Synthesis of Na^+/K^+ ATPase by the preimplantation rabbit blastocyst

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Summary. The rates of incorporation of [^{35}S]methionine into Na^+/K^+ ATPase, actin (β - and γ -isoforms), and total protein of the preimplantation rabbit blastocyst were determined between Days 4 and 7 of development. Blastocyst proteins were metabolically radiolabelled with [^{35}S]methionine and subsequently analysed by co-isolation with purified Na^+/K^+ ATPase using two-dimensional polyacrylamide gel electrophoresis, immunoprecipitation, immunoblotting, fluorography, and liquid scintillation spectroscopy. The rate of [^{35}S]methionine incorporation into acid-soluble total protein increased 24-fold between Days 4 and 6 *post coitum* (*p.c.*), then diminished $\sim 79\%$ on Day 7. In-vitro incorporation of [^{35}S]methionine was linear at each stage of blastocyst development. [^{35}S]methionine incorporation rates were unaffected by low free intracellular methionine concentration ($< 0.06 \text{ mM}$) and stage-related differences in blastocoele volume. Analysis of β - and γ -actin synthesis revealed patterns of [^{35}S]methionine incorporation rates which were similar to those of total protein. In contrast, synthesis of blastocyst Na^+/K^+ ATPase was characterized by a 90-fold increase ($P < 0.001$) in the rate of [^{35}S]methionine incorporation between Days 4 and 6 *p.c.* The results demonstrate that Na^+/K^+ ATPase is actively synthesized at a high and increasing rate during preimplantation development in the rabbit at a period which is characterized by rapid fluid accumulation by the blastocyst.

Keywords: rabbit; blastocyst; protein synthesis; Na^+/K^+ ATPase

Introduction

During mammalian embryogenesis, many distinct morphological and physiological changes occur. Among these is the formation of tight junctions between adjacent blastomeres (Ducibella *et al.*, 1975), which in the rabbit occurs at the 8- to 16-cell stage (Hastings & Enders, 1975). This process, termed compaction (Ducibella & Anderson, 1975; Ziomek & Johnson, 1980), establishes morphological and functional polarization of the embryonic cells and spatially segregates these cells into two subsets, the inner cell mass (ICM) and the trophectoderm. The inner cell mass subsequently differentiates to form the embryo proper in an environment isolated from direct maternal influence. The outer embryonic cells differentiate to form a functional unilaminar epithelium, the trophectoderm. As such, the trophectoderm acts as a selective barrier to solute exchange, thereby regulating the composition of extracellular fluid which bathes the developing inner cell mass (Biggers *et al.*, 1977). Cavitation occurs soon after tight junction formation and is characterized by the accumulation of variable amounts of blastocoele fluid within the embryo before attachment to the uterine

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epithelium (Burgoyne & Ducibella, 1977). Previous studies in the rabbit have reported that blastocoelic fluid volume increases ~1000-fold (70 nl to 80 µl) between Days 4 and 7 of preimplantation development (Daniel, 1964; Biggers *et al.*, 1977), a period of changing protein synthesis activity (Van Blerkom & Manes, 1974). Mechanisms of active transepithelial solute transport have been reported to mediate this process of blastocoele expansion (see Benos & Biggers, 1981; Benos *et al.*, 1985, for reviews). Specifically, there is increasing evidence that the ubiquitous membrane-bound enzyme Na^+/K^+ ATPase (EC 3.6.1.3) mediates transepithelial fluid transport in the preimplantation mammalian blastocyst (Biggers *et al.*, 1978).

In an effort to understand more fully the functional role of Na^+/K^+ ATPase in differentiation and expansion of the rabbit blastocyst, experiments of the present study were designed to identify blastocyst Na^+/K^+ ATPase by using procedures of metabolic radiolabelling and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE); and to determine the rate of synthesis of the active Na^+ transport enzyme between Days 4 and 7 of preimplantation development. Because rates of uptake and incorporation of radiolabelled amino acids by developing embryos can be significantly affected by endogenous amino acid stores and labelling conditions (Regier & Kafatos, 1971; Epstein & Smith, 1973; Schultz *et al.*, 1979), we measured the free intracellular methionine pool size in rabbit blastocysts at 4–7 days *post coitum* and characterized optimum conditions for [^{35}S]methionine radiolabelling. The rate of Na^+/K^+ ATPase synthesis is compared relative to the [^{35}S]methionine incorporation rates determined for total blastocyst protein and β - and γ -actin, two actively synthesized cytoskeletal proteins.

Materials and Methods

Collection and labelling of blastocysts. Sexually mature New Zealand White rabbits (Meadowbrook Farms, Amherst, MA, USA) were individually caged in a controlled environment (14 h light:10 h dark, lights on at 07:00 h, 23°C). Water and commercial rabbit food were available *ad libitum*. Animals were twice naturally mated to bucks of proven fertility with the day of mating considered to be Day 0 of pregnancy. On Days 4, 5, 6 and 7 *post coitum* (*p.c.*), female rabbits were killed by intravenous injection of pentobarbitone sodium (1 ml/kg body wt; Abbott Laboratories, Chicago, IL, USA). The reproductive tract was removed and each uterine horn was flushed with warmed Ham's nutrient medium F10 (F10, GIBCO, Grand Island, NY, USA) containing 20 mM-Hepes buffer adjusted to pH 7.4. Recovered blastocysts were immediately transferred to fresh F10 medium and incubated briefly (<15 min, 5% CO_2 in humidified air, 37°C) before radiolabelling. Because of their fragility, 7-day *p.c.* blastocysts were dissected from the uterine horns (Benos, 1981a). Blastocysts were incubated in 1–2 ml labelling medium for 3 h (5% CO_2 in humidified air, 37°C). The labelling medium consisted of F10 containing 200 µCi [^{35}S]methionine/ml (sp. act. >1200 Ci/mmol; Amersham/Searle, Arlington Heights, IL, USA). To increase the incorporation of label, the unlabelled methionine concentration of the labelling medium was reduced to 0.1 µM. After labelling, blastocysts were washed in ice-cold F10 medium containing 1 mM-phenylmethylsulphonyl fluoride (PMSF), transferred to a second cold wash and the zona pellucidae were removed using glass needles under $\times 20$ magnification (Wild M5A stereomicroscope). Blastocysts were gently dissected open to expel blastocoelic fluid, and the tissues were twice washed in cold medium F10 + PMSF. Tissues of 6–20 blastocysts were dissolved in 50 µl lysis buffer (O'Farrell, 1975), stored at -70°C and the polypeptides subsequently analysed by 2D-PAGE. Five replicate experiments were carried out for each developmental stage blastocyst (Days 4 to 7 *p.c.*).

Free intracellular methionine analysis. The procedure of Schultz *et al.* (1979) was used to quantify the free methionine pool size of 4-, 5-, 6- and 7-day *p.c.* rabbit blastocyst tissues by reaction with [^3H]dinitrofluorobenzene with the following modification; blastocysts were cultured in 1 ml KRBG containing 200 µCi [^{35}S]methionine and 20 µM unlabelled methionine (5% CO_2 in air, 37°C). Blastocysts were pooled per sample (18 for Day 4 to 4 for Day 7) and 4–6 samples were analysed for each day of development (Days 4–7 *p.c.*). [^3H]dinitrofluorobenzene (sp. act. >20 Ci/mmol) was purchased from New England Nuclear (NEN, Boston, MA, USA). DNP-methionine and polyamide plates were obtained from Sigma Chemical Company (St Louis, MO, USA) and Accurate Chemical and Scientific Co. (Westbury, NY, USA) respectively.

Kinetics of in-vitro [^{35}S]methionine incorporation. Blastocysts were collected and cultured in labelling medium containing 200 µCi [^{35}S]methionine/ml as previously described. After labelling for 0.5, 1, 2 and 3 h, groups of 3–5 blastocysts were removed and individually washed in 5 ml cold medium F10 + PMSF. Blastocoelic fluid samples (2–10 µl) were obtained by micropuncture from 6- and 7-day embryos for subsequent determination of ^{35}S radioactivity. Zona-free tissue of individual blastocysts was washed in cold F10-PMSF, solubilized in 25 µl SDS sample buffer (0.0625 M-Tris, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.6% SDS, pH 6.8) and subjected to 3 freeze-thaw cycles. The specific radioactivity present in acid-precipitable and acid-soluble tissue lysate was determined by the

procedure of Van Blerkom (1978) with modification. Briefly, duplicate 5 μl samples of tissue lysate were added to 1.5 ml microcentrifuge tubes containing 0.5 ml distilled water and 100 μl 0.2% bovine serum albumin (BSA). After the addition of 0.5 ml ice-cold 30% trichloroacetic acid (TCA), proteins were precipitated for 1 h at 4°C. Protein precipitates were centrifuged (12 000 g, for 10 min and twice suspended, washed with ice-cold 5% TCA and recentrifuged). The TCA-precipitable [^{35}S]methionine activity was determined by liquid scintillation spectroscopy (Beckman 6800). Acid-soluble radioactivity was determined by counting triplicate 10 μl samples of supernatant from the initial precipitate centrifugation.

Polyacrylamide gel electrophoresis. Labelled blastocyst samples were thawed and frozen 3 times, and the acid-precipitable ^{35}S radioactivity (d.p.m.) was determined as described. After solubilizing blastocyst tissue in lysis buffer, >94% of the total blastocyst radioactivity was present in the supernatant after centrifugation at 100 000 g for 45 min. Solubilized blastocyst proteins were separated by high resolution two-dimensional PAGE (O'Farrell, 1975) as modified for mammalian embryos by Van Blerkom (1978). Synthesized blastocyst Na^+/K^+ ATPase was identified by co-electrophoresis of [^{35}S]methionine-labelled blastocyst proteins with purified Na^+/K^+ ATPase α -subunit. Purified Na^+/K^+ ATPase (5 μg) was added to each blastocyst sample (3×10^6 d.p.m. of acid-precipitable ^{35}S activity) before isoelectric focussing (IEF, 5% polyacrylamide, 9.5 M-urea, 3.5% NP-40, 2% ampholines 50:50, pH 3–10:pH 4–6). Labelled blastocyst proteins and purified Na^+/K^+ ATPase were co-separated by IEF (6400 Vh) and subsequently resolved according to their molecular weights on SDS–polyacrylamide slab gels (7–15% exponential gradient). Gels were fixed (50% MeOH) and silver stained (Morrissey, 1981) to visualize Na^+/K^+ ATPase and other blastocyst proteins. Gels were dried under vacuum, exposed to unflashed XAR-5 film (Kodak) for 2–4 days at -70°C , and the resulting autoradiographs were developed by autoproccessing (Kodak X-OMAT M-60). Na^+/K^+ ATPase (α -subunit) and β - and γ -actin spots were excised from stained gels and solubilized overnight in glass minivials (Rochester Scientific Co., Rochester, NY, USA) containing 100 μl Protosol (NEN) at 60°C . After cooling, 3 ml Bioflour (NEN) were added to each vial and the sample radioactivity (expressed as d.p.m.) was determined using a Beckman LS-6800 liquid scintillation counter programmed for quench correction of ^{35}S in silver-stained polyacrylamide gel samples.

Purified Na^+/K^+ ATPase. The purified catalytic, α -subunit ($M_r \sim 100\,000$) of Na^+/K^+ ATPase, prepared from shark rectal gland, was kindly provided by Dr J. C. Skou (Skou & Esmann, 1979). Rabbit kidney Na^+/K^+ ATPase (α -subunit) was isolated and purified as described by Jorgensen (1974). Both purified enzyme preparations were stored in 30 mM-imidazole, 1 mM-EDTA (pH 7.4) and 25% (v/v) glycerol at -70°C . The purity of the Na^+/K^+ ATPase preparations was determined by one-dimensional SDS–PAGE as described by Van Blerkom (1978).

Immunoprecipitation of blastocyst Na^+/K^+ ATPase. Day-6 blastocysts were radiolabelled as described and trophoctodermal Na^+/K^+ ATPase was identified by immunoprecipitation (Tarone *et al.*, 1984). After labelling, the isolated tissues of 4–8 blastocysts were placed in 1.5 ml microfuge tubes containing 0.75 ml Tris-buffered saline (150 mM-NaCl, 20 mM-Tris-HCl, 5 mM-EDTA) containing 1 mM-PMSF, 0.5% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate (TDS buffer) for 30 min at 4°C . Samples were sonicated (3×10 sec, 4°C) and lysates were prepared by centrifugation (12 000 g, 10 min). To 0.5 ml samples of lysate were added 20 μl Na^+/K^+ ATPase antiserum. The polyclonal antiserum (rabbit) was raised against the purified α -subunit (catalytic subunit, $M_r \sim 100\,000$) of sheep kidney Na^+/K^+ ATPase (Ball *et al.*, 1983). After incubation (24 h, 4°C), 50 μl of a 10% suspension of fixed *Staphylococcus aureus* cells (IgG-sorb) were added and incubated for 30 min (4°C). Immunoprecipitates were centrifuged (10 000 g) and washed ($\times 3$) with ice-cold TDS buffer, solubilized in SDS sample buffer, heated (90°C , 3 min) and analysed by one-dimensional PAGE and autoradiograph. Treatment of labelled blastocyst lysate with non-immune rabbit serum served as a negative control.

Statistical analysis. The uptake of [^{35}S]methionine by groups of 4-, 5-, 6- and 7-day rabbit blastocysts was determined 5 times at random. Rates of incorporation into total protein, Na^+/K^+ ATPase and actin (β - and γ -) were measured on each sample of pooled blastocysts. The observations on each sample therefore formed a vector of correlated observations. A curvilinear regression analysis was performed on each of the sets of data to determine the lowest order polynomial needed to fit the observations. The analysis was done by applying a one-way ANOVA, and partitioning the sum of squares attributed to variation between days by means of orthogonal polynomial coefficients. This method of analysis was chosen, rather than a multiple regression analysis, because (1) the procedure provides independent tests of significance of the linear, quadratic and cubic components in testing for the required degree of curvilinearity (Fisher, 1920; Sokal & Rohlf, 1981), and (2) it provides a greater computing accuracy (Draper & Smith, 1981).

Results

Free intracellular methionine content

The concentration of free methionine in blastocyst tissue is shown in Table 1. The methionine pool size increased from 0.32 to 27.1 pmol per blastocyst between Days 4 and 7 of development, which reflects an 85-fold increase. However, when endogenous methionine pool values are

expressed on the basis of μg DNA or blastocyst cell number the concentration of intracellular methionine was essentially unchanged between Days 4 and 7 of development.

Table 1. Free intracellular methionine content in 4- to 7-day rabbit blastocysts

Day <i>p.c.</i>	No. of blastocysts	Methionine pool (pmol/blastocyst)	Blastocyst cell no.	DNA content (ng/blastocyst)	Methionine content	
					fmol/cell	fmol/ng DNA
4	58	0.32 ± 0.03	1 100*	7†	0.29	0.023
5	49	2.98 ± 0.23	12 000†	78‡	0.25	0.026
6	32	12.4 ± 1.18	52 000†	338‡	0.24	0.027
7	18	27.1 ± 2.35	115 000†	747‡	0.24	0.027

Values are mean $\pm$ s.e.m.

*Data from Daniel (1964).

†Estimate based on 6.5 pg DNA per cell.

‡Data from J. E. Butler & J. D. Biggers (unpublished).

Kinetics of [³⁵S]methionine incorporation into total blastocyst protein

As shown in Fig. 1, the acid-precipitable ³⁵S radioactivity incorporated into blastocyst tissue increased linearly during the 3-h labelling period at each stage of development. Correlation coefficients were 0.978, 0.989, 0.988, and 0.979 for 4-, 5-, 6- and 7-day blastocysts, respectively. Regression analysis showed that the rates of specific incorporation of [³⁵S]methionine ranged from 39 400 to 362 000 d.p.m./blastocyst/h.

Fig. 1. Time-course of [³⁵S]methionine incorporation by rabbit blastocysts *in vitro*. The rates of specific incorporation of ³⁵S radioactivity into 4-(▼), 5-(◆), 6-(●) and 7-(■) day blastocysts were determined by TCA precipitation and were linear over the 3-h labelling period. Non-specific uptake of ³⁵S radioactivity by tissue (○, acid soluble) and zonae pellucidae (△) is also plotted. Values represent the mean $\pm$ s.e.m. of 5 replicate experiments (4–7 embryos per time point).

Uptake of acid-soluble ^{35}S radioactivity was low, rapidly saturable (30 min) and remained constant during the labelling period (Fig. 2, open circles). Similarly, ^{35}S radioactivity associated with the zonae pellucidae was minimal, with values $<7\%$ of the total incorporated label (d.p.m./blastocyst) at each time period. Moreover, $>85\%$ of zona-associated radioactivity readily eluted from isolated zonae pellucidae when placed in KRBG overnight (4°C). Thus, by using the described labelling conditions in all subsequent experiments, rabbit blastocysts were radiolabelled to high ^{35}S specific activity during a 3-h incubation period.

Fig. 2. Time-course of ^{35}S methionine equilibration between labelling medium and blastocoelic fluid of 6- (●) and 7-day (○) rabbit blastocysts. Samples of labelling medium and blastocoelic fluid were obtained by micropuncture and sampling of the blastocoele. Values represent the mean $\pm$ s.e.m. of triplicate experiments. For each experiment, 5–8 blastocysts were used per time point.

Because of the large increase in blastocoele volume between Days 4 and 7 *p.c.*, it was possible that blastocoelic fluid may act as a substrate sink and thereby preclude accurate quantitative comparisons of ^{35}S methionine incorporation rates between blastocysts at different stages. The ^{35}S specific activity of blastocoelic fluid reached $>90\%$ of the external medium specific activity within 2 h (Fig. 3). These results demonstrate that no significant 'sink' effect exists when embryos are labelled for 3 h under the given conditions.

The arithmetic mean rates of ^{35}S methionine incorporation into total acid-precipitable blastocyst protein are shown in Table 2. Because the variances tend to vary directly with the means, all individual observations were transformed to natural logarithms to reduce this dependency. The transformation is appropriate because growth data tend to be distributed lognormally (Sokal & Rolf, 1981; Hines, 1983). Incorporation of ^{35}S methionine increased 9-fold between Day 4 and Day 5 of development and reached a maximal value just before the 6th day. Thereafter, specific uptake of ^{35}S methionine by 7-day embryos decreased $\sim 79\%$. On a per cell basis, ^{35}S methionine incorporation rates were similar in 4- and 5-day blastocysts, whereas 6- and 7-day blastocysts showed decreasing rates. Comparisons of free methionine pool size versus rate of ^{35}S methionine incorporation reveal that the endogenous pool of 4-, 5-, and 6-day blastocysts represented $<1\%$ of the ^{35}S methionine incorporated in 1 h, but $\sim 10\%$ of Day 7 blastocysts (Table 2).

Fig. 3. Fluorograph of dried polyacrylamide slab gel after two-dimensional PAGE separation of [^{35}S]methionine labelled Day-6 rabbit blastocyst proteins. Approximately 3×10^6 d.p.m. of acid-precipitable ^{35}S radioactivity and $5 \mu\text{g}$ purified Na^+/K^+ ATPase were applied to each gel. Blastocyst Na^+/K^+ ATPase (α -subunit) is protein B (broken lines indicate region occupied by purified Na^+/K^+ ATPase), and β - and γ -actin are proteins T and S, respectively.

Two-dimensional PAGE analysis of [^{35}S]methionine-labelled Na^+/K^+ ATPase and actin

Figure 3 shows a fluorograph developed from a representative two-dimensional PAGE gel of labelled Day-6 blastocyst proteins. In general, rabbit blastocyst proteins have molecular weights of 15 000 to 325 000 and have pI values of 4.5 to 7. Blastocyst Na^+/K^+ ATPase, identified by co-electrophoresis with purified enzyme (broken outline), and β -actin and γ -actin are proteins B, T and S, respectively.

The maximum rate of incorporation of [^{35}S]methionine and the time it is reached were calculated from the simplest polynomial equation which fitted the data for each protein (Table 3). The b_1 and b_2 regression coefficient values demonstrate that Na^+/K^+ ATPase, β - and γ -actin each

Table 2. Rates of [^{35}S]methionine incorporation by 4-, 5-, 6- and 7-day rabbit blastocysts

Day <i>p.c.</i>	[^{35}S]Methionine incorporation		Methionine pool/ incorporation rate (h)
	pmol/blastocyst/h	fmol/cell/h*	
4	57 $\pm$ 11	51.8	0.0056
5	512 $\pm$ 207	42.7	0.0058
6	1377 $\pm$ 462	26.5	0.0090
7	262 $\pm$ 67	2.3	0.1034

Values are mean $\pm$ s.e.m. for 5 experiments.

*See Table 1 for cell numbers.

Table 3. Estimates of the regression coefficients for the orthogonal polynomials required to summarize the incorporation of [^{35}S]methionine into Na^+/K^+ ATPase and actin by 4–7 day rabbit blastocysts

Protein	Regression coefficients			Maximum rate of incorporation (fmol/blastocyst/h)	Time maximum is reached (day <i>p.c.</i>)
	b_0	b_1	b_2		
Na^+/K^+ ATPase (B)	19.765	5.773***	-5.378***	308	6.0
γ -Actin (S)	22.192	3.618***	-4.253***	334	5.9
β -Actin (T)	32.866	2.581**	-3.332***	2629	5.8

All values of b_0 were highly significant ($P < 0.001$); all values of b_3 (the cubic component) were not significant ($P > 0.05$) and are not shown. **0.001 $< P < 0.01$; *** $P < 0.001$.

Fig. 4. Rates of incorporation of [^{35}S]methionine into blastocyst Na^+/K^+ ATPase (●). The means $\pm$ s.e.m. are based on 5 replicate observations.

displayed different patterns of [^{35}S]methionine incorporation during development, and that the ^{35}S incorporation rate for each protein increased significantly ($P < 0.01$ for actins; $P < 0.001$ for Na^+/K^+ ATPase) between Days 4 and 6 *p.c.*

The rate of incorporation of [^{35}S]methionine into Na^+/K^+ ATPase (α -subunit, B) is plotted in Fig. 4. Blastocyst Na^+/K^+ ATPase showed a ~ 90 -fold increase ($P < 0.001$) in [^{35}S]methionine

Fig. 5. Fluorograph of immunoprecipitated Na^+/K^+ ATPase from total Day 6 blastocyst proteins labelled with [^{35}S]methionine. Lane 1 shows blastocyst Na^+/K^+ ATPase, the holoenzyme (A) and the α -subunit (B) are seen as major bands of M_r 320 000 and 100 000, respectively. Actin (C) and unidentified background proteins were also precipitated. Lane 2 depicts immunoprecipitated Na^+/K^+ ATPase after prior precipitation of labelled blastocyst lysate with non-immune serum, while Lane 3 shows blastocyst proteins which precipitated with non-immune serum alone.

incorporation rate between Days 4 and 6 *p.c.* Na^+/K^+ ATPase is among a small subset of blastocyst proteins whose incorporation rates increase most sharply during this period (E. W. Overström, D. J. Benos & J. D. Biggers, unpublished).

The one-dimensional PAGE profile of immunoprecipitated Na^+/K^+ ATPase from ^{35}S -labelled Day-6 blastocysts is shown in Fig. 5. Anti- α serum precipitated Na^+/K^+ ATPase, both the

Fig. 6. Comparison of [^{35}S]methionine rates of incorporation into blastocyst β -actin (●) and γ -actin (◆) with development. Values are means $\pm$ s.e.m. of 5 replicate experiments. Also shown is the regression of the differences (○) between the proteins with respect to developmental age.

holoenzyme ($M_r \sim 320\,000$) and the α -subunit ($M_r\,100\,000$), and a heterogeneous population of minor proteins. Pretreatment with non-immune serum reduced non-specific precipitation while enhancing immunoprecipitation of Na^+/K^+ ATPase (holoenzyme and α -subunit).

The predominant polypeptide synthesized throughout the preimplantation period was β -actin (Fig. 6). The cytoskeletal protein displayed a maximum incorporation rate of 2629 fmol/blastocyst/h just before Day 6, a 19-fold increase from Day 4 *p.c.* In contrast, the maximum ^{35}S incorporation rate for the γ -actin isoform was 334 fmol/blastocyst/h which represents a 27-fold increase during the same period. Since the regression of the differences in incorporation rates on developmental age is significant, the temporal patterns of [^{35}S]methionine incorporation into β - and γ -actin are different during the preimplantation period.

Discussion

The results of the present study demonstrate that the rate of incorporation of [^{35}S]methionine into total blastocyst protein increases 24-fold then diminishes slightly during preimplantation development in the rabbit. The change in rate is the mean of changes for all individual polypeptides of the blastocyst. This general pattern is reflected in the specific rates of [^{35}S]methionine incorporation into blastocyst Na^+/K^+ ATPase and actin. Under defined conditions, the [^{35}S]methionine incorporation rates of individual proteins reflect an estimate of their independent rates of synthesis. Several factors, however, must be taken into account. For example, changes in the free endogenous intracellular amino acid pool have been shown to influence directly the rate of incorporation of radiolabelled amino acids by embryos undergoing high rates of differentiation (Regier & Kafatos, 1971; Epstein & Smith, 1973; Biggers & Borland, 1976). We report here that free intracellular methionine concentrations in 4- to 7-day rabbit blastocysts are uniformly low ($<0.06\text{ mM}$). This is in agreement with previous reports that free methionine concentration is very low and comparable in rabbit blastocyst tissue (Petzoldt *et al.*, 1973; Miller & Schultz, 1987), blastocoelic fluid (Lesinski *et al.*, 1967; Jaszcak

et al., 1972) and uterine fluid (Jaszczak *et al.*, 1972; Miller & Schultz, 1987). During radiolabelling, methionine is readily transported into blastomeres by Na^+ -dependent and Na^+ -independent mechanisms (Benos, 1981b; Bell *et al.*, 1986) and swamps out the comparatively small endogenous methionine pool. We demonstrate that free methionine rapidly equilibrates between the uterine and blastocoelec compartments which support the work of Miller & Schultz (1987). Collectively these results suggest that the specific activity of these extracellular compartments is essentially that of the endogenous pool. Although comparisons of [^{35}S]methionine incorporation rates may not reflect accurate comparisons of absolute synthesis rates between different blastocyst proteins because the numbers of methionine residues vary among polypeptides, under the described experimental conditions, the [^{35}S]methionine incorporation rate of an individual blastocyst protein, e.g. Na^+/K^+ ATPase, provides an accurate estimate of its changing rate of synthesis between Days 4 and 7 of development.

Changes in protein degradation rates may also influence the rate of [^{35}S]methionine incorporation into blastocyst proteins. Studies in the mouse (Brinster *et al.*, 1976, 1979) have demonstrated distinct differences in protein degradation rates of cleavage and blastocyst-stage embryos. While it is possible that there is a small population of rabbit blastocyst proteins that turnover at high rates between Days 4 and 7 *p.c.*, it is likely that most of the constitutive proteins have half-lives greater than 3 h since incorporation into total protein was linear over the 3-h labelling period. Protein degradation rates in preimplantation rabbit blastocysts have not been reported, but in other systems the half-life of Na^+/K^+ ATPase is greater than 5 h (Lo & Edelman, 1976; Cook *et al.*, 1985).

This study presents for the first time direct evidence that blastocyst Na^+/K^+ ATPase is being synthesized at a rapidly increasing rate between Days 4 and 6 of development in the rabbit. This observation is of particular significance because processes of blastocyst expansion and blastocoelec fluid accumulation proceed maximally during this period (Benos & Biggers, 1981). This finding corroborates several indirect lines of evidence which implicate trophoctodermal Na^+/K^+ ATPase in blastocyst formation (DiZio & Tasca, 1977; Wiley, 1984) and expansion (Borland *et al.*, 1977; Benos & Biggers, 1981; Benos *et al.*, 1985). As in other transporting epithelia, Na^+/K^+ ATPase functions to generate an electrochemical gradient across the trophoctoderm by actively transporting intracellular Na^+ out of the cell and into the blastocoelec, thereby facilitating the accumulation of blastocoelec fluid (Borland *et al.*, 1976, 1977). Previous reports have established that accumulation of rabbit blastocyst fluid, which increases > 1000-fold between Days 4 and 7 *p.c.* (Benos & Biggers, 1981), is coupled to active solute transport across the trophoctoderm (Gamow & Daniel, 1970; Smith, 1970; Cross, 1973, 1974; Borland *et al.*, 1977). For example, net transtrophoctodermal Na^+ influx increases ~ 10-fold during this period (Borland *et al.*, 1976). Ouabain, a specific inhibitor of Na^+/K^+ ATPase, has been shown to inhibit both the accumulation of blastocyst fluid (Biggers *et al.*, 1978) and > 50% of net trans-trophoctodermal Na^+ transport in preimplantation rabbit blastocysts (Benos, 1981b). Other studies have shown that the Na^+ pump is localized within the basal-lateral domains of rabbit and mouse trophoctoderm cells (Benos *et al.*, 1985; Watson & Kidder, 1988). Benos (1981b) has also reported that ouabain binding, a direct measure of Na^+ 'pump sites', increases dramatically in rabbit blastocysts between Days 4 and 6 *p.c.* This increase in binding parallels the 90-fold increase in Na^+/K^+ ATPase synthesis reported here.

The mechanism(s) which controls the rate of synthesis of trophoctodermal Na^+/K^+ ATPase during mammalian blastogenesis is not known. There has accumulated considerable evidence from work in other systems that genetic regulation of synthesis and degradation of the ubiquitous Na^+ pump (Boardman *et al.*, 1974; Pollack *et al.*, 1981; Edelman *et al.*, 1985) is modulated by hormonal factors (Jorgenssen, 1972; Geheb *et al.*, 1983; Paccolat *et al.*, 1987; McDonough *et al.*, 1988). Therefore, as the blastocyst undergoes progressively rapid and complex stages of differentiation, and as embryonic development proceeds in an environment of fluctuating hormonal conditions, we postulate that synthesis and functional expression of trophoctodermal Na^+/K^+ ATPase are genetically regulated via mechanisms of hormonal modulation.

The cause of the reduction in rate of incorporation of [^{35}S]methionine into blastocyst proteins on Day 7 is unclear. Several possibilities exist, including an increase in the rate of trophoblast cell death and slowing of the cell cycle (Daniel, 1964), and a decrease in overall oxidative metabolism (Benos & Balaban, 1980, 1983). Other physiological studies (Benos, 1981a, b; Benos & Biggers, 1981; Benos *et al.*, 1985) have identified differences in trophoctodermal solute transport properties between 6- and 7-day rabbit blastocysts; functional differences which coincide with decreasing blastocyst uptake and utilization of [^{35}S]methionine reported here. These developmentally regulated changes include: (1) the loss of a Na^+ -dependent methionine transport mechanism; (2) the appearance of an amiloride-sensitive component to Na^+ influx; and (3) the acquisition of a furosemide-sensitive NaCl co-transport system. In addition, Overstrom *et al.* (1983) have reported that the composition of iodinated plasma membrane proteins of Day-6 and Day-7 blastocysts vary qualitatively with regard to developmental age and their apical or basolateral domains. These observations suggest that dramatic changes in epithelial transport mechanisms reflect the changing developmental expression of trophoctodermal membrane constituents, in particular, Na^+/K^+ ATPase. Such changes may reflect a general 'uncoupling' of oxidative metabolism from ion transport and protein synthesis/degradation functions to other cellular activities commencing on Day 7 *p.c.*, the day that implantation is initiated.

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Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publ., NIH 78-23, revised 1978).

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