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EARLY GROWTH OF RABBIT TROPHOBLAST*

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Extensive studies have been made of the neoplastic character of trophoblast tissue as it occurs during implantation and placentation (Ober, 1959; Boving, 1959a; Kirby, 1962). Growth of pre-implantation trophoblast has received little attention. The rate of growth of this tissue and its contribution to conceptus growth in the first days after its differentiation, is the subject of this paper.

METHODS

Embryos were removed from female New Zealand white rabbits, at multiples of 24 hours after breeding. Previous to four days *post coitum* they were flushed from the oviduct, but thereafter, were found in the uterus. Flushing was accomplished by forcing a stream of warm physiological saline through the tubes with a hypodermic syringe, and collecting the irrigant in large watch glasses where the ova or blastocysts settle quickly to the bottom. Blastocysts older than five days of age do not flush readily from the uterus, so these were removed by carefully opening the uterus with pointed forceps and scissors while it was submerged in saline. Gentle agitation of the opened uterus washed the blastocysts free. Measurements of blastocyst diameter and of individual cells were made with an ocular micrometer. Cell counts and mitotic indices were determined from flat-mount preparations made according to the methods of Moog and Lutwak-Mann (1958) and the acetic orcein squash procedure of Tjio and Whang (1960).

RESULTS

Table 1 lists measured diameters, calculated volumes, and surfaces of conceptuses through the first ten days of development. Figures 1 and 2 show these data graphically. For these calculations the blastocysts were assumed to be perfect spheres, although in reality they are frequently ellipsoidal or compressed slightly along one axis. The diameters reported are averages between the shortest and longest measurements for these aspherical specimens. Because of the number of other papers that present size data on rabbit embryos, it was considered uneconomical to accumulate more data here than needed to establish the general growth pattern. Table 1, therefore, also lists the mean diameters of conceptuses at different stages as recorded by several other authors. The general agreement is apparent.

*This investigation was supported by a grant from the National Science Foundation (NSF GB-44).

TABLE 1

Days (*post coitum*) ± .5 hr	Number of conceptuses studied	Average diameter (mm)	Range of diameter (mm)	Average volume (mm^3)	Range of volumes (mm^3)	Average surface (mm^2)	Range of surfaces (mm^2)	Average diameters from other sources (mm)
1	10	.16	.15–.2					.163*
2	6	.16	.15–.19					.164*
3	14	.16	.13–.19	.0020	.0011–.0036	.0803	.0527–.116	.170*–.3†
4	12	.27	.2–.4	.0157	.0042–.0335	.304	.125–.502	.4†
5	19	1.02	.5–1.4	.565	.065–1.445	2.605	.784–6.16	1†
6	18	2.8	1.8–3.9	11.5	3.06–31.0	24.8	10.17–47.6	3†
7	10	5.01	4.5–5.9	66.06	47.8–92.2	78.9	63.5–112	5†–6.7‡
8	8	8.7	8–9.5	344.5	268–448	238	207–283	10.5‡
9	7	11.9	10.5–13	878.3	698–1152	444.5	346–530	12.5‡
10	6	16.9	10–20	2514.0	523–4189	896	314–1256	16.7‡

*Greenwald, 1957
†Boving, 1959 b
‡Adams, 1960

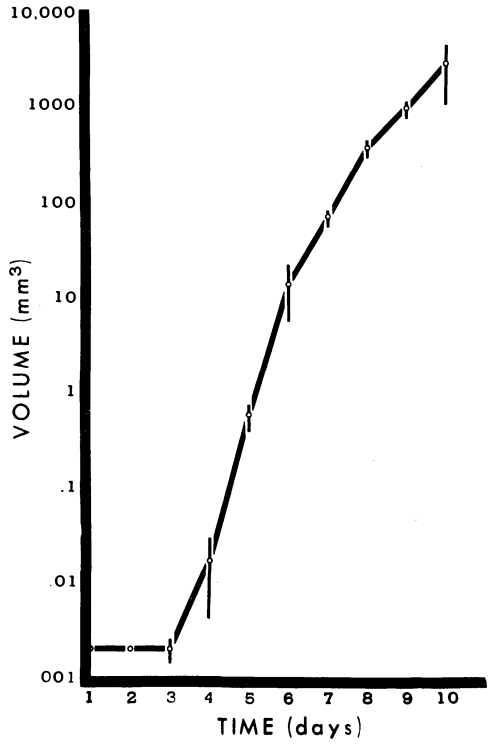

FIGURE 1. Volume growth of early rabbit blastocysts. The thin vertical line at each point represents the standard deviation.

Measurements of individual cells composing the trophoblast of blastocysts show that they cover approximately the same area, regardless of the age of the embryo (table 2). Embryos of eight, nine and ten days of age are already implanted so that it was not possible to measure trophoblast cells in their normal state of distension. However, embryos grown to these ages from seven days *post coitum*, in culture, have comparable sized cells.

Since the size of trophoblast cells stays relatively constant, the number of cells necessary to compose the spherical surface of a blastocyst of any

TABLE 2

Age of blastocyst (days *post coitum*)	Trophoblast cell area	
	Range $X10^{-4}$ mm²	Mean $X10^{-4}$ mm²
4	2.94–3.20	3.00
5	2.71–3.92	3.05
6	2.94–3.55	3.23
7	3.10–3.30	3.16
		Average: 3.11

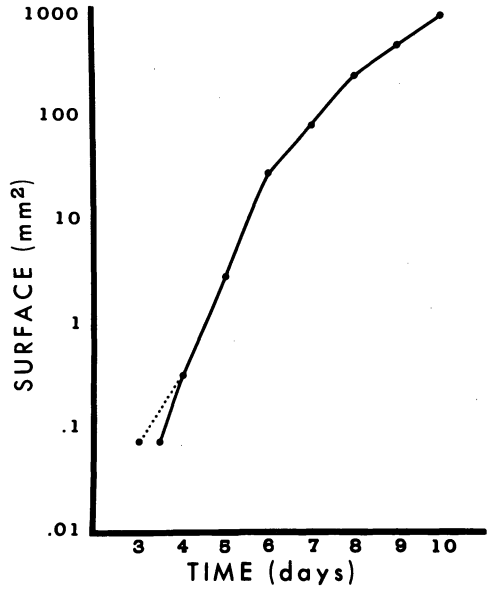

FIGURE 2. Surface growth of early rabbit blastocysts. Although measured at day three, actual expansion does not begin until at least 3½ days.

given size may be determined. From this, the doubling time can be calculated. Table 3 lists these data along with the number of cells and the doubling time of early cleavage stages. Doubling time is shown in figure 3. Table 4 shows the mitotic index of trophoblast.

DISCUSSION

Rabbit trophoblast is first visually differentiated when blastulation begins at about 3½ days *post coitum* (Lewis and Gregory, 1929; Gregory 1930; Purshottam and Pincus, 1961). At that time, the swelling of the spherical mass serves to separate those cells which will form the surface from the ones that will remain aggregated into the embryonically-oriented inner-cell-

TABLE 3

Days *post coitum*	Average number of cells by count	Number of cells needed to compose surface	Doubling time (hours)
1	2		
			7.9
2	18		
			8.1
3	141		
			8.1
4	1112	983	
			7.9
5	9000	8,930	
			7.8
6		80,259	
			15.1
7		255,339	
			15.9
8		770,227	
			25.6
9		1,436,893	
			24.0
10		2,899,676	

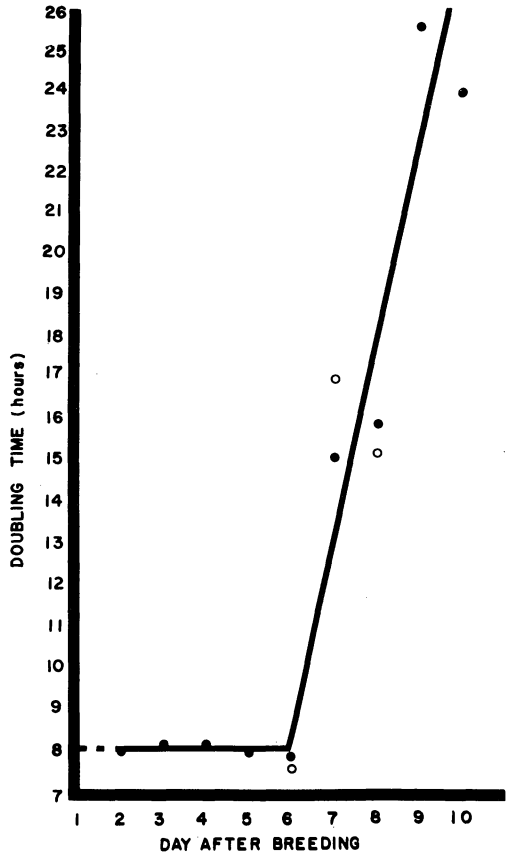

FIGURE 3. Doubling times for cleavage stages and early trophoblast cells. Open circles are points calculated from data of Adams, Hay and Lutwak-Mann (1961).

mass. This early tissue cannot be considered fully differentiated because, as Boving (1959a) pointed out, it does not yet invade, therefore lacking the main diagnostic quality of trophoblast. The growth potential of this precursor tissue is, however, exceptional.

TABLE 4

Days *post coitum*	Number of cells counted	Number of mitotic figures	Mitotic index (%)
3	300	13	4.33
4	1800	75	4.16
5	4700	203	4.32
6	4000	178	4.45
7	5000	219	4.45
8	2000	81	4.05
9	2000	98	4.90
10	4000	189	4.72
			Mean: 4.42 ± .28

The process of blastulation begins just prior to the time when the cells reach their smallest volume. During the first cleavage, the ovum divides into two daughter cells, each containing only half the volume of the parent cell. This process continues with successive cleavages; each time reducing the single cell volume by half while doubling the number of cells present, but not significantly altering the total embryonic volume. By four days *post coitum*, the single cell volume ceases to be reduced by each division and remains constant thereafter for trophoblast cells throughout the period studied (see figure 4). The average measurement of this minimal volume for trophoblast cells is 1.545×10^{-6} mm^3. Reference to table 2 shows that the surface covered by each cell also remains constant. Therefore, the surface of a blastocyst must increase by duplication of trophoblast cells unless new cells were to be supplied from lower layers. Additions from lower layers would gradually deplete these layers unless they too replicated at the same or at a higher rate. Cross sections of blastocysts show that the trophoblast remains one cell layer thick, except where it forms a continuum with the inner cell mass. Pictures published by Glenister (1961) support this observation.

The production of enough trophoblast cells to cover the surface of the rapidly expanding blastocyst requires a doubling time of about eight hours up to six days *post coitum*. The doubling time during cleavage is also

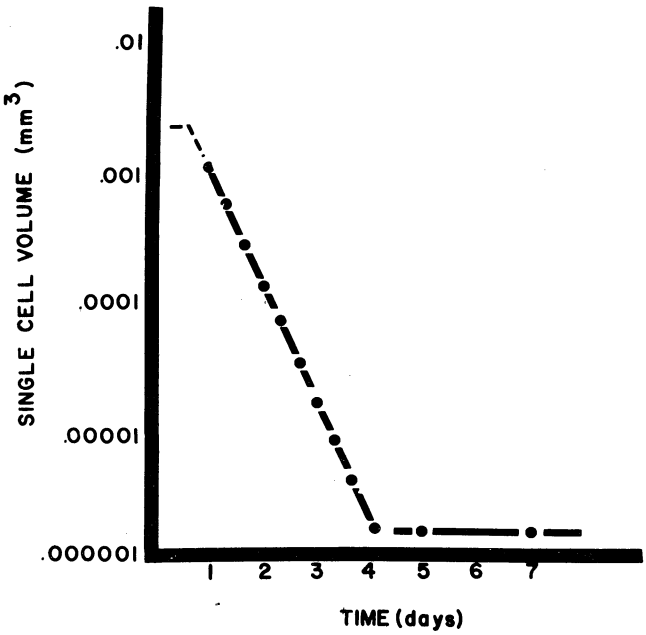

FIGURE 4. Changes in single cell volume of early rabbit embryos. At four, five and seven days *post coitum*, the measurements were made on trophoblast cells directly; the values for preceding days were calculated from the volume of the ovum and estimates of the number of cleavages.

noted to average about eight hours. (Lewis and Gregory, 1929; Gregory, 1930). Occasional "slow cleaving" embryos (Hafez, 1962) have been observed but they are not considered in this report.

When the doubling times, as recorded in table 3, are plotted against the day of development (figure 3) it may clearly be seen that the eight-hour doubling time changes dramatically on day six. It begins to lengthen at the average rate of five hours each day for the period studied. This change in growth beyond six days *post coitum*, reflected by doubling time, demands resolution.

Doubling time is a product of the effects of mitotic index and mitotic duration and as such may be altered by changes in either parameter. For the short doubling time of eight hours, a cell mass would be expected to have either a high mitotic index or a short mitotic duration or both (assuming that the cells were not phased). From table 4 it may be seen that the mitotic index of trophoblast tissue is remarkably constant for the period studied and that it averages 4.4 per cent. This figure is in good agreement with the observations of Adams, Hay and Lutwak-Mann (1961) who found an average mitotic index of 4.28 per cent in trophoblast cells of 5½ to 6½ day blastocysts. Using the formula $M = \frac{\tau}{T} \log_e 2$ (where M = mitotic index, τ = mitotic duration and T = doubling time) derived by Smith and Dendy (1962), it may be concluded that a mitotic duration of 30.5 minutes will permit a tissue having a mitotic index of 4.4 per cent to double the number of its cells in exactly eight hours. Experiments on mitotic inhibition support this observation. When cell division was blocked in six-day rabbit blastocysts by *in vivo* colcemide application (Adams, Hay and Lutwak-Mann, 1961) the greatest mitotic index (69.1 per cent) was reached when treatment was continued for nine hours. One would expect mitotically-inhibited cells to appear in greatest number at a time interval for continuous treatment, corresponding to their normal "turnover time." Whereas doubling time is the required for a cell population to double the number of cells, "turnover time" (Hooper, 1961) is the time needed for all the cells present to divide. Turnover time for a tissue having a mitotic index of 4.4 per cent and a mitotic duration of 30.5 minutes will be 11.5 hours. Therefore, where 100 per cent of the cells will have entered mitosis in 11.5 hours, after nine hours 69.1 per cent of them will have approached the mitotic stage. 69.1 per cent of 11.5 hours is 7.99 hours. If just one hour could be assumed as necessary for distribution and absorption of the drug in effective quantity, then the true effective time would be eight hours. A mitotic duration of about 30 minutes is therefore substantiated.

By the addition of five hours to the doubling time for each day after day six, (with the same mitotic index) the mitotic duration is forced to lengthen. One day thereafter, it will be 49.5 minutes, two days 68.5 minutes and continuing so that it may be seen to lengthen at the rate of 19 minutes per day. Using the basic formula $\left(\frac{M}{\tau}\right)$ of Evensen (1962), for mitotic rate (that is,

number of cells out of 100 that complete mitosis per unit time), this rate will, of course, drop from 8.8 cells/hour in six-day rabbit trophoblast to 2.48 cells/hour in the ten-day old tissue.

It is difficult to imagine that actively growing tissue would show a *severe* reduction in mitotic rate, especially when this reduction is achieved by prolongation of mitosis to where this process would require over 8½ hours for completion by the time of parturition on day 32.

Alternate explanations for the apparent growth changes initiated on day six are more reasonable.

The number of trophoblast cells present at each stage was calculated from those necessary to provide the spherical surface. In reality, more trophoblast cells exist because of the formation of trophoblast knobs (Schoenfeld, 1903) and ultimately of syncytiotrophoblast. Adams, Hay and Lutwak-Mann (1961) note that the knobs may first be seen in "some of the larger six day embryos" but their presence, even in larger embryos, is quite variable.

If one determines the difference between the potential number of trophoblast cells that would be present in a given conceptus if the original eight-hour doubling time were maintained after day six, and the number actually present, it may be seen that trophoblast knob formation accounts for a very small fraction of this difference. For example, an average seven-day old blastocyst which normally contains 255,339 trophoblast cells would contain about 600,000 cells if the original growth rate had continued; a difference of 344,661 cells. This same blastocyst contains about 1578 knobs over its surface, each knob composed of an average of 27 cells (or rather nuclei because definitive cell walls become less apparent as the syncytium develops) for a total of 42,606 cells. This number is only 12 per cent of the difference. Obviously the production of syncytiotrophoblast does not account for very much of the difference in cell number.

In the same paper cited above (Adams, Hay and Lutwak-Mann, 1961) it was noted that "nuclear degeneration was commonly observed in the embryonic area and a moderate amount of it was considered normal." Cell death, as a method of disposing of some fraction of the cells produced is thus considered as a factor in accounting for the cell reproductive changes initiated on day six. Occasionally necrotic cells were observed in the flattened trophoblast preparations but accurate counts were not possible. However the effect of cell death can be calculated. To determine the cell death rate one cannot use the potential number of trophoblast cells as described above because the death of even a small fraction of cells, due to the loss of their progeny, would have a large effect on the ultimate size of the mass. This rate was therefore determined for each day in relation to one hour of growth on that day. When the difference between the number of trophoblast cells that would be present on each day, if the mitotic rate of 8.8 per cent were maintained for one hour and the number existing at the observed rate, is considered as representative of the cells that die, a cell death rate may be calculated (table 5). For continued growth, the cell

TABLE 5

A On day *post coitum*	B One hour's growth at the observed rate results in cells numbering	C One hour's growth, if the 8.8% mitotic rate is maintained, will result in cells numbering:	D Difference (C – B) (deaths)	E Cell Death Rate (% deaths/hr.) $\left(\frac{D}{C}\right)$
6	87,029	87,209	180	.206
7	268,939	277,789	8,850	3.19
8	799,927	838,077	38,150	4.55
9	1,480,143	1,561,493	81,350	5.21
10	2,971,176	3,154,676	183,500	5.82
*16	4,207,206	4,523,556	316,350	7.0
*21	10,786,468	11,631,318	844,850	7.25
*29	22,074,637	23,875,337	1,800,700	7.52

*These figures are derived under the assumption that the same mitotic index exists and the trophoblast cells continue to retain a constant average size, but these observations were *not* made.

death rate can never equal or exceed the mitotic rate, but would be presumed to approach that rate on the flattened portion of the growth curve. The difference between the two rates at any one time would be a measure of net growth at that time. In the case of these trophoblast cells, where the mitotic rate is 8.8 per cent, the cell death rate always stays below this level but continues to approach it until parturition, by which time the existence of and necessity for trophoblast tissue is terminated. By this time, the difference between the two rates is about 1 per cent. Figure

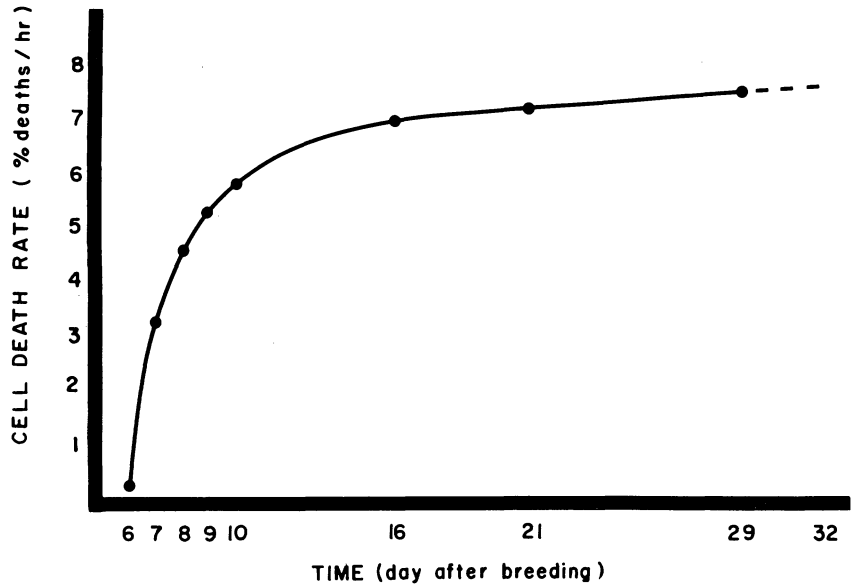

FIGURE 5. Changes in trophoblast cell death rate with advancing age of the embryo.

5 shows graphically the changes in cell death rate that can be calculated for rabbit trophoblast.

Any measure of mitotic index is subject to question because of the difficulty of recording cells in early prophase or late telophase. Most counts therefore, are made of cells in the obvious stages ranging from late prophase to early telophase. Because these terminal stages are frequently unusually long (Hooper, 1961; Moog and Lutwak-Mann, 1958) the *true* mitotic index may easily be double the observed value. Until further work can solve this problem, it is reasonable to accept the 4.4 per cent mitotic index recorded here as a measure comparable to those recorded by other investigators for use in similar studies.

The difference in doubling time of rabbit trophoblast cells that begins after six days *post coitum* is therefore believed to be the product of some change in mitotic duration, development of syncytiotrophoblast, and onset of cell death. These events are coincidental with the 200-fold increase in metabolic rate reported by Boving (1959a) but this relationship is not understood.

The mechanism for accumulation of fluids during blastulation is, as noted by Lutwak-Mann (1959), "entirely unknown." Trophoblast cells are believed to provide the blastocyst fluid (Blandau and Rumery, 1957). That inner-cell-mass-free fragments of blastocysts will reconstitute by fluid accumulation (Daniel, 1961) is support for this belief.

Comparison between the number of trophoblast cells and the change in volume of the blastocyst at any given time permits the establishment of a constant for the acceleration of the rate of blastocyst fluid provision from single trophoblast cells. Table 6 lists the essential data and calculations. Figure 6 shows the relationship between the increase in blastocyst volume, plotted as mm^3/hour/trophoblast cell and the time of development, plotted in days *post coitum*. The volume per cell per hour accelerates at a constant rate throughout the period studied. This constant is found to be $.222 \times 10^{-6}$ mm^3/cell/hour/hour.

TABLE 6

Day *post coitum*	Fluid is accumulating at the rate of (mm^3/hour)	Being provided by trophoblast cells numbering	Fluid-mm^3/hour/cell ($X10^{-6}$)	Same as preceeding column, but calculated from data of Adams, et al. (1961)
4	.002250	983	2.288	3.62
5	.068	8,930	7.610	7.04
6	.8	80,259	9.96	11.70
7	5.6	255,339	21.96	
8	18	770,227	23.4	
9	42	1,436,893	29.5	
10	96*	2,899,676	33	

*The volume of an 11-day blastocyst was acquired by extrapolation of the growth curve, and the rate of fluid accumulation at day 10 was based on that figure.

Extrapolation to zero puts the point of first fluid production at about 3½ days of development, the same time as blastulation has already been observed to start and immediately prior to the time when single trophoblast cell volume of the early embryo reaches its minimal and constant size. It is believed, therefore, that trophoblast cells become specifically differentiated in their capacity to provide a blastulating fluid at or about the time when the cells of the cleaving ovum reach their smallest size.

Some insight into the mechanism of blastocyst fluid accumulation is now possible. At the acceleration rate noted, a single cell would, on the average, be producing its own volume of fluid within three to four hours after the start of blastulation and would be producing almost 20 times its own volume of fluid each hour by nine days *post coitum*. The process, therefore, would not seem to be a secretion in the usual sense. An osmotic system is ruled out because osmotic rate would *decelerate* as the surface-volume ratio of a sphere decreased and as the internal pressure increased, unless

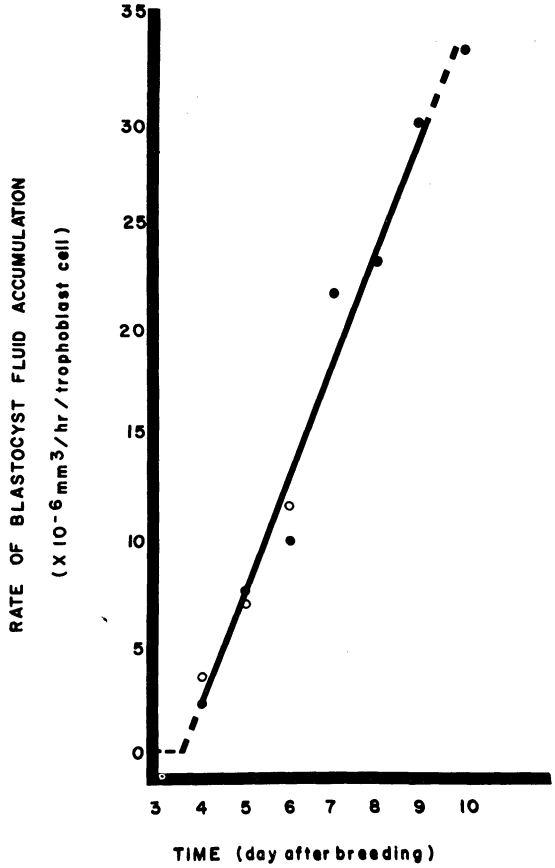

FIGURE 6. Acceleration of rate of blastocyst fluid accumulation. Open circles are points calculated from data of Adams, Hay and Lutwak-Mann (1961).

the composition of the hypertonic phase changed radically and continuously. When one considers the multiple membranes represented by cell surfaces of all forming germ layers and the internal and external changes mediated by the metabolizing cells, it is apparent that a blastocyst is not a simple osmotic system. But if the expansion is to be credited to osmotic action then, within the period studied, the relative concentration of one or all of the essential solutes in the innermost blastocyst fluid as compared to that in the uterine fluid must have progressively increased until over 20 times the original gradient exists. Studies by Lutwak-Mann (1954, 1959, 1962) and others (Gregoire, Gongsakdi and Rakoff, 1961) have shown that this is not the case. These observations do not exclude the possibility that *some* fluid enters by osmosis and that *some* secretion (for example, lactic acid) occurs, but neither of these phenomena alone, or in combination, could be considered sufficient to supply the fluid at the rate needed.

Lutwak-Mann, Boursnell and Bennett (1960) presented evidence to support a concept of "active transport" as opposed to diffusion. The observations recorded above further support this belief. The trophoblast cells are presumed to possess a mechanism whereby some of the components of the fluid surrounding the developing blastocyst are actively transported at a fast and accelerating rate to permit the rapid expansive growth. Such a mechanism might be described as a transtrophoblast pump.

SUMMARY

Several indices of growth are recorded for early trophoblast tissue, as calculated from measurements made on rabbit blastocysts throughout the first ten days of development.

Doubling time of trophoblast surface cells, up to six days *post coitum*, is about eight hours, extending thereafter at the average rate of five hours per day until it is greater than 24 hours by nine to ten days *post coitum*.

The mitotic index stays relatively constant throughout this period, averaging 4.42 per cent.

The duration of mitosis in early trophoblast tissue is shown to be about 30 minutes up to six days *post coitum*. It *may* lengthen beyond that time to partially account for the change noted in doubling time. Production of syncytiotrophoblast and cell death are also considered to contribute to this change.

Calculations are made for the rate of blastocyst fluid accumulation. An acceleration rate constant is observed equal to $.222 \times 10^{-6}$ mm^3/trophoblast cell/hour/hour. The existence of some type of transtrophoblast pump is inferred to account for this rapid rate.

Correlations between trophoblast cell size, onset of fluid accumulation, and blastulation are noted.

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