

Amino acid transport in mouse blastocyst compartments

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SUMMARY

Properties of uptake and exchange of L-methionine associated with the compartments of the blastocyst were examined in the mouse embryo. The inner cell mass (ICM) was derived from the 102 h blastocyst by immunosurgical procedures and studied in isolation. The ICM possessed strong exchange and efflux transport character. The V_{\max} of transport was about one-fifth that of the intact blastocyst, but the K_m (660 μM) was about 30 times as high as that of the intact blastocyst. Consequently, at a concentration of about 100 μM -methionine, the velocity of uptake into the ICM is about one-thirtieth of that into the intact blastocyst. Uptake of amino acid into the intact blastocyst was resolved into cellular and cavity components by mechanically collapsing the blastocyst following uptake of radiolabelled methionine. By this method, it was found that about 70 % of the label accumulated by the blastocyst was in the cavity.

INTRODUCTION

An increased capacity to take up amino acid accompanies an increased programme of protein synthetic activity as the fertilized egg develops to the blastocyst stage (Kaye *et al.* 1982). In preimplantation mouse development, leucine (leu) and methionine (met) uptake is mediated by carrier-sponsored processes and qualitative and quantitative changes (particularly an increase in V_{\max} and an onset of Na^+ -dependence) in uptake occur over this early developmental period (Borland & Tasca, 1974, 1975). Methionine is a useful probe for studies of neutral amino acid transport in mammalian cells because it is taken up by both the exchangeable, Na^+ -independent L (leucine-preferring) system and the more broadly reactive, weakly exchangeable, Na^+ -dependent A (alanine-preferring) systems defined by Christensen (1984).

Embryonic development is marked, prior to implantation, by the formation of the blastocyst structure and the concomitant differentiation of its cellular components to yield the trophoectoderm and the inner cell mass (ICM, for a general discussion see Johnson, 1981). The trophoectodermal surface of the blastocyst is the membrane between the cellular cytoplasm and the uterine luminal environment. The distribution of amino acids taken up from the uterine fluid by the trophoectoderm interfacial system is of significance to the support of the ongoing development of the blastocyst and its cellular components.

Key words: amino acid transport, mouse, blastocyst, methionine, kinetics.

In the rabbit blastocyst, by far the bulk of amino acids are accumulated into the cavity (Miller & Schultz, 1983). Evidence has been presented for the existence of an exchange transport system on the inner trophectodermal face which would be capable of mobilizing these amino acids. Exposure of the inner trophectodermal surface of mouse blastocysts to incubation media by treatment with cytochalasin D also has suggested the presence of an exchange system in mouse embryos (Kaye *et al.* 1982).

In the mouse system, the ICM can be dissected free of the blastocyst by the technique of immunosurgery. After this treatment, amino acid transport properties of the isolated ICM can be studied. In addition, a technique has been developed to separate the amino acid accumulated by the cellular compartment from that accumulated by the cavity compartment. This physical separation of blastocyst compartments has allowed a more detailed study of amino acid transport at this stage of development than has been previously reported.

MATERIALS AND METHODS

Superovulation and embryo culture

Seven-week-old female random-bred Swiss Albino CD1 mice (Charles River, St. Constant, Quebec) were induced to superovulate as described previously (Kaye *et al.* 1982). At 84 h postfertilization, mice were killed by cervical dislocation and embryos were flushed from the uterus, and transferred through several washes of fresh Medium 2 (M2, Whittingham, 1971) containing 0.4 % (w/v) bovine serum albumin (Pentex, Miles Laboratories, Rexdale, Ontario). All embryos of developmental stage equal to or more advanced than early blastocyst were collected and then transferred through two washes of BMOC-3 (Brinster's medium for ovum culture) before being placed in 50 μ l droplets of BMOC-3 under silicon oil in 35 \times 100 mm disposable Petri dishes (Falcon, Oxnard, CA). About twenty blastocysts were placed in each drop. Embryos were cultured overnight at 37°C under an atmosphere of 5 % CO₂, 5 % O₂, with the balance N₂.

Expanded blastocysts were recovered from overnight culture in BMOC-3 as described above. In addition blastocysts were recovered by flushing the uterus with M2 at 96 to 98 h postfertilization. These were used in an experiment to compare uptake of embryos cultured *in vivo* to those cultured *in vitro*. Blastocysts that were not fully expanded or that had collapsed during manipulation were discarded.

Isolation of inner cell mass

At 96 to 98 h postfertilization, expanded blastocysts were recovered from overnight culture and placed in M2 at ambient temperature (approximately 22°C). Embryos were subjected to immunosurgery essentially as described by Handyside & Barton (1977). Following trophectodermal lysis, ICMs were isolated and transferred through several washes in M2. A 3 h recovery period in M2 at 37°C was allowed prior to transport experiments.

Two techniques were chosen to examine the viability of ICMs and to assess the integrity of ICM membranes following immunosurgery. The ICMs exhibited bright fluorescence as described by Mohr & Trounson (1980) and excluded trypan blue when incubated for 5 min in a 0.5 % solution of the dye in M2 medium without BSA.

Uptake of methionine by blastocyst and by inner cell mass

Radiolabelled met (L-[³⁵S]methionine, aqueous solution of approximately 13 mCi ml⁻¹, specific activity approximately 1300 Ci mmol⁻¹, Amersham, Oakville, Ontario) was used as a tracer. It was concentrated by lyophilization or diluted with stocks of unlabelled L-met (Sigma

Chem. Co., St. Louis, MO) to yield solutions of the required concentration and specific activity when added to M2 medium. Incubations were carried out in droplets of medium of sufficient volume (20 to 200 μ l) to render substrate depletion, substrate efflux or carry-over volumes during manipulations insignificant with respect to experimental requirements. These droplets were maintained under silicon oil in 35 \times 100 mm plastic Petri dishes. Following labelled uptake, ICMs or blastocysts were transferred through four 1.5 ml washes of M2 at 4°C to remove free L-[³⁵S]met. Experiments in which ICMs or intact blastocysts were placed in medium containing radiolabelled L-[³⁵S]met for a few seconds, washed, and assayed for radioactivity demonstrated that adsorption and carry-over of radioactivity was negligible and not measurable above background.

To examine the Na⁺-dependence of methionine uptake, ICMs and intact blastocysts were labelled in medium containing 50 mM-Na⁺ prepared by the equimolar substitution of sodium chloride by choline chloride (BDH Laboratories, Edmonton, Alberta).

Counting procedures

Acid-precipitable radioactivity was insignificant relative to total intracellular radioactivity such that the total amount of L-[³⁵S]met measured was used to represent uptake into the acid-soluble pool. A typical experiment on ICMs included three to four replicates of groups of five or more ICMs. After the wash regime, embryos were transferred in a 2–3 μ l droplet of M2 to a scintillation vial, lysed by addition of 200 μ l of distilled water and counted in a toluene-based scintillation cocktail containing 10% (v/v) Bio-Solv 3 (Beckman, Fullerton, CA) at a counting efficiency of 67%.

In experiments on intact blastocysts, three or four groups of three to five blastocysts were counted in scintillation cocktail following lysis with distilled water as above. A scheme to measure the label accumulated in the cellular compartment was also devised. Blastocysts were labelled and then transferred directly to a 1.5 ml M2 wash at 4°C. Upon transfer to this wash, blastocysts were mechanically collapsed by drawing them into a soda lime glass capillary (20 μ l) pulled to a bore size of about two thirds blastocyst diameter. The cellular material was transferred through three more washes and then inserted into scintillation vials. Water (200 μ l) was added to the vials prior to the addition of scintillation cocktail. Intact blastocysts, subjected to the same experimental conditions except for mechanical collapse, were counted so that cavity accumulation could be calculated from the difference between total and cellular counts.

Data handling procedure

Estimation of kinetic parameters was made using a direct fit non-linear least-squares curve-fitting procedure based on Cleland (1967) as described previously (Miller & Schultz, 1983).

RESULTS

The accumulation of L-[³⁵S]met in inner cell masses obtained by immunosurgery and placed in medium containing radiolabelled substrate (100 μ M) was observed to increase rapidly over the initial 5 to 10 min and then to increase less rapidly over the ensuing 2 h incubation period (Fig. 1). The amount of uptake at 60 min in 100 μ M L-[³⁵S]met was calculated to be 60 fmol per ICM and very much lower than that for an intact blastocyst where the value was measured to be 5 pmol under the same conditions.

Initial velocity of uptake as a function of substrate concentration was plotted in double log form for both ICMs (Fig. 2A) and intact blastocysts (Fig. 2B). Previous studies of amino acid transport in mammalian development systems have used a least-squares analysis of the linear transformation of the Michaelis–Menten equation to derive estimates of the kinetic constants, K_m and V_{max} . However, this is not

the best statistical approach when unweighted data pairs are used (Cleland, 1967) nor does it allow for contributions to initial velocity by other than a single Michaelis–Menten system. The shape of the curve for amino acid uptake in both experimental situations (Fig. 2) was observed to be compatible with a Michaelis–Menten (saturable) system and a linear (non-saturable) system acting in parallel. A saturable system would have led to a curve with a plateau at high substrate concentrations. Rather, a biphasic shape was observed with a linear component accounting, in most part, for the resurgent increase in rate of L-[³⁵S]met uptake at high substrate concentrations for both the ICM (10^{-2} to 10^{-1} M, Fig. 2A) and intact blastocysts (greater than 10^{-3} M, Fig. 2B). Hence, kinetic parameters were calculated by using a direct non-linear least-squares curve-fitting approach modified from Wentworth (1965*a,b*) and Cleland (1967) as applied previously to amino acid transport studies in preimplantation rabbit embryos (Miller & Schultz, 1983).

The estimates for V_{\max} , K_m and P obtained from the data in Fig. 2 are presented in Table 1 in comparison with estimates of K_m and V_{\max} determined by Miller (1984) for unfertilized eggs. The V_{\max} for met transport into the 102 h blastocyst is at least four-fold higher than that of the egg, while the K_m is about three-fold lower

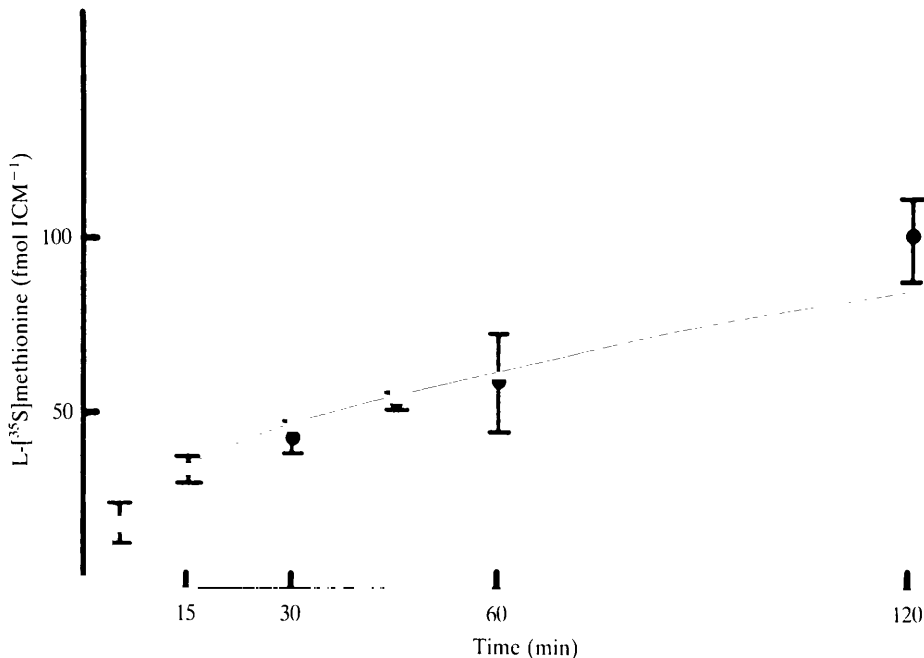


Fig. 1. Time course of uptake of L-[³⁵S]met into mouse ICMs. ICMs were incubated in $100 \mu\text{M}$ L-[³⁵S]met at a specific activity of 13 Ci mmol^{-1} at 37°C . At the times indicated, ICMs were removed from this medium, washed, and counted for radioactivity. Radioactivity taken up was converted directly to mol L-[³⁵S]met taken up through measurements of the specific activity of the incubation medium. Each point is the mean of three experiments containing 10 to 12 ICMs. The standard deviations of the means are indicated by error bars.

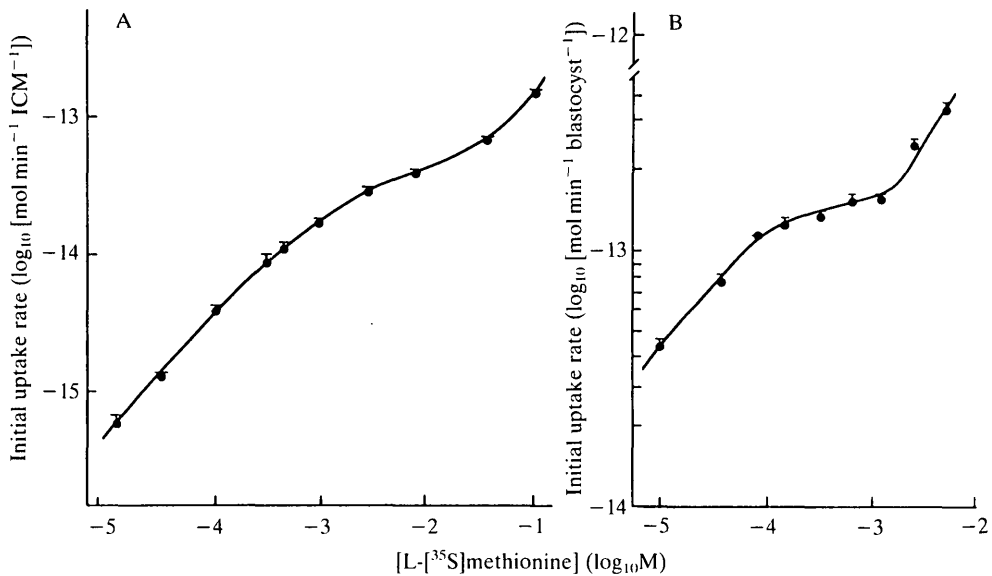


Fig. 2. Kinetics of uptake of L-[³⁵S]met in ICMs and intact blastocysts. (A) ICMs were placed in selected concentrations of L-[³⁵S]met (specific activity of a 100 μM solution was 13 Ci mmol⁻¹; all others were 13 Ci mmol⁻¹ times the quotient of 100 μM and the concentration of interest) at 37°C for 5 min. Following these incubations ICMs were washed and assayed for radioactivity. Radioactivity taken up was converted to mol L-[³⁵S]met based on the specific activity of the incubation medium. Velocity of uptake was this value divided by the duration of incubation, 5 min. Each point is the mean derived from a minimum of four experiments containing about 10 ICMs. The standard deviation of the mean is represented by error bars. (B) Blastocysts (102 h) were placed in selected concentrations of L-[³⁵S]met (specific activity of a 100 μM solution was 2.6 Ci mmol⁻¹; all others were 2.6 Ci mmol⁻¹ times the quotient of 100 μM and the concentration of interest) at 37°C for 5 min. Measurements and calculations were made as for ICMs in (A). Each point is the mean derived from three experiments containing two groups of five blastocysts per group. The standard deviation of the mean is indicated by error bars.

Table 1. Kinetic constants for uptake of methionine in preimplantation mouse embryos

Stage	K_m (μM)	V_{\max} (pmol min ⁻¹)	P (pl min ⁻¹)
Egg*	69	0.032	3.49
102 h blastocyst†	22	0.133	4.39
102 h ICM†	660	0.028	0.81

* Data from Miller (1984)

† Kinetic constants calculated from data in Fig. 2. Values of 5 min uptake for cultured blastocysts were not statistically different from those of blastocysts of the same age but freshly flushed from the uterus.

(Table 1). The K_m for uptake in the isolated ICM, however, is an order of magnitude greater than in the egg although the V_{max} is similar.

Uptake of L-[³⁵S]met in ICMs was reduced when experiments were conducted in the presence of the competing amino acids alanine and leucine (Table 2). The reduction in uptake in the competition experiments was observed at both relatively low concentrations (100 μ M L-[³⁵S]met and 500 μ M competitor) operating in a region for the saturable uptake system and at higher concentrations (20 mM L-[³⁵S]met and 20 mM competitor) capable of affecting the linear (non-saturable) component of uptake.

When ICMs were incubated in either 1 μ M or 1 mM L-[³⁵S]met for 5 min in medium with reduced Na⁺ content, uptake was reduced by 30 to 40 % in ICMs in Na⁺-depleted (50 mM) medium compared to those in control (149 mM) medium.

Experiments were also conducted to evaluate efflux and exchange properties of met transport in isolated ICMs. The ICMs were incubated in medium containing 300 μ M-L-[³⁵S]met (specific activity of 4.3 Ci mmol⁻¹) for 1 h at 37°C to preload them with radiolabelled methionine. The average uptake value was measured to be 955 \pm 25 c.p.m. L-[³⁵S]met per ICM (mean \pm s.e.m., four experiments on pools of five to six ICMs per experiment). Preloaded ICMs were divided into two groups. One group was placed in medium containing 300 μ M-met and a parallel group in medium lacking amino acid. After a 40-minute efflux or exchange period, ICMs were washed and counted for radioactivity to calculate the amount of L-[³⁵S]met remaining. It was reduced to 216 \pm 6 c.p.m. in ICMs placed in medium containing met and 414 \pm 84 c.p.m. in medium without methionine. Thus, while loss of labelled methionine from ICMs was greater than into amino-acid-free medium, it was observed to be substantial under either condition.

Another experiment to measure exchange properties was performed by pre-loading ICMs with substrate by incubation in medium containing 1 mM-unlabelled methionine. After this period, ICMs were rapidly washed and placed in medium

Table 2. *Uptake of methionine by mouse ICMs in the presence of competing amino acids*

L-[³⁵ S]met/competitor (mM/mM)	Competitor	Uptake of L-[³⁵ S]met (Percent of control)*
20/20	ala	83 \pm 7
20/20	leu	71 \pm 6
0.1/0.5	ala	66 \pm 4
0.1/0.5	leu	50 \pm 9

* ICMs were incubated at 37°C in 0.1 mM L-[³⁵S]met (specific activity of 13 Ci mmol⁻¹) either containing (competed) or lacking (control) 0.5 mM of leu or ala, or in 20 mM L-[³⁵S]met (specific activity of 0.065 Ci mmol⁻¹) either containing (competed) or lacking (control) 20 mM of leu or ala. After five min ICMs were removed, washed, and counted for radioactivity. The mol of L-[³⁵S]met taken up was calculated on the basis of specific activity measurements. The ratio of competed to control uptake was expressed as a percentage of control uptake. Each value is the mean \pm s.e.m. derived from three experiments consisting of 10 to 12 ICMs per experiment.

containing 100 μM -L-[³⁵S]methionine (specific activity of 13 Ci mmol⁻¹). Uptake of radiolabelled methionine after 5 min at 37°C was measured and compared to that in a control set of ICMs which had not been preloaded with unlabelled substrate. Uptake of L-[³⁵S]met into preloaded ICMs was about two and one-half times greater than uptake into control ICMs. The measured values were 960 ± 91 c.p.m. ICM⁻¹ 5 min⁻¹ (mean ± s.e.m., four experiments containing five to six ICMs per experiment) for preloaded ICMs and 384 ± 19 c.p.m. ICM⁻¹ 5 min⁻¹ for controls.

The distribution of accumulated L-[³⁵S]met under several conditions in intact blastocysts was also examined (Table 3). The ratio of L-[³⁵S]met distribution between cavity and cellular compartments (70:30) was essentially the same under each set of conditions.

DISCUSSION

The rate of uptake of methionine in isolated ICMs incubated in 100 μM -methionine decreases with increasing time (Fig. 1). The shape of this uptake curve

Table 3. *Compartmental uptake of methionine into the mouse blastocyst*

Experimental conditions	Accumulation of L-[³⁵ S]met (Percent of total)*	
	Cell	Cavity
5 min uptake†	30 ± 1	70
1 h uptake†	30 ± 1	70
2 h uptake†	34 ± 6	66
Competition, 5 min‡	34 ± 6	66
Competition, 1 h‡	30 ± 5	70
Efflux§	35 ± 1	65
Exchange++	42 ± 8	58

* At the conclusion of these incubations, some of the blastocysts were subjected to the compartmental separation described in Materials and Methods while others were left intact for whole blastocyst uptake values. The intact blastocysts and the cellular compartments were washed and counted for radioactivity. This radioactivity was directly converted to mol L-[³⁵S]met accumulated on the basis of specific activity measurements of the incubation drops. Cellular uptake was expressed as a percentage of uptake into the intact blastocyst and cavity uptake was calculated as the difference between intact and cellular uptake and expressed as a percentage of uptake into the intact blastocyst. These cellular values represent the mean ± s.e.m. of three experiments containing three groups of four blastocysts per group.

† Uptakes were conducted in 100 mM L-[³⁵S]met (specific activity of 2.6 Ci mmol⁻¹) for the times indicated.

‡ Uptakes were conducted in 100 mM L-[³⁵S]met (specific activity of 2.6 Ci mmol⁻¹) either lacking (control) or containing (competition) 500 μM -leu for the time indicated.

§ Efflux experiments were conducted by loading blastocysts in 100 μM L-[³⁵S]met (specific activity 2.6 Ci mmol⁻¹) for one hour, washing once in M2 at 37°C and then incubating in 100 μM unlabelled met for 1 h (control uptakes without efflux were also done).

** Exchange experiments were conducted by preloading blastocysts in 100 μM unlabelled met for 1 h and then placing in 100 μM L-[³⁵S]met (specific activity of 2.6 Ci mmol⁻¹) for 1 h (control experiments without preloading were also done).

is, in this respect, midway between that observed for eggs and that observed for 4-cell cleavage-stage embryos (Kaye *et al.* 1982). This type of behaviour is often associated with the existence of an exchange system that has not yet reached a steady state or the co-existence of an exchange system and an accumulating system with the exchange system somewhat more dominant at the concentration of amino acid used.

When ICMs are preloaded with radiolabelled methionine, efflux of this amino acid is observed. Efflux is greater in ICMs placed in medium containing methionine than in the ICMs placed in amino-acid-free medium although efflux is strong under both conditions. That a vigorous exchange system is present in the mouse ICM is also shown by the high degree of trans-stimulation of uptake of L-[³⁵S]met for ICMs preloaded with unlabelled met. On the basis of experiments in other mammalian cell types, the exodus of preloaded amino acid from intact cells into amino-acid-free medium is also mediated by an exchange system (Christensen & Handlogten, 1968; Christensen, 1977*a,b*; White & Christensen, 1982). Hence, the high degree of exchange-mediated efflux suggests that the uptake component of transport in the ICM is more active than the uptake curve indicates.

The kinetics of uptake into the ICM are compatible with the parallel activity of a saturable and a non-saturable system (Fig. 2, Table 1). The V_{\max} of transport is within the midrange of values for V_{\max} of the egg and other developmental stages (see Borland & Tasca, 1974) but the P value is much lower (than in egg) and the K_m much higher than the kinetically dominant component in other stages (Table 1, Borland & Tasca, 1974). Therefore the initial velocity of uptake for the ICM, at 100 μM -met, is about 30-fold and 5-fold less than that of the intact blastocyst and the egg, respectively (calculated from Table 1). The time period used for these ICM incubations (5 min) and the length of the washing regime (5 min) was as short as practicable but it may be that efflux did occur so that the V_{\max} may be an underestimate. In any case, it is clear that the low uptake at 1 h is, in part, a consequence of the great efflux and exchange character of transport in the ICM rather than lack of influx activity.

The high K_m value observed for met transport in the ICM and the exchange behaviour noted above are suggestive of system L (Christensen, 1984). The coexistence of an ASC or A type system (also a high K_m system; Oxender & Christensen, 1963) contributing to uptake in ICMs is a possibility for two reasons. Firstly, alanine (transported primarily by the A and ASC systems) appears to have similar competitive potency to that of an L-system amino acid, leucine (Table 2). The non-saturable component of transport also can be competed by ala and leu at high concentrations of L-[³⁵S]met and competitor amino acids (Table 2) and thus probably is also system-mediated. Secondly, the marked reduction of L-[³⁵S]met uptake in Na⁺-depleted medium is highly suggestive of the presence of the Na⁺-dependent A and ASC systems.

In summary, the isolated mouse ICM displays transport characteristics consistent with the existence of L and A/ASC systems. A dominant feature of transport in the ICM is strong efflux and exchange behaviour. The latter may be

important for nutrient acquisition within the intact blastocyst since the blastocyst cavity fluid may function as a reservoir of transported amino acids.

A direct method of assaying the distribution of transported amino acids in the blastocyst is to separate the compartments after incubation in radiolabelled amino acid. This has been done in the rabbit (Miller & Schultz, 1983) and, although technically more difficult, has also been analysed in this study on mouse embryos. The results of such a mechanical separation of compartments in the mouse blastocyst reveal that about 70 % of the uptake of label under a variety of uptake conditions resides in the cavity (Table 3).

The 102 h blastocyst is more active in amino acid accumulation than the early blastocyst (Table 1; Borland & Tasca, 1974). In parallel, the rate of protein synthesis increases progressively from early to late blastocyst stages (Brinster, Wiebold & Brunner, 1976; Braude, 1979). Both ICM and the trophectoderm are active in this synthesis with each compartment exhibiting the production of some tissue-specific polypeptides (Van Blerkom, Barton & Johnson, 1976; Handyside & Barton, 1977). Since the blastocyst cavity contains amino acids, transport systems (of the type presented here for methionine) may operate to maintain or expand the cavity pool. Perhaps both the trophectoderm which has an exchange system on its inner (cavity-facing) surface (Kaye *et al.* 1982), and the strongly exchanging ICM can use these pools as an amino acid reservoir to provide the substrate needs for these actively growing and differentiating tissues. Alternatively, Wheatley & Robertson (1981) contend that the protein synthetic apparatus of the cell draws on freshly transported amino acids rather than the total acid soluble pool that can be measured in cells. If this is true, perhaps the co-existence of a system with strong accumulation properties in the trophectoderm and a system with strong exchange properties in the ICM is a viable strategy for charging the protein synthetic apparatus without incurring an osmotic debt.

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