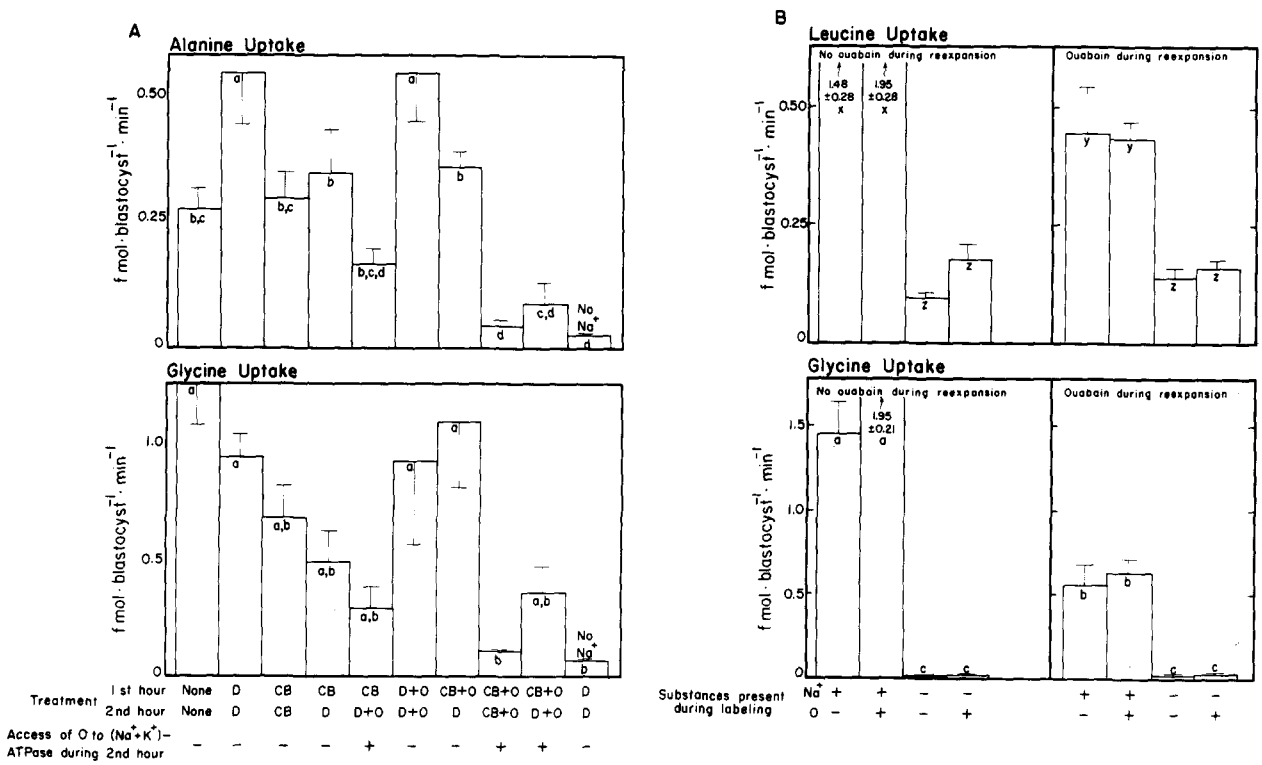

Fig. 9. Effects of cytochalasin B and ouabain on uptake of amino acids by two-cell conceptuses and blastocysts. Conceptuses were incubated with ouabain ('O', 1.0 mM in panels A and B and 10 mM in panel C) and/or cytochalasin B ('CB') for 1 h and then the same or different combinations of the same substances for an additional hour in Brinster's medium as indicated in the figure. CB was dissolved in dimethylsulfoxide ('D', 1.0 mg/ml) and then diluted to a final concentration of 5 μg/ml of medium, so some 'control' treatments contained 0.5% dimethylsulfoxide. After the indicated treatments, conceptuses were incubated with 2.0 μM [^{3}H]glycine, 0.9 μM L-[^{3}H]alanine, or 0.8 μM L-[^{3}H]leucine for 5 min to estimate their mean ± S.E. uptake. Treatments that resulted in statistically indistinguishable levels of transport ($P > 0.05$), as determined utilizing analysis of variance, are marked with the same letters at the top of the bars. (A) The means of four replicate determinations (about four blastocysts/determination) obtained in three independent experiments are reported after labeling blastocysts in Brinster's medium also containing 1.0 mM ouabain. In one case (column 10), choline was substituted for Na^+ in the medium used for labeling. (B) All blastocysts were treated with cytochalasin B and ouabain for 1 h before being allowed to reexpand (i.e., reform their blastocyst cavity) in Brinster's medium or this medium containing 1.0 oubain, but neither cytochalasin B nor dimethylsulfoxide for 70 min. The means of four replicate determinations (about six blastocysts/determination) obtained in two independent experiments are reported after labeling blastocysts in Brinster's medium or this medium in which choline had been substituted for Na^+ and in which 1.0 mM ouabain was or was not present as indicated in the figure. The results were similar when albumin in the medium was replaced with ficoll, 10 mM oubain was used instead of 1.0 mM oubain, or blastocysts were labeled in modified Brinster's medium or this medium in which Li^+ had been substituted for Na^+ (data not shown). (C) Two-cell conceptuses were incubated in Brinster's medium or this medium containing dimethylsulfoxide, cytochalasin B, 10 mM oubain, or cytochalasin B plus 10 mM oubain for 130 min before labeling them in Brinster's medium for 5 min. The means of twelve replicate determinations (about four conceptuses/determination) obtained in three independent experiments are reported.

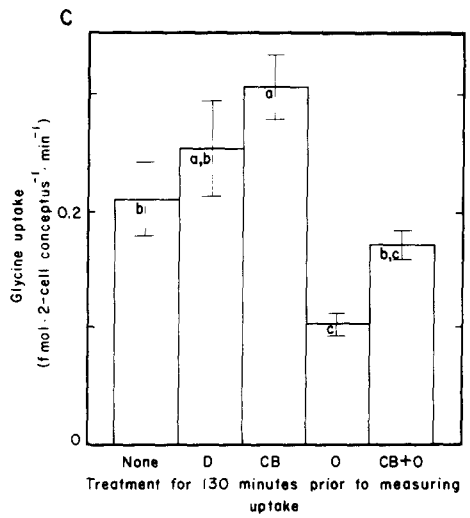

Fig. 9 (continued).

pendent amino acid transport can be tested by collapsing these conceptuses with cytochalasin B [26]. Transport of glycine or alanine was slower in blastocysts treated with both cytochalasin B and ouabain for 2 h prior to measuring uptake (column 8, Fig. 9A). When cytochalasin B was removed during the second hour of treatment (column 9, Fig. 9A), or ouabain was not included during the first (column 5, Fig. 9A), transport was not affected in a statistically significant manner. Hence, ouabain did not appear to inhibit completely the Na^+-dependent component of glycine and alanine transport (difference between column 10 and the other columns in Fig. 9A) whenever collapsed blastocysts were incubated with this compound. Nevertheless, uptake by ouabain-treated, collapsed blastocysts (i.e., those groups marked with '+' under Fig. 9A) also could not be distinguished on statistical grounds from Na^+-independent transport in these initial studies (Fig. 9A). Similar results were also obtained when amino acid uptake by blastocysts was measured in phosphate-buffered NaCl after treatments such as those indicated in Fig. 9A (data not shown). To determine whether or not ouabain would inhibit Na^+-dependent transport almost completely in collapsed blastocysts exposed to this compound, these conceptuses were collapsed with cytochalasin B in the presence of ouabain and then allowed to re-expand (i.e., reform their blastocyst cavity) in Brinster's medium or this medium containing ouabain prior to assessing their Na^+-dependent amino acid transport activity. (These treatments are the same as those indicated under columns 7 and 9 in Fig. 9A except that dimethylsulfoxide was not included in the medium during the second hour.) Although the Na^+-dependent component of transport of both glycine and leucine was reduced when ouabain was included in the culture medium during re-expansion, this transport was far from completely inhibited by either 1.0 or 10 mM ouabain (Fig 9B and data not shown for the same experimental design, 10 mM ouabain, and the use of Li^+ instead of choline to replace Na^+). Results similar to those reported in Fig. 9B were also obtained for glycine transport when ficoll (Pharmacia; Piscataway, N.J.) was substituted for albumin in Brinster's medium (data not shown). Inclusion of ouabain in the culture medium during re-expansion did not affect the Na^+-independent component of leucine transport (Fig. 9B), and most of the latter transport is mediated [14]. Transport of glycine was also inhibited in two-cell conceptuses treated with ouabain for about 2 h

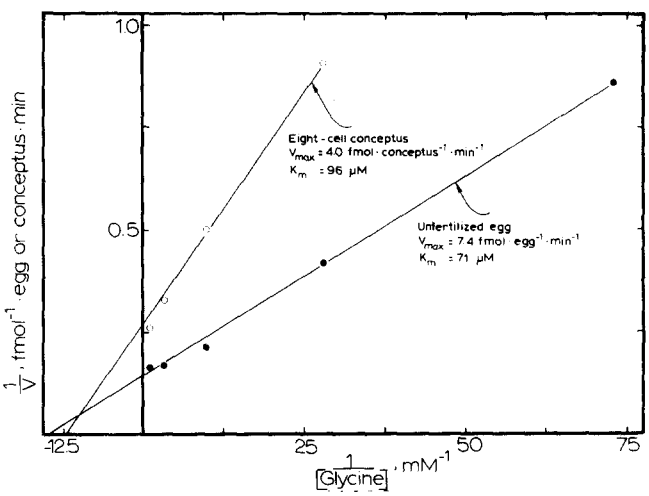

Fig. 10. Double-reciprocal relationship between mediated glycine influx in unfertilized eggs and eight-cell conceptuses and the glycine concentration. Eggs or conceptuses were incubated with the indicated concentration of substrate (2.5 μM [^{3}H]glycine) for 15 min in modified Brinster's medium. Each point represents the reciprocal of the mean uptake of four or eight determinations (approx. ten eggs or conceptuses/determination) obtained in two or four independent experiments for eight-cell conceptuses and unfertilized eggs, respectively. Nonsaturable uptake has been subtracted from the data presented. The correlation coefficient for each line is 0.999.

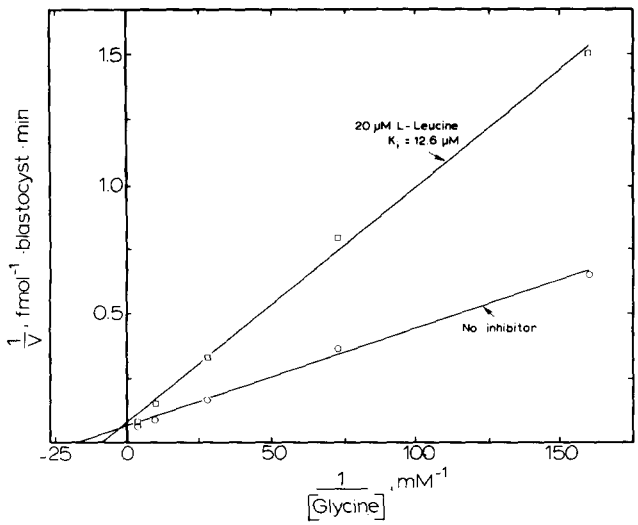

Fig. 11. Effect of 20 μM L-leucine on the double reciprocal relationship between Na^+-dependent glycine uptake by blastocysts and the glycine concentration. Blastocysts were incubated with the indicated concentrations of substrate (2.5 μM [^{3}H]glycine) for 5 minutes in phosphate-buffered NaCl. Each point represents the reciprocal of the mean uptake of five replicate determinations (about seven blastocysts/determination) obtained in two independent experiments. The lines represent transport after subtraction of nonsaturable uptake and the correlation coefficients are higher than 0.996. Each line would be shifted to make the apparent K_m and V_{max} values about 10% higher if nonsaturable uptake had not been deducted.

(Fig. 9C), although glycine transport was still predominantly Na^+-dependent in these conceptuses after they had been treated as described in Fig. 9B with 10 mM ouabain (data not shown).

Determination of kinetic parameters

The decrease in the rate of glycine uptake that occurred during development of eight-cell conceptuses from eggs (Fig. 1) reflected both a decrease in the apparent V_{max} value and an increase in the apparent K_m value for glycine transport (Fig. 10). Conversely, more rapid uptake of glycine in blastocysts than in eight-cell conceptuses (Fig. 1) was attributable to an increase in the V_{max} value and a decrease in the value of K_m (Table IV). Glycine and leucine appeared to compete with each other for Na^+-dependent uptake in blastocysts (Figs. 11 and 12), and the K_m and K_i values for these interactions were each about 50 μM for glycine and approx. 10 μM for leucine (Table IV; 'AB' portion of the 'ABC' test which is considered necessary to show that two amino acids compete for the same transporter; Refs. 1, 8, 15). Furthermore, the K_i values for inhibition of Na^+-dependent transport by lysine were about the same when either glycine or leucine was the substrate (Table IV; one instance of the 'C' part of the 'ABC' test).

Discussion

Qualitative changes in the characteristics of glycine transport during preimplantation development

We detected only one mediated process for glycine transport in unfertilized eggs, fertilized eggs and two-cell conceptuses. This sarcosine-sensitive Na^+-dependent process resists inhibition by

TABLE IV

KINETIC PARAMETERS FOR Na^+-DEPENDENT TRANSPORT AND INHIBITION OF TRANSPORT OF GLYCINE AND L-LEUCINE IN BLASTOCYSTS

Experiments were performed as described in the legends of Figs. 11 and 12 for parameters determined in phosphate-buffered media and as described in the legend of Fig. 10 for parameters determined in modified Brinster's medium. The mean K_i values reported were calculated from K_i values estimated at several substrate concentrations, such as those in Figs. 11 and 12, utilizing the formula: $K_i = (V_i/(V - V_i)) \cdot (K_m[I]/([S] + K_m))$; where V = substrate influx, V_i = substrate influx in the presence of inhibitor, [I] = inhibitor concentration and [S] = substrate concentration [13,14].

Substrate	Medium	Value of V_{max} (fmol · blastocyst^{-1} · min^{-1})	K_i or K_m value for each inhibitor [a]		
			glycine (μM)	L-leucine (μM)	L-lysine (μM)
Glycine	modified Brinster's	20	30 [a]		70
Glycine	phosphate-buffered	16	60 [a]	12.6	
L-Leucine	phosphate-buffered	7.5	46	5.0 [a]	50

[a] Values of K_m are reported when an amino acid is listed as its own 'inhibitor'.

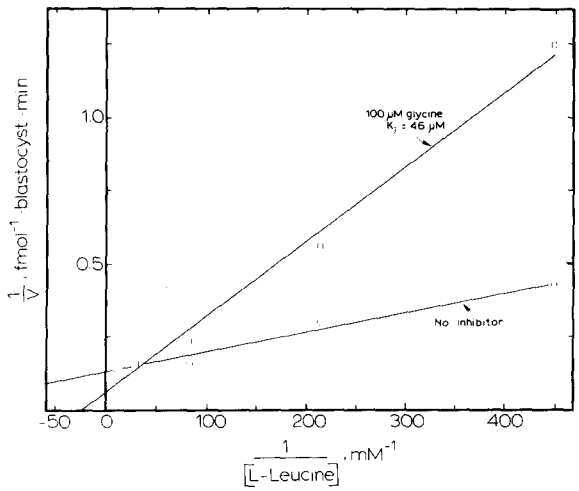

Fig. 12. Effect of 100 μM glycine on the double-reciprocal relationship between Na^+-dependent L-leucine influx in blastocysts and the leucine concentration. Blastocysts were incubated with the indicated concentrations of substrate (1.0 μM [^{3}H]leucine) for 5 min in phosphate-buffered NaCl or this medium in which Na^+ was replaced with Li^+. Transport in phosphate-buffered LiCl was subtracted from uptake in phosphate-buffered NaCl to yield net Na^+-dependent transport. Each point represents the reciprocal of the mean uptake of six replicate determinations (six or seven blastocysts/determination) obtained in three independent experiments. The correlation coefficients were 0.95 and 0.995 for the lines in the absence and presence of glycine, respectively. The large amount of error in the data presented here relative to our more usual results is probably attributable to the need to subtract a rather substantial Na^+-independent influx, with the greater absolute amount of error concomitant with its estimation, from total uptake to obtain net Na^+-dependent leucine transport. (The sum of the V_{max} values for Na^+-independent leucine transport [14] is about 5-fold higher than the V_{max} value for Na^+-dependent leucine transport reported here.) In addition, the K_m value for Na^+-dependent leucine transport was so low in the absence of an inhibitor (i.e., about 5 μM) that it might better have been estimated by measuring uptake at concentrations down to about 1/2 of the lowest leucine concentration used (i.e., down to about 1.0 μM leucine). The accuracy we might have gained, however, by repeating these studies with this lower concentration did not seem great enough to warrant the cost. The results reported in the figure are consistent with the interpretation that inhibition is competitive not noncompetitive and the estimate of the K_m value appears to be good enough for the purposes of ABC testing (see text).

lysine and most other amino acids, and it remains the most conspicuous route of glycine flux in conceptuses through the eight-cell stage (see summary in Table V). A less conspicuous, sarcosine-resistant Na^+-dependent component of mediated glycine transport is also present in eight-cell conceptuses, and it appears to be inhibited completely by alanine and partially by lysine (Fig. 8). Na^+-dependent alanine uptake by eight-cell conceptuses also appears to be inhibited incompletely by lysine (data not shown). Therefore, the less conspicuous portion of glycine transport in eight-cell conceptuses may have at least two components. One of the latter components seems to resemble an inconspicuous, alanine-sensitive, lysine-resistant component of transport in blastocysts (Fig. 7 and Table V). On the other hand, the apparently small lysine-sensitive portion of glycine transport in eight-cell conceptuses may become the predominant system in blastocysts. Lysine strongly and competitively inhibits the most conspicuous component of Na^+-dependent glycine transport in blastocysts, while sarcosine seems to be only a weak inhibitor of transport at this stage (Fig. 6). In fact, the sarcosine-sensitive transport process, which is so conspicuous in eggs and cleavage-stage conceptuses, was not detected in

TABLE V

SUMMARY OF CHANGES IN Na^+-DEPENDENT TRANSPORT ACTIVITIES DETECTED DURING DEVELOPMENT OF PREIMPLANTATION MOUSE CONCEPTUSES

Stage of development	Activities detected		
	system Gly (sarcosine-sensitive)	sarcosine- and lysine-resistant	system $B^{0,+}$ (lysine-sensitive)
Unfertilized egg	+ +	−	−
Fertilized egg	+ +	−	−
Two-cell conceptus	+ +	−	−
Eight-cell conceptus	+ +	+ [a]	+ [b]
Blastocyst	−	+	+ +

−, not detected; +, detected; + +, most conspicuous activity detected.

[a] The presence of this activity in eight-cell conceptuses is based on data presented in Fig. 8 and on a similar study of alanine uptake (data not shown).

[b] The substrate selectivity of the lysine-sensitive portion of transport has not been studied at the eight-cell stage of development. Therefore, we attribute it only tentatively to system $B^{0,+}$.

blastocysts (Fig. 7). Even when these conceptuses are collapsed with cytochalasin B in order to detect more easily transporters that may be present in the membrane next to the blastocyst cavity, most of the mediated glycine transport is sensitive to inhibition by lysine. The only known Na^+-independent transport processes for zwitterionic substrates in pre-implantation conceptuses prefer bulky amino acids [1,14]. This strong preference for bulky substrates probably explains why virtually all of the lysine- and sarcosine-resistant Na^+-independent glycine transport detected in conceptuses appears to occur via a nonsaturable route (Figs. 7 and 8).

Evidence for the presence of system Gly in eggs and cleavage-stage conceptuses

As expected for System Gly [8,10,29], sarcosine interacts strongly with the most conspicuous Na^+-dependent transport process detected in eggs and cleavage-stage conceptuses, whereas most amino acids with side chains larger than that of sarcosine inhibit glycine transport weakly if at all. Proline is the strongest inhibitor among the larger amino acids tested (Figs. 1, 4 and 5) and this is also the case for other occurrences of system Gly [29]. Moreover, although Li^+ can stimulate amino acid transport via at least two Na^+-dependent systems [9,30], it does not substitute appreciably for Na^+ in stimulating glycine uptake via system Gly in pigeon erythrocytes [31] or in mouse eggs and conceptuses (Figs. 1 and 2). Finally, like the familiar system Gly [32], the transport activity in cleavage-stage conceptuses is more rapid at higher Cl^- concentrations. The cost of mouse eggs and early conceptuses influenced us not to perform 'ABC' tests [1,8,15] to help determine more definitively whether glycine, sarcosine and proline compete for the same transporter. Even if the system in mouse eggs and cleavage-stage conceptuses is not precisely the same as other occurrences of system Gly, however, these systems appear to be sufficiently alike to have similar physiological functions in cells in situ.

Effect of Na^+ concentration on glycine transport

It has been concluded from the relationship between Na^+ concentration and glycine influx that two sodium ions are transported with each glycine molecule in cleavage-stage conceptuses [4]. Based on similar kinetic data Vidaver suggested that two sodium ions participate in the rate-limiting step catalyzed by system Gly [31]. Recently, data such as these were used to draw the same conclusion for glycine transport in the pig kidney brush border [33]. In contrast, the best linear transformation of our glycine transport data for two-cell conceptuses appears to be achieved when the numerical value of the Na^+ concentration is left at its first power (Fig. 2B). Interestingly, the stoichiometry observed for the associated fluxes of Na^+ and glycine via system Gly in the pigeon erythrocyte is approx. 1.53 : 1 [6,34]. Utilizing the original data [34], we calculated the 95% confidence interval for the latter ratio to be between 1.34 and 1.72. Therefore, the conclusion that two sodium ions participate in the rate-limiting step for glycine uptake via system Gly [31] may imply a level of understanding of the transport mechanism that is not yet warranted.

It also has been concluded from the relationship between Na^+ concentration and glycine influx that two sodium ions are transported by blastocysts with each glycine molecule they take up [4]. In general, however, the stoichiometry of the associated fluxes of Na^+ and amino acids cannot be deduced reliably from such relationships [6,34]. We have found that Na^+-dependent glycine transport is inhibited by choline in blastocysts (Tables I and II) and choline was used to replace Na^+ when its concentration was varied in previous experiments [4]. Hence the changes in glycine transport observed at different Na^+ concentrations [4] may be attributable to coincident variations in both the choline and Na^+ concentrations. A more nearly linear relationship between Na^+ concentration and glycine uptake by blastocysts is obtained when Na^+ is replaced by Li^+ instead of choline (Fig. 2A). Therefore, one reason why the relationship between Na^+ concentration and amino acid flux does not always reflect the associated fluxes of these two substances [6,34] may be that some of the osmolites substituted for Na^+ to vary its concentration themselves have undetected effects on these fluxes.

Nature of the most conspicuous transport activity in blastocysts

Our new data are difficult to reconcile with prior conclusions [3,4] that glycine is transported

in blastocysts mainly by a process which resembles system A. Mutually strong competitive inhibition of Na^+-dependent transport between glycine and leucine and K_m values for transport of each substrate similar to their K_i values for inhibition of transport (Table IV) supports the notion that these two substrates share the same transport sites. The strong reactivity of leucine with the transporter as indicated by the relatively low K_m and K_i values for this interaction is not characteristic of system A. Furthermore, transport of leucine and glycine are each strongly inhibited by lysine with about the same K_i value, and this is also uncharacteristic of System A. Similarly, BCO, serine and alanine inhibit Na^+-dependent glycine uptake almost completely, whereas 10 mM MeAIB, usually regarded as a system A-specific substrate [8,10], appears to have no effect on transport (Fig. 1). Moreover, Li^+ does not seem to substitute for Na^+ in stimulating glycine transport in blastocysts (Figs. 1 and 2A), although this ion substitutes well for Na^+ in stimulating glycine transport by system A in the Ehrlich cell [30]. The broad scope of amino acid structures that appear to compete strongly with glycine for transport in blastocysts is consistent with the conclusion that transport system $B^{0,+}$ [12,13] is primarily responsible for mediated glycine uptake by these conceptuses on day 4 post coitum. Although the presence of system $B^{0,+}$ in implanting [12] and delayed implanting [13] blastocysts has been documented, this is the first report that the system is present in blastocysts shortly after they form.

Lack of a clear relationship between glycine transport and Na^+/K^+-ATPase activity

Na^+-dependent alanine or glycine uptake was reported previously to be inhibited almost completely in blastocysts exposed to both cytochalasin B and 1.0 mM ouabain for a total of 2 h [26], and we were able to reproduce this result (Fig. 9A). We were unable to show, however, that a similar reduction in glycine or alanine transport occurs under all conditions in which 1.0 mM ouabain should have access to Na^+/K^+-ATPase for 1 or 2 h prior to measuring uptake. For example, when blastocysts are collapsed in the presence of cytochalasin B and ouabain, but then cytochalasin B is removed after 1 h by placing these conceptuses in fresh medium containing 1.0 mM ouabain, the effect on transport is smaller and not statistically significant after four or seven replicate determinations (Fig. 9A and data not shown for seven determinations in phosphate-buffered NaCl). As expected from the latter results, a large Na^+-dependent component of glycine or leucine uptake remains in blastocysts exposed to either 1.0 or 10 mM ouabain throughout treatment to first collapse these conceptuses and then allow them to re-expand in the presence of ouabain (Fig. 9B and data not shown for 10 mM ouabain). We interpret these data to mean that the synergistic effect of cytochalasin B and ouabain on Na^+-dependent transport might not be wholly attributable to the greater access ouabain may have to Na^+/K^+-ATPase after blastocysts are collapsed utilizing cytochalasin B. Moreover, ouabain inhibits Na^+-dependent glycine uptake in two-cell conceptuses (Fig. 9C), but Na^+/K^+-ATPase has not been detected at this stage of development utilizing cytochemical and immunocytochemical techniques [27,28]. García-Sancho and associates [35] also concluded that the effect of ouabain on amino acid transport in Ehrlich cells extends beyond inhibition of Na^+/K^+-ATPase. For all of these reasons, determination of the extent to which amino acid transport depends on the Na^+-gradient produced by Na^+/K^+-ATPase [36] in two-cell conceptuses and blastocysts must await development of an assay sensitive enough to determine routinely the level of this enzyme activity in preimplantation mouse conceptuses. Furthermore, the level of Na^+/K^+-ATPase activity may increase as a result of more rapid amino acid-dependent Na^+ uptake and the concomitant need for more rapid Na^+ extrusion [37]. Therefore, the interrelationship between these two transport processes, rather than dependence of only one on the other, probably should be studied when it becomes technically feasible to do so.

It is also not clear whether Na^+/K^+-ATPase is needed by the mouse conceptus to form and maintain its blastocyst cavity. Collapsed blastocysts re-expanded in the presence of 1.0 or 10 mM ouabain when cytochalasin B was removed from the incubation medium (Table VI, Fig. 9B, and data not shown), and other results support the conclusion that cavitation can occur when

TABLE VI

EFFECT OF CYTOCHALASIN B AND OUABAIN ON THE ABILITY OF BLASTOCYSTS TO MAINTAIN OR REFORM THEIR BLASTOCYST CAVITIES

Experiments were performed as described in the legend of Fig. 9 and the fractions of blastocysts that were collapsed after the first or second hour of treatment were recorded. The number of blastocysts collapsed over the total number of blastocysts observed is shown in parentheses. D, dimethylsulfoxide; CB, cytochalasin B; O, ouabain. Statistically significant re-expansion during the 2nd hour is indicated by double asterisks ($P < 0.01$; two-by-two contingency table).

Treatment during 1st hour	Percentage collapsed after 1st hour	Treatment during 2nd hour	Percentage collapsed after 2nd hour
None	0 (0/82)	None	0 (0/82)
D	0 (0/90)	D	4 (4/90)
CB	84 (69/82)	CB	100 (82/82)
CB	96 (79/82)	D	0 (0/82) **
CB	95 (78/82)	D+O	0 (0/82) **
D+O	0 (0/82)	D+O	0 (0/82)
CB+O	83 (68/82)	D	10 (8/82) **
CB+O	92 (85/92)	CB+O	100 (92/92)
CB+O	92 (77/84)	D+O	25 (21/84) **

Na^+/K^+-ATPase is not detectable with immunofluorescence and polyclonal antibodies against the Na^+/K^+-ATPase catalytic subunit [28]. In another study, however, mouse blastocysts that were collapsed in the presence of cytochalasin B and 1.0 mM ouabain did not re-expand unless both of these substances were removed from the medium [26]. Therefore, as is true for the relationship between amino acid transport and Na^+/K^+-ATPase, involvement of the latter enzyme in formation and maintenance of the mouse blastocyst cavity may remain unclear until it is feasible to assay the Na^+/K^+-ATPase activity actually present in preimplantation mouse conceptuses.

Relationship between transport activity and the glycine content of eggs and conceptuses

The decrease in system Gly activity (as measured by the V_{max} value) between the unfertilized egg and eight-cell stages (Fig. 10) correlates with, and could contribute to, the decline in the glycine content from 60 to 33% of the total α-amino acids in these cells during this period of development [23]. The increase in the apparent K_m value for glycine transport between these stages (Fig. 10) may be another reason for the decline in glycine content. The high substrate selectivity of system Gly and the slow efflux and exchange of glycine [3] in comparison to other amino acids [5] in eggs and cleavage-stage conceptuses may help account for their relatively high glycine content. Since system $B^{0,+}$ transports a broad scope of substrates, several amino acids may compete strongly with glycine for transport by blastocysts in situ. This competition could help produce the further decrease in the glycine content of conceptuses to about 7.2% of the total α-amino acids between the eight-cell and blastocyst stages [23]. Moreover, the relative glycine content of blastocysts may decrease because exodus of this amino acid is faster from blastocysts than from conceptuses at earlier stages of development [3], whereas exodus and exchange of other amino acids is slower in blastocysts than at earlier stages [5]. The total content of amino acids in preimplantation mouse conceptuses decreases by 50% or more when they are grown in vitro for a day or more in medium devoid of added amino acids [38]. Therefore, firm conclusions concerning the relationship between the glycine content of eggs and conceptuses and their amino acid transport activities will be possible only after the concentrations of amino acids in secretions of the reproductive tract have been determined. For example, if these concentrations in the mouse resemble those in fluid absorbed with Whatman GF/C paper from the rabbit uterus on day 6 post coitum (more than 75% glycine; Ref. 39), then system $B^{0,+}$ would function to take up mainly glycine in mouse blastocysts in situ. Systems similar to $B^{0,+}$ also may be present in immature *Xenopus* oocytes and fertilized sea urchin eggs, but the relationships between these transport systems, and intra- and extra-cellular amino acid concentrations are also matters of conjecture [1]. Nevertheless, if system $B^{0,+}$ and the $B^{0,+}$-like transport processes present at different stages of development in sea urchins, frogs and mice are eventually shown to be homologous [1], then elucidation of the mechanisms that control expression of these transport systems may help us learn how heterochrony occurs. Heterochrony, which is a change in the chronology of phenotypic expression is believed to be an important process by which species evolve [40].

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