Calcium Transport in the Early Conceptus and Associated Maternal Tissues in the Rabbit

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1. The kinetics of calcium transport were studied in unmated (oestrous) and pregnant rabbits in the first half of gestation, with the aim of establishing evidence of hormonal (ovarian) influence on the pattern of transport. 2. The following tissues were examined at short- (45 min and 2 h) and long-duration (4, 16 and 48 h) intervals after parenteral administration of ^{45}Ca or ^{47}Ca : maternal blood plasma, endometrium, uterine fluid, placental tissues, two developmentally disparate stages of rabbit conceptus, namely the unattached blastocyst and the early post-implantation foetus, and bone (femur). 3. Marked variability in calcium content characterized rabbit tissues and body fluids. 4. Compartmental analysis was applied to measurements of specific radioactivity. Oestrous endometrium had the largest rapidly exchanging calcium fraction (turnover time of 12 min) and the highest value for calcium flux ($500\text{ }\mu\text{g}$ of Ca exchanged/h per g fresh wt. of tissue). A marked downward gradient in values of flux existed between the progesterational endometrium, uterine fluid and blastocyst; there was a similar gradient between placental tissues and foetus. 5. An hormonal influence on calcium transport was evident in (i) the decrease in specific radioactivity of rabbit blood plasma with advancing pregnancy, (ii) the extraordinarily rapid calcium transport between blood plasma and endometrium, especially in the oestrous stage, and (iii) the effectiveness of ovarian hormone substitution in ovariectomized rabbits. 6. The very low specific radioactivity recorded for bone indicated that only a minute fraction of its calcium was exchanging with that of blood plasma under the experimental conditions examined. 7. The rate of uptake of ^{45}Ca by rabbit blastocysts growing *in vitro* was one-tenth of that of ^{22}Na , or that recorded for calcium *in vivo*. 8. Inhibition of carbonic anhydrase activity with acetazolamide *in vivo*, in maternal erythrocytes, endometrium and placental tissues, produced no appreciable changes in calcium uptake in these tissues or other systems examined as a routine on either day 6 or days 12–14 of gestation.

As part of long-term investigations on the physiology and biochemistry of the early mammalian conceptus and its environment, while studying the kinetics of zinc transport in certain tissues and body fluids in non-pregnant and pregnant rabbits (McIntosh & Lutwak-Mann, 1972*a,b*), we made preliminary observations on the uptake of calcium by the pre-implantation embryo and associated maternal tissues (Lutwak-Mann & McIntosh, 1971).

The experiments with calcium have been extended, and below we report measurements of specific radioactivity of ^{45}Ca or ^{47}Ca in relation to time after tracer administration, made on the unattached pre-implantation embryo (blastocyst) and the post-implantation foetus, as well as maternal tissues, in intact and ovariectomized animals, and also in ovariectomized animals treated with ovarian hormones. Compartmental analysis has been used, enabling us to fit mathematical models to the experimental results, and estimates have been made of the sizes of the calcium compartments, their turnover times and the rates of

flux of calcium in the systems examined. In addition, experiments were done on the uptake of calcium by rabbit blastocysts surviving in a nutrient medium under conditions *in vitro*; in most instances the rate of ^{45}Ca uptake by the embryos was compared with that of ^{22}Na .

Materials and Methods

Animals and tissues examined

Non-pregnant and pregnant rabbits from a cross-bred high-fertility colony were used; all were multiparous animals aged 1–2 years, with a mean weight of 3.7 kg. The first half of gestation extending from the day of mating (day 0) to day 14 (roughly mid-term) was investigated. Radioactive calcium injections were given to 157 rabbits; 24 animals, not given labelled calcium but matched in respect of reproductive stage, were used for determining the total content of calcium.

Ten rabbits were ovariectomized; after an interval of 2 weeks, when their reproductive tracts showed a

marked degree of regression, these animals received daily the following hormone treatment: (i) 14 injections of $10\mu\text{g}$ of oestradiol benzoate or (ii) one or two priming injections of $5\mu\text{g}$ of oestrogen followed by six injections of 5mg of progesterone. Tracer experiments were done within 1 day of cessation of hormone treatment.

Four rabbits (three on day 6, and one on day 14, of pregnancy) were given subcutaneous injections (50mg/kg body wt.) of acetazolamide sodium salt (Diamox; Lederle Laboratories, Pearl River, N.Y., U.S.A.), the first at 20h and the next at 1h before tracer administration. Assays of carbonic anhydrase activity (Philpot & Philpot, 1936) in erythrocytes and endometrial tissue were done at the same time as measurements of radioactivity.

The following materials were examined, depending on the reproductive condition of the animals: blood plasma, uterine fluid, free-lying 6-day-old blastocysts, endometrium, early undifferentiated placental tissue (days of gestation 7–9), maternal and foetal placental tissue and coeval foetus (days of gestation 12–14), and bone (femur). Tissues and fluids were recovered as described previously (Lutwak-Mann *et al.*, 1960).

Injections of animals with radioactive calcium (^{45}Ca) and duration of experiments

$^{45}\text{CaCl}_2$ ($10\text{--}25\text{mCi/mg}$ of Ca) in aqueous solution or $^{47}\text{CaCl}_2$ (0.2mCi/mg of Ca) in 0.9% NaCl were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Each animal was injected subcutaneously with $10\text{--}30\mu\text{Ci}$ of ^{45}Ca or $3\text{--}10\mu\text{Ci}$ of ^{47}Ca in 1.8ml of 0.9% NaCl.

In the experiments reported earlier (McIntosh & Lutwak-Mann, 1972a), radioactive zinc and calcium were administered together in a sodium citrate buffer, pH7 (because the presence of citrate was essential for the experiments with zinc). Analysis of results obtained with calcium indicated that specific-radioactivity equilibrium of the tissues was apparently not attainable in the presence of citrate; therefore in subsequent experiments with ^{45}Ca , the citrate was omitted from the injection solution. It was assumed that ionic tracer rapidly reached equilibrium with the various fractions of calcium in tissues and body fluids.

The amount of calcium injected was a negligible proportion of the total calcium content of the tissues and the steady state was therefore not disturbed. Tissues and fluids were sampled at 45min and 2h (short-duration) and 4, 16 and 48h (long-duration) intervals after administration of tracer. About a quarter of the rabbits were used to obtain results on the 45min interval, in the non-pregnant animals and on each day of pregnancy up to day 14; the remaining animals were used for the other post-injection time-intervals, when the experiments were limited to stages

that are representative of well-defined hormonal and developmental conditions of rabbit reproduction, namely (i) non-pregnant (oestrous), and day of mating (both referred to as day 0); (ii) day 6, that is the so-called progestational phase, which coincides with the free-lying blastocyst stage; (iii) days 12–14, or post-implantation phase, concurrent with the early foetal stage.

Determination of ^{45}Ca

Freshly dissected tissue ($20\text{--}80\text{mg}$) was chopped up, weighed in liquid-scintillation vials and digested for 24h at 40°C in a mixture of Triton X-100 and Nuclear-Chicago Solubilizer (G.D. Searle and Co. Ltd., High Wycombe, Bucks., U.K.) diluted with toluene. To each sample 5ml of Triton X-100-toluene (2:5, v/v) containing PPO (2,5-diphenyl-oxazole) (4g/l) and POPOP [1,4-bis-(5-phenyl-oxazol-2-yl)benzene] (0.1g/l) was added and the samples were counted for radioactivity with an efficiency of about 80% in a Packard liquid-scintillation spectrometer, model 3380. Duplicates were determined with a mean s.d. of 5.4%, a minimum of 5000 counts being collected for each sample.

Determination of ^{47}Ca

Tissue ($50\text{--}500\text{mg}$) was transferred to plastic vials and counted for radioactivity directly in a Wallac gamma-ray spectrometer having a well-type NaI(Tl) crystal, with an efficiency of about 12%. Discriminator settings were selected carefully to avoid including the less energetic disintegrations from ^{47}Sc . Results were independent of tissue mass. At least 5000 counts were collected for every sample and duplicates were determined with a mean s.d. of 4.2%.

Determination of calcium

Calcium was determined in samples of soft tissues and fluids by atomic-absorption spectrophotometry as described for zinc by McIntosh & Lutwak-Mann (1972a), but with the addition of LaCl_3 . Sections of bone were cleaned by dissection, weighed, and ashed at 700°C in a furnace with a good flow of air. The ash was dissolved in 0.1M-HCl and diluted with LaCl_3 for determination as above.

Calculation of specific radioactivities

Measurements of radioactivity, expressed as d.p.m./g fresh wt. of tissue, were used to calculate uptake ratios, defined as (d.p.m./g)/(d.p.m. injected/mass of animal). Small corrections were made for residual blood in the tissues (McIntosh & Lutwak-Mann, 1972a). Specific radioactivities were calculated in the form of ratios by dividing the uptake ratio for a particular tissue by the concentration of calcium in that tissue, which was itself expressed as a ratio.

Uptake of ^{45}Ca and ^{22}Na by rabbit blastocysts in vitro

Six-day-old free-lying blastocysts were removed from the uterine horns and placed in weighed vials containing warmed and gassed (air+CO₂, 95:5) medium (Medium 199 with Earle's salts; Flow Laboratories, Irvine, Ayrshire, U.K.), supplemented with 1.5% (w/v) bovine serum albumin [Fraction V; Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.], adjusted with Tris to pH 7.4; gentamycin (Flow Laboratories), 5 µl/ml of medium, was added; incubation was carried out at 37°C. In a parallel study (C. Lutwak-Mann, unpublished work) it was ascertained that under the above conditions rabbit blastocysts continue to grow and differentiate normally for up to 48 h.

Sufficient ^{45}Ca and/or ^{22}Na ($^{22}\text{NaCl}$, 25 µCi/mg of Na; The Radiochemical Centre) was included to give about 200 000 d.p.m. of each radioisotope/ml. After incubation at either 37° or 19°C, the blastocysts were washed four times with tracer-free medium not containing serum albumin. The final wash was free of significant amounts of either tracer. The blastocysts were carefully drained, re-weighed and transferred to fresh vials for determination of radioactivity. When the radioisotopes were determined simultaneously by using the liquid-scintillation spectrometer, ^{22}Na radioactivity was counted with an efficiency of about 30% in a window at higher energy than the most energetic ^{45}Ca disintegrations; a correction was made for the ^{22}Na in the ^{45}Ca -counting window.

Compartmental analysis

The method of applying compartmental analysis to the measurements of specific radioactivity was described in detail by McIntosh & Lutwak-Mann (1972a). By assuming that the steady state was maintained and that the experimentally observed exchange of tracer between the blood plasma and the other tissues could be described in terms of simple two- or three-compartment open systems, it was possible to derive estimates of the calcium fluxes (exceptions to this are referred to in Table 2). For the blastocysts, which do not possess direct links with the maternal blood plasma, the endometrium was assumed to act as the reservoir of tracer. The models were valid descriptions of the experimental data, as judged by the values of reduced χ^2 obtained (Table 2).

Results*Calcium concentration in rabbit tissues*

Most values have been reported previously (Lutwak-Mann & McIntosh, 1971). No significant differences in calcium concentrations were found at the various reproductive stages investigated. Results for tissues not reported by Lutwak-Mann & McIntosh

(1971), expressed as µg of Ca/g fresh wt., $\pm$ S.E.M. ($\pm$ S.D.) were: maternal placenta, 89 ± 5 (± 18); foetal placenta, 90 ± 7 (± 23); foetus, 75 ± 3 (± 11); bone, $2.64 \times 10^5 \pm 0.09 \times 10^5$ ($\pm 0.29 \times 10^5$). Generally, there was considerable individual variation in the concentration of calcium in rabbit tissues in comparison with that of zinc (McIntosh & Lutwak-Mann, 1972a).

Specific radioactivity of ^{45}Ca in rabbit tissues at 45 min after tracer administration, in relation to blood plasma, during days 0–14 of pregnancy, and in tissues from ovariectomized and hormone-treated animals

Certain aspects of the results can best be illustrated by comparing the specific radioactivities of the tissues 45 min after injection of tracer (Fig. 1). Up until day 2 of gestation, values registered for blood plasma and endometrium were nearly the same, indicating a remarkably rapid exchange between these two systems. In fact, equilibrium between the non-pregnant oestrous endometrium and blood plasma was already attained at 25 min. By days 2–3 values for the endometrium began to decrease; this trend was maintained (except for minor peaks on days 5 and 9) as gestation developed, indicating a slowing down of the rate of calcium exchange, which therefore appeared to be related to the hormonal condition of the animal.

Experiments with ovariectomized rabbits provided further evidence of influence exerted by hormones on calcium transport in the endometrium. The specific radioactivity in the endometrium of ovariectomized or ovariectomized and subsequently oestrogen-treated animals corresponded to that established for non-pregnant rabbits, whereas endometrium recovered from ovariectomized progesterone-treated rabbits showed values matching those recorded for this tissue in the progestational phase (days 5–6 of gestation), which is characterized by a high concentration of circulating progesterone. Unlike in the endometrium, there was no change in the specific radioactivity of the blood plasma of ovariectomized and untreated or hormone-treated animals.

Early undifferentiated placental tissue (days 7–9) attained higher values than the contiguous endometrial tissue, reaching a peak at 9 days. In fully differentiated maternal and foetal placental tissue (days 12–14) values were lower.

Specific radioactivities of ^{45}Ca in rabbit blood plasma, endometrium, uterine fluid, blastocysts, placental tissues and foetus as a function of time after injection of tracer

Results to which compartmental analysis was applied, expressed as variation in radioactivity of ^{45}Ca in relation to time after injection of tracer, are presented in Fig. 2; it is evident that at 48 h values of specific radioactivity have reached equilibrium in the systems illustrated.

Fig. 1. Specific radioactivity of ^{45}Ca in tissues at 45 min after injection of tracer, during days 0–14 of pregnancy in the rabbit

Measurements were made in rabbit blood plasma ($\circ$), endometrium (Δ), undifferentiated placental tissue ($\blacktriangledown$) and maternal ($\square$) and foetal ($\blacksquare$) placenta. Included are values for ovariectomized rabbits (untreated, U), oestrogen-treated (Oe) and progesterone-treated (P); blood plasma ($\bullet$), endometrium ($\blacktriangle$). The uncertainties, represented as vertical bars, are equal to S.E.M. Abbreviation: NP, non-pregnant.

Blood plasma. The results of measurements on samples of blood plasma (Fig. 2) were fitted satisfactorily by a function consisting of the sum of two exponential terms. The passage of tracer into the bloodstream was slower than with ^{65}Zn (McIntosh & Lutwak-Mann, 1972a) but was sufficiently fast to allow the analysis to be carried out.

The parameters derived are summarized in Table 1. There was little difference between the results obtained for the three disparate reproductive stages investigated. Close examination of the fitted models indicated, however, that with advancing pregnancy the

specific radioactivity in blood plasma at each post-injection interval was lowered, suggesting that the rate of calcium exchange between plasma and the other tissues was enhanced as pregnancy progressed.

Endometrium. The difference in specific radioactivity between oestrous and progestational endometrium at 45 min (also seen above in Fig. 1) was obvious, but at long-duration intervals this difference became progressively less.

Compartmental analysis (Fig. 2a, Table 2) indicated that it was possible to fit a three-compartment model to the data obtained for both non-pregnant

Fig. 2. Variation in specific radioactivity of ^{45}Ca in relation to time after injection of tracer

Values are shown for tissues from rabbits at different stages of pregnancy; values for non-pregnant rabbits are also included. The curves represent the fitted models. (a) Non-pregnant (NP) and 6-day pregnant (P) rabbits. $\circ$, NP and $\bullet$, P blood plasma; $\square$, NP and $\blacksquare$, P endometrium; $\triangle$, uterine fluid; $\blacktriangledown$, 6-day-old blastocysts. (b) 12–14-day pregnant rabbits. $\bullet$, Blood plasma; $\square$, maternal and $\blacksquare$, foetal placenta; $\circ$, foetus. The uncertainties, represented as vertical bars, are equal to S.E.M.

and progestational endometrium. The flux in both these reproductive stages was very high in endometrial tissue, especially in the oestrous condition. Comparison of the two hormonal stages shows that the turnover times of the fast and slow compartments were roughly the same, whereas the relative compart-

ment sizes were different: in the non-pregnant endometrium the fast compartment represented nearly 90%, as against 60% in the progestational endometrium, of the tissue calcium.

Uterine fluid. In the uterine fluid the specific radioactivity was much lower than in the endometrium in short-duration experiments, and reached a maximum at about 4h. A two-compartment model sufficed to describe the results; calcium flux in uterine fluid was a small fraction of the endometrial value and the turnover time was 4.6h, as against 12min for the major compartment of the oestrous endometrium.

Blastocyst. In the 6-day-old blastocysts tracer was present at 45min; in fact, tracer was detectable in these unattached embryos already at 25min from the time of administration. The blastocyst values were well below those of the uterine fluid and also reached a maximum at 4h. Both these systems came into equilibrium with the endometrium between 16 and 48h after injection.

At this stage of rapid embryonic growth, when rabbit blastocysts increase their mass 25-fold within 48h, the system was not amenable to steady-state compartmental analysis. Therefore results from long-duration experiments were not analysed. However, values from short-duration experiments were used to calculate the turnover time and compartment size of the more rapidly exchanging blastocyst fraction. These values, as well as those obtained for calcium flux, were similar in blastocysts and foetus.

Table 1. *Parameters in the function fitted to the values of specific radioactivity of ^{45}Ca in blood plasma in relation to the time interval after injection of label*

The function $p_t^* = A_1 \exp(-A_2 t) + A_3 \exp(-A_4 t)$ was fitted to the pooled specific radioactivities in each group of experimental animals: non-pregnant, day 6 and days 12–14 of pregnancy. p_t^* is the specific radioactivity at time t after injection. The function consists of a term describing a fast process of calcium exchange (A_1 and A_2) and a term describing a slow process (A_3 and A_4). When fitting models for the other tissues it is necessary to relate these to the decrease with time in the specific radioactivity of the blood plasma, because it is assumed that blood plasma acts as a reservoir of labelled calcium. The rate constants (A_2 and A_4) only are given because the two other parameters have little practical significance here. Uncertainties are expressed as s.d.

	A_2 (h^{-1})	A_4 (h^{-1})
Non-pregnant (oestrous)	0.43 ± 0.05	0.065 ± 0.004
6-Day pregnant	0.63 ± 0.15	0.089 ± 0.006
12–14-Day pregnant	0.45 ± 0.04	0.061 ± 0.002

Table 2. *Results of fitting the models to the measurements of specific radioactivity in the tissues, expressed in terms of compartment size, turnover time and flux of calcium*

Turnover time is the time taken for an amount of calcium equal to the total calcium in a compartment to pass in one direction across its boundary. For the three-compartment model, the tissue compartment exchanging calcium more rapidly is listed first. Numbers in parentheses after the names of tissues indicate the day of gestation. Uncertainties are expressed as s.d.

Tissue	Compartment size (% of tissue Ca content)	Turnover time (h)	Flux (μg of Ca transported to or from compartment/h per g fresh wt. of tissue)	Type of model (and reduced χ^2)
Non-pregnant endometrium (0)	88 ± 8 12 ± 8	0.20 ± 0.12 12 ± 6	540 ± 320 1.1 ± 0.6	Three-compartment (1.1)
Pregnant endometrium (6)	58 ± 2 42 ± 2	0.22 ± 0.09 9.1 ± 0.3	370 ± 150 5.1 ± 0.7	Three-compartment (1.3)
Oestrous uterine fluid	100	4.6 ± 0.3	21 ± 1.4	Two-compartment (0.86)
Blastocysts (6)* (endometrium serving as reservoir)	66 ± 7 34 ± 7	7.1 ± 0.9 —	8.0 ± 1.4 —	Three-compartment (1.1)
Maternal placenta (12–14)	71 ± 4 29 ± 4	0.73 ± 0.1 230 ± 88	87 ± 16 0.11 ± 0.03	Three-compartment (0.86)
Foetal placenta (12–14)	62 ± 3 38 ± 3	0.77 ± 0.1 148 ± 38	72 ± 13 0.23 ± 0.08	Three-compartment (1.6)
Foetus (12–14)*	65 ± 13 35 ± 13	7.6 ± 1.7 201 ± 63	6.5 ± 2.1 0.13 ± 0.05	Three-compartment (5.2)

* The steady state cannot be assumed for either the blastocyst or foetus owing to their rapid growth over the experimental period (see the text). It was not possible to derive meaningful values for either the turnover time or the flux of the second blastocyst compartment.

Placental tissue. In tissues examined at the post-implantation stage (Fig. 2b and Table 2) the maternal placenta exchanged calcium with blood plasma only slightly more rapidly than did the foetal portion. In fact, insofar as 12–14 rabbit placentae are concerned, little difference was noted between the maternal and foetal portions with respect to flux, turnover time and relative compartment sizes; compared with the endometrium, placental tissue had a much smaller calcium flux.

Foetus. As indicated by the large value for reduced χ^2 obtained in analysing the data recorded for the foetus (Table 2), the three-compartment model did not satisfactorily describe the variation of experimental values of specific radioactivity with time. This may have been because the model was inadequate or because the steady state was not achieved in the fast-growing conceptus. At the same time, however, it is noteworthy that the flux to the foetus was a small percentage of that established for placental tissue. The turnover time of the slow compartment and the relative sizes of the two compartments of the foetus resembled those of placental tissue.

Bone. The very low specific radioactivity recorded for bone indicated that only a minute fraction of the bone calcium was exchanging with that of blood plasma in the experimental interval imposed by our experiments. At 48 h the specific radioactivity of bone was one-twentieth of that of the other tissues. Analysis of results for bone was not attempted. It may be mentioned here that measurements on bone removed from a rabbit at a much more advanced gestational stage, namely day 24, gave substantially the same results; in contrast, values 3–4 times higher were obtained in bone from a fast-growing 4-month-old female given tracer under identical experimental conditions.

Effect of a carbonic anhydrase inhibitor on ^{45}Ca uptake

In view of the occurrence of carbonic anhydrase in the endometrium and placental tissue of the rabbit (Lutwak-Mann, 1955), and the apparent involvement of this enzyme in movements of tissue electrolytes

(Maren, 1972), it was decided to examine the influence of a carbonic anhydrase inhibitor such as acetazolamide (Diamox) on the uptake of calcium on days 6 and 14 of gestation, representing respectively the progestational phase, which coincides with maximal carbonic anhydrase activity in the rabbit endometrium, and the early post-implantation stage, in which placental tissue is fully operative.

It was found that in four rabbits that had been pre-treated with acetazolamide in such a way that the carbonic anhydrase activity was completely inhibited both in the erythrocytes and in the uterine tissues referred to above, the pattern of ^{45}Ca uptake 45 min after tracer injection on either day 6 or 14 was not significantly altered in any of the systems investigated at these two gestational stages.

Uptake of ^{45}Ca and ^{22}Na by rabbit blastocysts in vitro

Blastocysts incubated in the nutrient medium for more than 3 h increased in size, as judged by increase in weight. The results of fresh-weight measurements on 53 blastocysts before and after incubation are given in Table 3. At 37°C, but not at 19°C, the increase in mass of a blastocyst after 18 h incubation was considerable. After 18 h of incubation at 37°C, 63% of the mass of the blastocyst represented fluid taken up by the embryos from the surrounding medium, because, although embryonic cell numbers increased at the same time, this would have had no significant influence on blastocyst mass at this developmental stage.

The results of tracer experiments are shown in Fig. 3. At 37°C, after 18 h of incubation, the concentration of ^{45}Ca in blastocysts was not more than 15% of that in the medium, and at 19°C the uptake was negligible. Therefore it was evident that optimal conditions for penetration of calcium into the blastocoelic cavity were not met. Moreover, when blastocysts were removed after 18 h of incubation, thoroughly rinsed, and replaced in fresh, tracer-free medium and incubated for a further 2–3 h, practically no tracer was lost from the blastocysts to the medium.

To test whether experimental conditions for uptake of ions by blastocysts *in vitro* were adequate, we repeated the experiments, incubating the embryos in the presence of radioactive sodium. Unlike calcium, sodium was rapidly taken up by the blastocysts, reaching a concentration of about 70% of that contained in the medium after 18 h at 37°C; this value was in line with the observed uptake of fluid by the embryos. At 19°C the uptake of ^{22}Na was very slight.

By drawing tangents to the curves at zero time, estimates were obtained of the rate constants for uptake of tracer at 37°C. These were 0.01 h^{-1} for calcium and 0.12 h^{-1} for sodium. Thus the rate of uptake of sodium was about 10 times faster than that of calcium. At 19°C this differential was maintained even though the rates of uptake were markedly diminished.

Table 3. Increase in mass of rabbit blastocysts on incubation *in vitro*

Blastocysts were weighed before and after incubation. The mass-increase factor was obtained by dividing the post-incubation mass by the initial mass of the blastocysts. Uncertainties are expressed as S.E.M.

Conditions	Mean mass-increase factor	No. of blastocysts
18 h, 19°C	1.24 ± 0.02	7
3 h, 37°C	1.22 ± 0.01	17
18 h, 37°C	2.65 ± 0.10	29

Fig. 3. Uptake of ^{45}Ca and ^{22}Na by 6-day-old rabbit blastocysts *in vitro*

Uptake of radioactivity is expressed as the ratio between the radioactivity (c.p.m.) found in unit weight of entire incubated blastocyst and the radioactivity (c.p.m.) in unit weight of incubation medium. The uptake of radioactivity was calculated for the weight attained by the embryos at the end of incubation. ○, Calcium and △, sodium incubated at 37°C (○, △) and at 19°C (●, ▲). Each point is the mean of measurements made on between 3 and 29 blastocysts. The uncertainties, represented as vertical bars, are equal to S.E.M.

The concentrations of calcium and sodium in the medium (72 and 143 mmol/litre respectively) were comparable with those in the blastocoelic fluid itself (87 and 138 mmol/litre). Doubling the concentration of calcium in the medium failed to enhance the rate of uptake. Comparison with results arrived at by compartmental analysis indicated that the rate of calcium uptake by rabbit blastocysts under conditions *in vivo* was approximately 10 times higher than that recorded in experiments *in vitro*.

Discussion

It was the object of this study to measure the kinetics of calcium transport in the reproductive tract of the rabbit, and at the same time pinpoint the hormonal aspects of calcium movement, in the oestrogen-dependent stage of the non-pregnant animal and the progesterone-dominated conditions prevailing in pregnancy. We have limited our observations to early gestation and two different phases of development of the rabbit conceptus, namely the blastocyst stage, when the embryos lie free in the uterine lumen and receive nutrients via the secretory products of the endometrium, and the post-implantation stage, when the young foetus is supported by placental tissues.

A difficulty experienced when assessing specific radioactivities for the various systems examined was

the marked variability in the concentrations of calcium encountered in rabbit body fluids and tissues. This produced a relatively large variance for the measurements concerned. As no definite seasonal variation emerged, it must be assumed that the calcium fluctuations in rabbit tissues are characteristic of this species. It is conceivable that up to a point they may be responsible, for some at any rate, of the minor peaks in specific radioactivity evident, for instance, in blood plasma and uterine tissues between the day of mating and day 14 of gestation, seen in Fig. 1.

However, we were able to detect a correlation between calcium transport and hormonal conditions, such as the decrease in specific radioactivity of the blood plasma, and consequently the rise in calcium exchange, parallel with advancing pregnancy (Figs. 1 and 2). Another phenomenon suggesting hormonal dependence was the strikingly rapid mixing of systemically administered tracer between blood plasma and the endometrial tissue, especially marked in the oestrous condition (Fig. 1). Findings made with ovariectomized animals further strengthened the evidence of a hormone-linked relationship in endometrial tissue: results established for non-pregnant rabbits could be reproduced by oestrogen treatment of ovariectomized animals, whereas values typical of the progestational stage (days 5–6 of gestation) were obtained by administering progesterone.

Compartmental analysis showed that the total calcium flux per g of fresh tissue was highest in the endometrium, and there was a pronounced downward gradient from the latter towards the uterine fluid and the blastocysts. A similar, though less steep, gradient was evident between placental tissue and foetus. Comparing total fluxes for calcium with values for flux previously established for zinc (McIntosh & Lutwak-Mann, 1972a), it was found that the former exceeded the latter by a factor of about 400 in the endometrium, 150 and 30 in maternal and foetal placenta, respectively, and 30 in the foetus.

The turnover times of the compartments that exchanged calcium rapidly ranged from 45 min to 7 h, except for endometrial tissue, which displayed a turnover time of only 12 min. The remarkably long turnover times for the slowly exchanging compartments of placental tissue and foetus were beyond the time-scale of our longest experimental interval. Therefore within the framework of our experiments they must be regarded as representing virtually unexchangeable calcium (Van Breemen *et al.*, 1966), amounting to 30–40% of the tissue calcium.

With respect to relative compartment size, in each instance the fast compartment was larger than the slow one by a factor of 3–9. Without a detailed investigation of the state of calcium in each tissue, it was not feasible to assign to these compartments specific physiological function or significance.

Concerning the rabbit conceptus and taking first the unattached blastocysts, it was clear that under conditions *in vivo* penetration by ^{45}Ca occurred rapidly. It was therefore noteworthy that the specific radioactivity of uterine fluid, that is the sole vehicle of tracer for the blastocysts, was markedly higher than that of the embryos themselves. Rapid penetration of unimplanted embryos has also been observed for certain other ions (Lutwak-Mann *et al.*, 1960) as well as a large number of drugs (Lutwak-Mann, 1973), underlining thereby the role of endometrial secretory products as the intermediaries between the maternal body and the pre-implantation conceptus.

The results obtained *in vivo* contrasted with findings made on blastocysts growing *in vitro*. Although we were able to demonstrate that, under our experimental conditions, ions such as sodium freely entered the blastocoelic space, obviously the culture medium lacked some as yet unidentified but essential carrier, presumably emanating from the endometrium under physiological conditions, which facilitates the passage of calcium into the blastocysts, across barriers which at this stage consist of the albuminous coat, zona pellucida and trophoblast layer. In the absence of such a hypothetical factor, possibly a calcium protein, the uptake of calcium by the blastocysts *in vitro* was one-tenth of that recorded for blastocysts *in vivo* or sodium *in vitro*.

Although the 12–14-day-old rabbit foetus possesses fully developed blood links with maternal circulation, the specific radioactivity of the foetus was not higher than that of the blastocysts.

Bone is the main reservoir of calcium in mammals and it was therefore important to include this tissue in our measurements. However, the involvement of bone in respect of calcium movement during the time-interval imposed by our experiments was slight. This may have arisen partly because we were concerned with the first half of gestation, and partly owing to the age of the animals, which were fully grown rabbits; when a fast-growing rabbit was studied under our

routine conditions, uptake of ^{45}Ca by bone was several times higher.

It has been known for some time that the peak of carbonic anhydrase activity in the rabbit endometrium coincides with the blastocyst stage of embryonic development, and that the enzyme is also present in placental tissue. Attempts made in the past to establish a link between carbonic anhydrase activity and embryonic survival were unsuccessful. Similarly, in the present study, we have failed to note any significant departure from the pattern of ^{45}Ca uptake in the systems examined as a routine on either days 6 or 12–14 of gestation, when using for tracer experiments rabbits in which carbonic anhydrase activity had been completely inhibited in erythrocytes and uterine tissues by treatment with Diamox. Whatever the role of uterine carbonic anhydrase may be in relation to reproductive processes, it does not appear to be involved in calcium transport.

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