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Some Physiological and Biochemical Properties of the Mammalian Blastocyst

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I wish to express to the organizers of this international Symposium my sincere appreciation for having extended to me an invitation to participate in the proceedings.

This gives me a most welcome opportunity of renewing old friendships as well as meeting colleagues whom, so far, I had known by name only.

I am a frequent visitor to this beautiful country and always take away with me the happiest of memories. I know that the present Symposium will also stand out in my memory as an occasion scientifically rewarding and at the same time most enjoyable socially.

Research in the physiology and biochemistry of the mammalian embryo is expanding at present with great rapidity. To review and discuss adequately the numerous contributions is no longer feasible within the confines of a short article. It has therefore been decided to restrict comments to findings made by the author and her coworkers.

The study of the pre-implantation mammalian embryo and its relation to the maternal environment has for some years formed an integral part of our investigations, in Cambridge, on the physiology and biochemistry of animal reproduction. For the study of the early mammalian embryo we have chosen the rabbit blastocyst, which represents a most useful object of study owing to its remarkably large size and numerical availability in fertile animals. The rabbit uterus too, lends itself admirably to biochemical studies, not least because of the case with which the endometrium and early placental tissue can be separated for experimental purposes, by simple dissection.

In the rabbit the blastocoelic cavity begins to form on day 4 of gestation (the day of mating being named day 0). This is the time when the embryos begin to enter the uterine horns, in which early on day 5 they are found, usually in a cluster, at the ovarian uterine end. By about day $5^3/_4$, their diameter is 0.5–1.0 mm and their fresh weight 1–2 mg each. Early on day 6 the blastocysts' diameter increases to about 3 mm, and they weigh on the average 25 mg each. In the course of day 6, a series of consecutive changes takes place, culminating in the formation of the primitive streak and the appearance of trophoblastic knobs and extra-embryonic mesoderm. Late on day 6, the blastocysts are found evenly spaced along the uterine horns, they are elipsoid in shape and weigh about 100 mg each. Their zona pellucida becomes very

thin and ruptures easily. The impressive increases in weight during the blastocyst development, in the rabbit, are almost entirely due to water uptake, as the dry mass remains very low. But in the rabbit blastocyst this developmental phase is also accompanied by a steep increase in cell numbers: whereas the 5-day-old blastocysts are made up of some 5 000 to 10 000 cells, towards the end of day 6 the cell number reaches 60 000.

So as to be able to assess quickly and simply the condition of the embryos after their exposure either in vivo or in vitro, we have worked out a histological method, the so-called flat-mount (Mooc and Lutwak-Mann 1958), which in essence consists of the following few steps. The blastocysts are removed directly from the exposed surface of nembutalized rabbits, without rinsing or flushing, and fixed several hours in methanol. The flat-mount is prepared so as to place the embryonic disk, i. e. the embryo proper, centrally, in correct relationship to the trophoblast, and the preparation is stained. Unlike other fixatives, methanol does not cause shrinkage of the rabbit blastocysts, therefore the method permits, among others, quantitative assessment to be made of the overall dimensions of the embryos, as well as measurement of the surface area of the entire blastocyst, and the area of the embryonic disk separately. Application of this technique to many hundreds of blastocysts recovered from perfectly normal, untreated rabbits, has enabled us to classify empirically the developmental progress of the rabbit embryo into a series of 'stages'. These stages form a base-line for the evaluation of blastocysts obtained from animals that had been treated by exogenous agents; equally well, the method can be applied to blastocysts that had been cultured in vitro. When examined by the flat-mount technique, the 5-day-old blastocysts are seen to have a thin embryonic disk of irregular outline; in our classification this is stage A. Early day-6 blastocysts are classified as stage B. The steps that lead to the formation of the primitive streak and up to day 7 of gestation, we refer to as stages C–G (Lutwak-Mann and Hay 1965a).

The rapid growth of the blastocysts during days 5–7 of pregnancy is clearly reflected in the dimensions of the entire blastocyst surface (S), and rather less impressively, of the area of the embryonic disk (ED), expressed as sq. mm, range in brackets: at 6 days 5 h., which is equivalent to our stages B–C, S = 39.45 (24.33–57.91), ED = 0.74 (0.50–1.26); however, at 6 days 17 h., that is our stages F–G, S = 87.26, (71.77–117.91), and ED = 1.27 (0.78 to 1.93). These values illustrate not only the overall increase of rabbit blastocysts emphasized earlier, but help to underline a most important point, namely the considerable range of variability in size, evident among blastocysts from normal, highly fertile animals. This is a significant physiological feature of the mammalian blastocyst, especially marked in polytocous laboratory animals. Failure to take into account this variability which exists among coeval blastocysts normally, can lead to serious errors in the evaluation of experimental results concerned, for instance, with blastocysts obtained after maternal exposure to drugs etc.

As is well known, on day 7 of gestation precisely, the rabbit blastocyst

attaches antimesometrially to the uterus, whereas the embryonic disk faces the mesometrium. The phenomenon of disk orientation is among the most interesting features of uterine attachment, but as yet its mechanism is not understood. Subsequent to the antimesometrial attachment there follows, during days 7–9 of gestation, in the rabbit, the period of maximum permeability of the blastocoelic cavity, which enables the entry into blastocyst fluid of maternal blood proteins, and various foreign substances, such as e. g. the azo-dye trypan blue, which are kept out at earlier or later stages. The fact that the attachment occurs in the rabbit with great punctuality on day 7, gives us a most valuable criterion of the action of various substances, some of which, as will be seen later, are capable of preventing blastocyst attachment.

Our own experimental work on rabbit blastocysts has been chiefly concerned with the chemical composition of the embryos and the entry into the embryos of certain maternally transmitted agents. Below it is proposed to discuss some aspects of this experimental approach.

1. Some chemical constituents of the rabbit blastocyst

Most of our data on rabbit blastocysts pertain to the 6–7-day-old embryos. In the course of analytical determinations on blastocysts we have generally endeavoured to relate embryonic values to those in the maternal body. Determinations were therefore extended to maternal body fluids such as blood plasma or serum, and peritoneal fluid, but special attention has been devoted to the immediate environment of the free-lying blastocysts, that is the endometrium and its secretion.

It is not universally known that in the rabbit the endometrial mucosa produces two distinct types of secretion, depending upon ovarian hormonal control, during the early stages of pregnancy with which we are concerned (Lutwak-Mann 1962 b). In the oestrous phase, which coincides with the pre-ovulatory stage, the lumen of the rabbit uterus is distended with a variable quantity of thin fluid. However, from about the 4th day onwards, that is during the so-called progestational stage of endometrial proliferation, no free fluid is ever found under physiological circumstances, but the endometrium produces a scanty, thick and remarkably sticky secretion, a film of which also coats the surface of the blastocysts. For experiments one can readily collect, by aspiration of the uterine horns, as much as 1–2 ml or more, of the oestrous uterine fluid; on the other hand, not more than 20–50 mg per uterine horn can be obtained during the progestational phase, preferably by uptake into absorbent filter paper, followed by elution. For most experimental purposes we have generally made use of the more readily available oestrous uterine fluid, the more so as we have also elaborated a simple experimental means for inducing the production of uterine fluid in non-pregnant rabbit, namely by injection of watersoluble oestrogens (Leone et al. 1963).

Table 1

Some chemical constituents of the rabbit blastocyst (6 days 4 hrs)

Constituent	*Content per single blastocyst* (average wet weight 25 mg)
Total N	38 µg
Amino acid N	10 µg
DNA	0.55 µg
RNA	2.0 µg
Cell number	about 59,000
Na	79 µg
K	11 µg
Cl	64 µg
Bicarbonate (CO_2)	36 µg
Glucose	5 µg
Lactic acid	7 µg
Nicotinic acid	32 mµg
Thiamine	1.9 mµg
Riboflavin	0.6 mµg
Cyanocobalamine (vitamin B_{12})	8 mµg
Folinic acid	0.2 mµg
Inositol	trace ?
Sialic acid	trace

Table 1 lists the content per single blastocyst (aged 6 days 4 hrs) of certain important embryonic constituents, of which we shall discuss only some, namely nucleic acid; amino acids; glucose, lactic acid and bicarbonate; coenzymes and growth factors.

a) Nucleic acid

Analyses of DNA content in rabbit blastocysts have been carried out by LØVTRUP (1963), using his highly specific method of microbiological assay (LØVTRUP and ROOS 1963). LØVTRUP established that in the course of days 5–7 of rabbit blastocyst development there is a marked increase in total DNA content (Table 2). He also noted variability in DNA content of chronologically identical embryos, in confirmation of our morphological findings. In 6-day-old blastocysts which have been recovered from rabbits treated with 6-mercaptopurine, a treatment which invariably destroys the disk (see p. 111), LØVTRUP found DNA values considerably below those set up for coeval normal blastocysts. Thus, in each instance, determinations of DNA in rabbit blastocysts served to underline histologically observed phenomena.

Analyses of RNA were done by EDSTRÖM (1963), separately on material from the embryonic disks, and from the trophoblast area. Because the technique (EDSTRÖM 1958, 1960) is worked out for the determinations of ultra-microquantities of RNA, the trophoblast parts dissected from that layer for analysis had to be estimated by cell count; the values obtained in this way are considered to possess a certain error. On the other hand, in the case

Table 2

DNA content in rabbit blastocysts (LØVTRUP)

Blastocyst age	μg DNA per single blastocyst
5 days 2 hrs	0.031
6 days 2 hrs	0.403
6 days 2 hrs	0.468
6 days 2 hrs	0.679
6 days 3 hrs	0.523
6 days 6 hrs	1.400
6 days 16 hrs	1.232
6 days 16 hrs	1.440
6 days 18 hrs	3.159

of embryonic disk, the total RNA content per disk was obtained by adding values established for successive measured pieces; these values are thought to be much more accurate. The analytical findings (Table 3) indicate that during the time-span 5–6½ days of pregnancy, the total RNA level of the rabbit blastocyst increases about 3 times; of the two blastocyst components, the disk showed the largest relative increase, in agreement with morphological changes. In spite, however, of the larger relative RNA increase in the embryonic disk, the total content in the disk was lower than that of the trophoblast layer. Microelectrophoretic RNA analyses of the disk and trophoblast did not reveal any significant differences between these two regions (Table 4). Ribonuclease extracts obtained from the two blastocyst components contained some contaminating material which interfered with the determination of the pyrimidine nucleotides, so that reliable values were obtained only for the purines. Nevertheless, it was possible to judge from some particularly good separations that the base composition is the one typical for mammalian ribosomal RNA, with adenine and uracil around 10%, and guanine and cytosine around 30%. No significant differences could be recorded in the adenine to guanine ratio, between the disk and trophoblast, nor was there evidence of any significant change during days 5–6 of development of the embryos.

Table 3

Content of RNA in rabbit blastocysts (EDSTRÖM)

Blastocyst age	RNA content (μg)		
	Embryonic disk	Trophoblast	Entire blastocyst
5 days 5 hrs	0.09	0.61	0.7
5 days 5 hrs	0.02	0.55	0.6
6 days	0.14	2.20	2.3
6 days 7 hrs	0.29	1.60	1.9

Table 4

Adenine/guanine ratio in RNA extracted from rabbit blastocyst disk and trophoblast (EDSTRÖM)

Blastocyst (age)	Embryonic disk	Trophoblast	No. of determinations
5 days 5 hrs	0.62	0.58	2, 3
5 days 5 hrs	0.59	0.65	4, 2
5 days 5 hrs	—	0.56	–, 7
6 days	0.61	0.59	5, 2
6 days	0.58	—	14, –
6 days 7 hrs	0.61	0.66	5, 6
6 days 8 hrs	0.69	—	7, –
6 days 4 hrs* ...	0.56	0.58	6, 7
6 days 4 hrs* ...	0.60	0.58	4, 4
6 days 4 hrs* ...	0.59	0.55	4, 4
6 days 4 hrs* ...	—	0.62	–, 5
6 days 4 hrs* ...	—	0.57	–, 7

* These blastocysts have been recovered from thalidomide-treated rabbits.

In an isolated experiment analogous RNA determinations were made in blastocysts recovered from thalidomide-treated rabbits, but the values recorded appeared essentially the same as those registered in embryos from untreated rabbits.

Recently we have been doing some experiments on the content and nature of RNA in the uterine endometrium of the rabbit (VITTORELLI et al. 1966), and find among others, that the amount of RNA, when correlated either to protein or to the DNA content of the tissue, increases between days 0 and 3 of gestation, and declines thereafter, to form a plateau at days 5–8.

b) Amino acids

At 6½ days of pregnancy the rabbit blastocyst contains about 40 μg total nitrogen, of which ¼th is amino acid N. Using the Technicon Autoanalyser procedure for chromatographic separation and detection of amino acids (ROTTENBERG and LUTWAK-MANN 1964), we have found that rabbit blastocyst fluid at 6 and 7 days is a rich source of free amino acids, especially glycine and alanine. An abundance of free amino acids in blastocyst fluid was in itself quite interesting, but we were reluctant to ascribe special significance to this phenomenon until similar analyses were also made in maternal blood plasma and peritoneal fluid, and, of course, in the uterine fluid. Moreover, we have made analyses of blastocyst fluid obtained from embryos recovered from rabbits that had been treated with either mercaptopurine or thalidomide, so as to see whether such maternal treatments were capable of modifying the amino acid pattern in the embryos. Somewhat to our surprise, we found that except for minor differences, the amino acid pattern was nearly identical for blastocyst fluid and the maternal body fluids;

furthermore, in blastocysts from the drug-treated animals there was no major change either, in this respect.

Fig. 1 reproduces typical measurements of the amino acid ninhydrin-complex at 570 mμ, made in directly comparable quantities of 6-day-old rabbit blastocysts, oestrous uterine fluid, and maternal peritoneal fluid, respectively; the key to the amino acid peaks is in Table 5; norleucine (13) was used as marker.

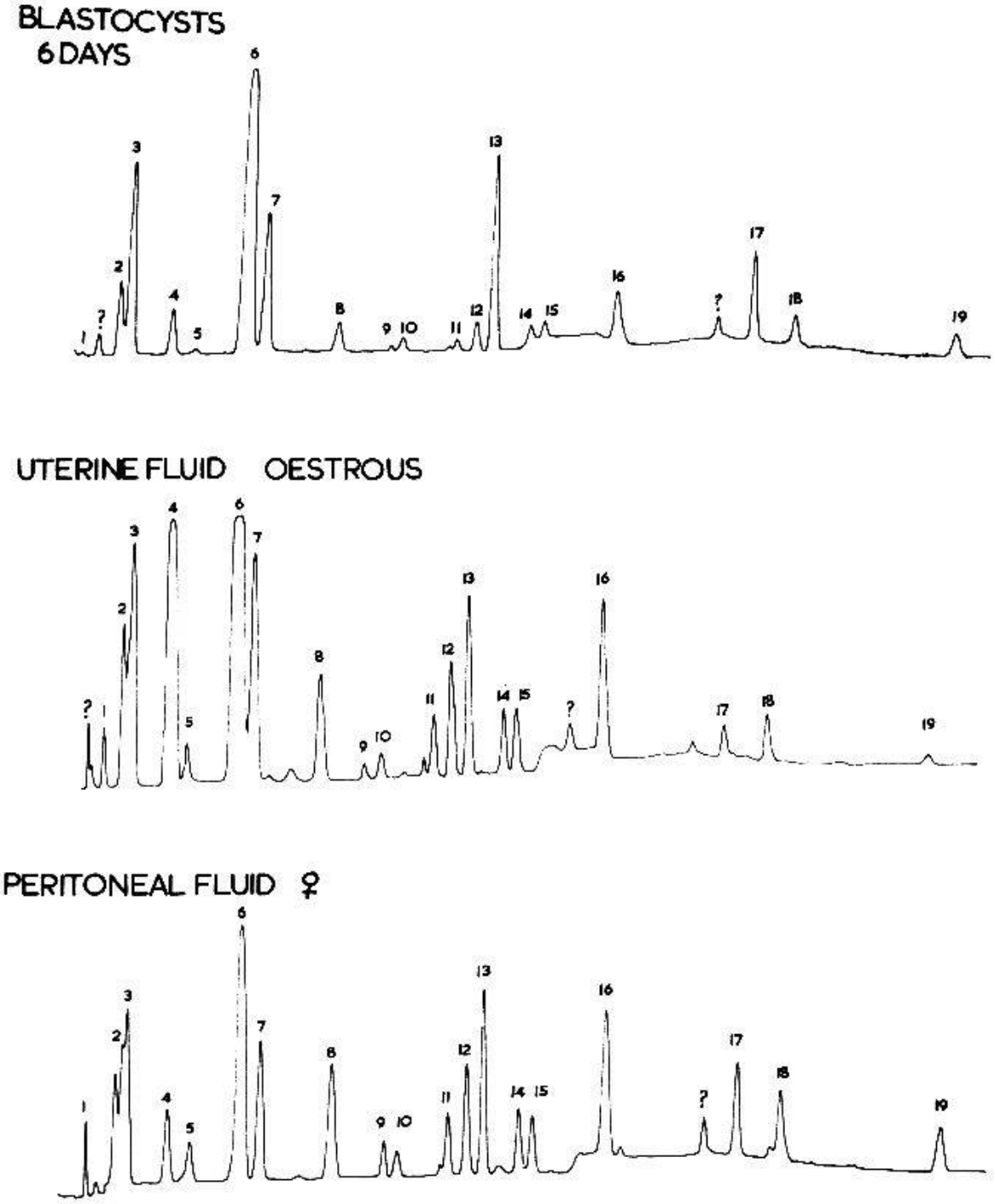

Fig. 1. Measurements of the amino acid ninhydrin-complex at 570 mμ (Technicon Autoanalyser), in directly comparable quantities of 6-day-old rabbit blastocysts, uterine oestrous fluid, and maternal peritoneal fluid. Table 5 gives the key to amino acid peaks; norleucine (13) served as marker.

Table 5
Key to amino acids in Fig. 1

1	Aspartic acid	8	Valine	14	Tyrosine
2	Threonine	9	Cystine	15	Phenylalanine
3	Serine	10	Methionine	16	NH_3
4	Glutamic acid	11	Isoleucine	17	Lysine
5	Proline	12	Leucine	18	Histidine
6	Glycine	13	Norleucine (marker)	19	Arginine
7	Alanine				

It seems thus that the amino acid composition recorded is generally representative of the rabbit as an animal species, rather than specifically of the embryos. Also, it is clear that markedly embryotropic drugs such as mercaptopurine or thalidomide, do not tend to affect the amino acid 'profile' in the blastocysts. It is as yet uncertain what role to ascribe to the many amino acids found in the blastocoelic cavity. The absence of a distinct gradient between their level in blastocyst fluid and uterine fluid, suggests that, unlike some other constituents, the amino acids enter the blastocoele by a process of diffusion, rather than more complex forms of active transport.

c) Glucose, lactic acid and bicarbonate

These three metabolites are of interest because their concentration undergoes characteristic changes in the course of days 5–7 of blastocyst development (LUTWAK-MANN 1962 a). In contrast to the free amino acids, these metabolites occur in the embryo in concentrations strikingly different from maternal body fluids. Glucose is very low in embryos between day 5 and 6½; it begins to rise late on day 6, but even on day 7 its level still remains distinctly below maternal blood glucose. Lactate concentration is also low on day 5 and 6, but towards day 7 the blastocoelic lactate rises to a level of 80–100 mg%, thus exceeding many times peripheral maternal values. Bicarbonate on day 5 and 6 shows a concentration several times higher than that of maternal blood plasma, but from day 7 onwards the embryonic bicarbonate values begin to coincide with those in maternal body fluids.

We have been interested in comparing the concentration of glucose, lactic acid, and bicarbonate, as well as the ions Na, K and Cl, in the 6-day-old blastocyst and the oestrous uterine fluid, as well as maternal plasma and peritoneal fluid; Table 6 shows the good agreement, in respect of these constituents, between the uterine fluid and blastocyst fluid.

d) Coenzymes and growth factors

The occurrence of nicotinic acid, thiamine, and riboflavin, all three of which have been determined by microbiological assay (KODICEK and LUTWAK-MANN 1957), indicates the presence in blastocysts of essential coenzymes. Of special interest to us was the extraordinarily high content in

Table 6

Concentration (mEq/l) of bicarbonate, glucose, lactate, K, Na, and Cl, in rabbit blastocysts (at 6 days 4 hrs of pregnancy), rabbit uterine fluid (10–12 hrs after ovulating injection), and maternal blood plasma and peritoneal fluid (average values).

	Blastocysts	Uterine fluid	Blood plasma	Peritoneal fluid
Bicarbonate	47	44.50	22	20
Glucose	0.55	0.48	5	6
Lactate	2.0	1.8	5	3
K	11.1	10.7	6.7	—
Na	138	110	150	—
Cl	72	55	105	—

blastocyst fluid as well as the endometrial progestational secretion, of vitamin B_{12} (JACOBSON and LUTWAK-MANN 1956). The amount of vitamin B_{12} in the rabbit blastocysts exceeds values established in liver, and is several times higher than the content of this growth factor in rabbit blood plasma. Its role in blastocyst development is unknown. Inositol, which is known to occur in rabbit foetal fluids at advanced stages of gestation, is not present in the blastocyst fluid, except perhaps in traces. Folinic acid was detected in the blastocysts, its presence in the uterine secretion has not been investigated.

Enzymes in blastocysts and maternal environment

In the course of some of our experiments we have had the opportunity to compare the occurrence of certain enzymes in the embryos and the uterine endometrium as well as early placental tissue. We have found that ATPase is readily detectable in rabbit blastocysts as well as in the endometrial mucosa and secretion, but the 5-nucleotidase is absent in the embryos, though very active in the uterus. Purine nucleosidase present in rabbit blood plasma and peritoneal fluid, as well as endometrial mucosa and secretion, is absent in the free-lying blastocysts (LEONE et al. 1963). RNase is widely distributed in rabbit body fluids and tissues, and is also active in the blastocysts (VITTORELLI et al. 1966).

Another enzyme in the female reproductive tract to which at one time we have devoted attention, is the endometrial (and placental) carbonic anhydrase, the dependence of which upon progesterone and luteoids generally, we have demonstrated several years ago (LUTWAK-MANN 1955, 1957). When the discovery of this enzyme was made in the rabbit endometrium, it seemed that there might be a close connection between the activity of uterine carbonic anhydrase on one hand, and the elevated bicarbonate content of blastocyst fluid, on the other, but so far, it has been difficult to provide a direct proof of such a link. Studies on uterine carbonic anhydrase in many mammals, including the opossum, also in birds, vivi-

parous fishes and certain reptiles (LUTWAK-MANN 1956, 1960; MANN and LUTWAK-MANN 1963), have established the ubiquitous occurrence of this enzyme in the female genital tract and its strict dependance upon hormonal, chiefly ovarian, control, but as yet, no clear-cut relationship to embryonic development has been demonstrable. Nevertheless, the endometrial-placental carbonic anhydrase merits further study, especially in respect of iso-enzyme pattern and the mechanism of the hormonal influence on its activity in the female reproductive tract.

2. Passage of exogenous agents into blastocysts in vivo

a) Simple sugars

The intravenous administration of a hypertonic glucose or fructose solution to the pregnant rabbit on day 6, fails to raise the concentration of either sugar in the free-lying blastocysts, concomitantly with prevailing maternal hyperglycemia or fructosemia. It may be added in parentheses, that similarly, parenteral administration of glucose or fructose has no effect upon the level of these sugars in the oestrous uterine fluid.

However, from day 7 of pregnancy onwards, coinciding with the uterine attachment, maternally introduced hexoses substantially increase reducing sugar concentration in blastocyst fluid of the attaching embryos. Moreover, in experiments of longer duration, the blastocyst sugar levels remain still elevated at a time when the maternal levels have already returned to normal (LUTWAK-MANN 1962 a).

It is not easy to explain why the glucose concentration in free-lying blastocysts is not affected by maternal hyperglycemia, in view of the fact that, as will be shown later, the unattached embryos react promptly to a large variety of maternally transmissible agents, among them the glucose analogue, 2-deoxyglucose. However, on the basis of purely chemical determinations of glucose or fructose concentration, one cannot really judge whether or not these metabolites are capable of entering the unattached embryos. To solve the problem one would have to give, parenterally, labelled glucose, and follow up its distribution in relation to the embryonic space. At present, we also lack an adequate explanation of the lag in clearance of sugars (or other substances) from the blastocyst cavity, after maternal administration.

The relative stability of embryonic glucose level to changes in the maternal organism, is also underlined by experimental results with insulin. In short-term experiments maternal insulin-hypoglycemia does not perceptibly depress the glucose concentration of the 8 9-day-old blastocysts. It is, however, probable that more prolonged insulin treatment of the pregnant animal would ultimately affect the embryonic glucose content.

b) Labelled ions

Interesting and informative patterns of maternal-embryonic distribution were obtained following the administration of certain labelled ions, such as

^{32}P, ^{35}S, ^{42}K, ^{24}Na, ^{131}I, to the pregnant rabbit during the 6–10 day interval of pregnancy (LUTWAK-MANN et al. 1960). The systems used for evaluation of embryonic-maternal ion uptake comprised, among others, the endometrium and its secretion as the immediate environment of the free and attaching embryos. In experiments routinely terminated 45 min after ion injection, we found that all the ions listed were capable of entering freely the endometrium. They were also present in the uterine secretion, in relatively high and roughly equal amounts, for each of the ions investigated. In contrast, however, to the secretion, the pattern of ion uptake into the blastocyst fluid not only differed markedly for each ion, but also varied in extent, depending upon the day of the gestational span examined. Probably the most significant observations related to the entry of labelled phosphate from the maternal organism into the embryos: on day 6, in experiments of limited duration, there was practically no ^{32}P uptake by the free-lying blastocysts. In this respect therefore, the behaviour of maternally transmitted ^{32}P resembled that of glucose. However, concomitantly with the progress of uterine attachment, ^{32}P was able to enter the blastocoelic cavity. In recent experiments of extended duration (VITTORELLI et al. 1966) it was found that maternal administration of ^{32}P on day 2–3 of gestation, resulted in marked incorporation of phosphate into the nucleic acid fraction of the 6-day-old embryos. As yet, we have no information in what form phosphate actually reaches the embryos, whether in inorganic form, or built into phosphorylated organic derivatives.

c) Foreign substances such as antimetabolites, hormones and drugs

As a profitable side-line of our main studies on maternal-embryonic interaction, we have examined, by means of the flat-mount procedure, the morphological changes induced in rabbit blastocysts by various agents passed on via the mother. Our findings have been published in extenso elsewhere (ADAMS et al. 1961; LUTWAK-MANN and HAY 1962). We give therefore only a few illustrative examples to demonstrate the applicability of this simple technical approach, by describing briefly results which were obtained in blastocysts following treatment of pregnant rabbits with (i) 6-mercaptopurine (6-MP), (ii) 2-deoxyglucose (2-DG), (iii) water-soluble oestrogens, (iv) salicylate, and (v) thalidomide.

(i) When rabbits are given a single dose of 150–200 mg/kg of 6-MP parenterally, at any time between day 1 and late 4 of gestation, it will be found that whereas the entire dimensions of blastocysts remain unchanged, their embryonic disks are practically completely destroyed, and the cells of the trophoblast, though less conspicuously, are also deleteriously affected. Moreover, in a high proportion of blastocysts the antimesometrial uterine attachment on day 7 is prevented by maternal treatment with this purine analogue. How 6-MP acts on the cells of the disk is unknown, but presumably the analogue is preferentially taken up by the fast-metabolising disk cells, to form faulty and thus lethal, nucleic acids. We have some indirect evidence

(ADAMS et al. 1961) that 6-MP can be taken up by the zygote in the oviduct, without however causing demonstrable effects until the blastocyst stage is reached. Essentially similar effects on rabbit blastocysts were achieved by maternal treatment with 8-azaguanine; bromouracil and 2-deoxyuridine gave less clear-cut results.

(ii) Our interest in 2-DG stemmed from the observations on the behaviour of parenterally administered glucose outlined above. We have found (LUTWAK-MANN and HAY 1965 b) that 2-DG, in contrast to 6-MP, given in single 200–300 mg/kg doses to rabbits, produces entirely different results, depending upon the timing of the maternal treatment. Thus, when 2-DG was injected at any time between the day 0 and day 4 of gestation, there was no embryonic loss, and the blastocysts presented a perfectly normal appearance. However, injection of 2-DG either on day 5 or 6, invariably caused serious damage to the embryonic disk, without producing any obvious changes in the trophoblast cells. In addition, there was failure of attachment of blastocysts on day 7, probably due to a disturbance in the development of the trophoblastic knobs. The most striking finding in these experiments was the speed with which 2-DG was capable of exerting its damaging effect on the disk: we could observe marked abnormalities as early as 2 hrs after i. v. injection of this compound.

The results on blastocysts were borne out by the progress of entire pregnancies, because no litter loss was encountered when 2-DG was injected during days 0–4, whereas its administration on either day 5 or 6 was followed by early foetal resorption.

We have attempted, but so far only with partial success, to reverse the embryotoxic action of 2-DG, by giving it together with an excess of glucose. We hope that a better reversal will be obtained with an ATP-precursor such as adenine, as we believe that the damaging effect of 2-DG on the disk cells may be due to rapid depletion of ATP. It remains to be seen why the cells of the trophoblast are resistant to maternally transmitted 2-DG. It is conceivable that, unlike the cells of the embryo proper, the cells of the trophoblast possess an enzyme capable of dephosphorylating 2-DG-6-phosphate, which presumably is the derivative responsible for the metabolic interference. Equally, it will be interesting to examine whether results analogous to those recorded in vivo, will also be obtained in experiments with 2-DG acting on blastocysts in vitro. We have experiments of this kind in mind, also we plan to study the action of 2-DG-6-phosphate on the disk and trophoblast, separately.

It may be added that in preliminary experiments we have also tested the effect on blastocysts in vivo, of DL-glyceraldehyde, and glucose-6-phosphate. The poor solubility of DL-glyceraldehyde makes experimentation with this glycolysis inhibitor difficult; it is, however, possible that if it is used in experiments in vitro, this obstacle will be overcome. So far, we have not seen any deleterious effects on the blastocysts, with either of these substances.

(iii) As described in the foregoing, two entirely different metabolic ana-

logues, such as 6-MP and 2-DG, can prevent uterine attachment on day 7. Suitably timed ovariectomy also prevents blastocyst attachment (LUTWAK-MANN et al. 1962), but at the same time it causes a loss of embryos which escape via the cervix, owing to altered myometrial tonus. We have managed to replace ovariectomy by i. v. administration of water-soluble oestrogens, polyoestriol phosphate and stilboestrol diphosphate (LUTWAK-MANN and HAY 1964). If these compounds are given to the pregnant rabbit in small doses, preferably not earlier and not much later than 6 days 4 hrs, then blastocyst attachment on day 7 is to a large extent prevented, and no damage at all is evident in either the disk or the trophoblast area. Treatment of rabbits with these water-soluble oestrogens offers thus a useful supply of free-lying 7-day-old blastocysts, in good yield and perfect condition, for experiments in vitro or transplantation.

(iv) Of the many drugs the embryotropic properties of which we have studied, only two will be mentioned, namely salicylate and thalidomide.

Salicylate is of interest because it is a remarkably penetrative substance and its presence can easily be detected by chemical means. We have found that if salicylate is given in non-toxic doses, it exerts no untoward effect on rabbit blastocysts. We have purposely desisted from using excessive amounts of salicylate, because from our own older work (LUTWAK-MANN 1942) and recent research on this drug, it is evident that it acts as a phosphorylation-oxidation uncoupling agent, so that near-toxic doses are likely to affect the embryos not primarily, but in a secondary manner, probably by interfering with essential carbohydrate metabolism. The presence of salicylate in free-lying 6-day-old blastocysts can easily be detected and determined quantitatively, soon after injection into the mother. It is interesting, however, to add that salicylate continues to be detectable in blastocyst fluid for several hours after it is no longer demonstrable in maternal peripheral body fluids.

(v) Our experiments on the effect of maternally mediated thalidomide have now been outdistanced by the spectacular advances of the two major research groups, led respectively by KEBERLE in Basle, and WILLIAMS in London. However, it may be recalled that, in actual fact, we were the first clearly and unequivocally, and by very simple means, to demonstrate that in the rabbit characteristic morphological changes take place in the pre-implantation blastocysts, limited to the area of the disk, after maternal ingestion of thalidomide (LUTWAK-MANN and HAY 1962). The changes seen in the blastocysts as the result of thalidomide treatment of rabbits appear unique: no other drug among the many that we have tested so far, has produced anything like it. In contrast to alkylating agents, or the metabolic analogues referred to above, thalidomide does not cause degeneration in the disk, but alters its appearance in an unmistakable manner, probably by interference with cell movement which goes on very actively during the stage approaching formation of the primitive streak. Maternal thalidomide delays the appearance of the primitive streak in rabbit blastocysts as well as their uterine attachment;

these changes, however, are not lethal and are overcome by the surviving blastocysts, which proceed to term, a small percentage showing certain skeletal abnormalities (HAY 1964).

Concluding remarks

So long as the information on the metabolism of the mammalian embryo remains fragmentary as at present, no more than a few general statements can usefully be made concerning maternal-embryonic interdependence and exchange, two processes of great complexity but also of paramount medical significance. Using the rabbit blastocyst as a model, we observe that in this species, in agreement with morphologically evident advanced organization, metabolically also the pre-implantation embryo represents a markedly differentiated entity. It seems clear on the basis of experimental findings discussed above, that the cells of the embryo proper and those of the trophoblast possess a fundamentally different type and rate of metabolism. Perhaps when these differences are clarified in the future, it will be easier to understand why the trophoblast cells can withstand insults that threaten and effectively prevent the survival of the embryo proper. To solve this intriguing question it will be necessary to resort to analytical techniques more refined and searching than those hitherto applied in experiments with the scarce mammalian material. From the preliminary data of EDSTRÖM it appears that RNA in the two main regions of the blastocyst is identical insofar as base composition is concerned, but further research on the nature of nucleic acids and nucleoproteins may uncover some inherent characteristics that are responsible for the differential behaviour of morphologically distinct areas in the young embryo.

It has now been amply demonstrated that, even when it lies free in the uterine lumen, the mammalian embryo is rapidly reached by diverse exogenous factors emanating from the maternal organism. It depends upon circumstances, dosage, timing, maternal metabolism etc., how the embryo responds to such agents. Embryonic elimination, of presumably inadequate individuals, is indeed well known to be greatest at the blastocyst stage of gestation, when it can proceed with a minimal trauma to the mother. Equally, however, it is certain that up to and including the blastocyst stage, the conceptus can safely tolerate moderate degrees of injury, such as e. g. transient arrest of mitotic divisions, delay in the formation of primitive streak, interference with formative cell movement in the disk area, probably also localized minor cell losses, all apparently amenable to efficient intra-uterine repair, which permits the resumption of normal growth. Embryonic tolerance of environmental change, marked in the trophoblast but less pronounced in the embryo proper, also finds its expression in the replaceability, for extended periods, of the maternal milieu by extra-uterine sites, as well as artificial incubation media. Thus, the sensitivity of the mammalian embryo, recently much emphasized, appears to be well matched by the

young embryos' power of adaptation and recuperation, presumably reflecting metabolic flexibility inherent in embryonic development.

A major gap in the present-day knowledge of maternal-embryonic interrelations, is our ignorance concerning the mode of entry into and exit from, the unattached embryos, first of all in respect of physiological metabolites, hormones, vitamins etc., and next of pharmacological agents and their derivatives. Should one attempt to solve these problems by experimentation in vivo or in vitro? Much exiting work on embryos, particularly of the invertebrates but recently also on embryos of certain mammalian species, is being done in vitro. This type of research permits an approach at cellular and molecular level and has been yielding important results. But, however attractive per se the isolated embryonic systems may be and however commendable from the purely scientific angle, the medical viewpoint definitely demands an *integrative* elucidation of the maternal-embryonic partnership as a whole, under experimental conditions approximating those encountered in vivo. From where we stand today a complete solution of the problems involved appears as a distant goal, but we must continue our efforts to reach it ultimately.

Summary

In recent years blastocysts of various small laboratory mammals have been used extensively for experimental studies. We have chosen for our work the rabbit because in this species the blastocyst as object of study offers certain advantages, chief among them being the precision with which one can determine the age of the early embryos, their exceptionally large size at the blastocyst stage, and numerical availability in fertile animals.

Our investigations at the Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, University of Cambridge, have been directed mainly along two lines, namely (i) compilation of data on the *chemical composition of the blastocyst*, and (ii) elucidation of certain aspects of *maternal-embryonic relationships*, involving the study of the endometrium and its secretion, and the entry into the blastocyst of exogenous agents passed on via the mother.

The chemical analyses of rabbit blastocysts have provided values for the content of protein, amino acids, DNA, RNA, certain coenzymes, ions such as Cl, Na and K, and three metabolites, viz. glucose, bicarbonate and lactic acid, the level of which was found to vary in a characteristic manner with the progress of blastocyst development.

The study of the rabbit endometrium, and placental tissue, led to the discovery in these tissues of the hormonally controlled enzyme, carbonic anhydrase, which subsequently was also found to occur in the female reproductive tracts not only in numerous mammals, including the opossum, but also in birds, viviparous fishes and reptiles. Recently the nature of nucleic acids in the endometrial mucosa of the rabbit has been studied by

gradient centrifugation, and very characteristic patterns were found to accompany the early stages of gestation.

The study of the occurrence and nature of the endometrial secretion in the rabbit revealed two distinct types, representative respectively of the oestrous and progestational stage of pregnancy. A simple means was evolved to produce experimentally in the rabbit oestrous secretion in amounts sufficient for chemical experimentation, without ligation of the uterus, by intravenous administration of synthetic water-soluble oestrogens such as polyoestriol phosphate or stilboestrol phosphate. Several chemical components of the rabbit blastocyst fluid and the endometrial secretion have been found to occur in similar concentrations, expecially the amino acids, glucose, bicarbonate and lactate.

The investigation on maternal-embryonic relationships involved experiments a) on the passage and distribution in blastocysts and the uterine environment, of simple sugars, also labelled ions (P, S, K, Na, I), and b) administration to the pregnant rabbit of various drugs, metabolic analogues, vitamins, hormones, etc., followed by microscopic examination of blastocyst morphology, by a simple and rapid method, the so-called 'flat-mount' technique. This histological technique has been adapted so as to permit not merely qualitative but also quantitative, evaluation and comparison of rabbit blastocysts. The method was first applied to large numbers of perfectly normal blastocysts, and next to blastocysts recovered from treated rabbits. In this way it was possible to elucidate both physiological and pathological features of the blastocysts. Study of normal blastocysts underlined the existence of marked developmental variability, within and between coeval litters, and the occurrence in high-fertility animals of 5–10% of non-viable embryos. The most striking features revealed by experiments on blastocysts obtained from treated animals, were:

- the speed with which exogenous agents reach free-lying mammalian embryos;
- the delay which occurs in drug clearance of the blastocoelic space as compared with peripheral maternal body fluids;
- the differential sensitivity of morphologically distinct areas of the blastocyst, to maternally transmitted agents;
- the individual variability among litter-members, in the extent of drug response;
- the capacity for intra-uterine repair following minor degrees of injury, and subsequent resumption of apparently normal growth.

Some of the embryotropic substances investigated by us, if given at the right time and in appropriate quantities, were found to possess the ability to prevent the antimesometrial uterine attachment of blastocysts, which physiologically takes place on day 7 precisely of rabbit pregnancy. The prevention of the uterine attachment of rabbit blastocysts on day 7,

has been utilized as an additional, most useful, criterion for the testing of embryonic sensitivity to certain maternally-transmissible drugs, antimetabolites and hormones.

Zusammenfassung

In den letzten Jahren wurden Blastocysten verschiedener kleiner Laboratoriums-Säugetiere in umfassender Weise für experimentelle Studien verwendet. Wir haben für unsere Arbeit das Kaninchen gewählt, denn bei dieser Species bieten die Blastocysten als Studienobjekt gewisse Vorteile, insbesondere (i) die Genauigkeit mit welcher sich das Alter des frühen Embryo bestimmen läßt, (ii) die Größe der Embryonen im Blastocystenstadium, und (iii) deren Anzahl in fortpflanzungsfähigen Tieren.

Unsere Untersuchungen in der Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, Universität Cambridge, erfolgten hauptsächlich in zwei Richtungen, nämlich (i) im Sammeln von Daten über *die chemische Zusammensetzung der Blastocysten*, und (ii) in der Aufklärung über gewisse Aspekte *mütterlich-embryonaler Beziehungen*, welche die Erforschung des Endometriums und seiner Sekretion, und des Eindringens von Fremdstoffen in die Blastocysten via das Muttertier umfaßt.

Die chemische Analyse der Kaninchen-Blastocysten lieferte Werte über den Gehalt an Protein, Aminosäuren, DNS, RNS, gewissen Koenzymen, Ionen wie Cl, Na, und K, und auch Glukose, Bikarbonat und Milchsäure, drei Metaboliten, deren Menge in charakteristischer Weise mit der fortschreitenden Entwicklung der Blastocysten variiert.

Die Untersuchung des Endometriums und des Placentargewebes beim Kaninchen führte zur Entdeckung eines hormonal gesteuerten Enzyms, der Kohlensäureanhydrase, welche nachher nicht nur in den weiblichen Fortpflanzungsorganen zahlreicher Säuger, einschließlich des Opossum, festgestellt wurde, sondern auch bei Vögeln, lebendgebärenden Fischen und Reptilien. Kürzlich wurden die Nucleinsäuren in dem Kaninchen-Endometrium mittels fortschreitender Zentrifugierung untersucht, und es wurde festgestellt, daß das Früh-Stadium der Schwangerschaft durch ein charakteristisches Muster gekennzeichnet ist.

Die Forschung über die Sekretion des Endometriums beim Kaninchen führte zur Entdeckung zweier verschiedener Typen, die für den Oestrus und das progestative Stadium der Trächtigkeit repräsentativ sind. Eine einfache Methode wurde entwickelt, um beim Kaninchen auf experimentellem Wege die Oestrus-Sekretion in einer für chemische Versuche genügenden Menge zu erzeugen, und zwar – ohne Unterbindung des Uterus – durch intravenöse Verabreichung synthetischer wasserlöslicher Oestrogene, wie Polyoestriol-phosphat oder Stilboestrolphosphat. In der Blastocystenflüssigkeit des 6-Tage alten Embryo und in der endometrialen Sekretion wurden in ähnlichen Konzentrationen vorkommende Substanzen gefunden, insbesondere Aminosäuren, Glukose, Bikarbonat und Lactat.

Die Untersuchungen über mütterlich-embryonale Beziehungen schließen folgende Experimente ein:

a) über den Durchgang und die Verteilung einfacher Zucker, und markierter Ionen (P, S, K, Na, I) in Blastocysten und im mütterlichen Milieu, sowie

b) über die Verabreichung verschiedener pharmakologisch wirksamer Substanzen, Stoffwechselanalogen, Vitaminen, Hormonen usw., an das trächtige Tier, im Anschluß an eine mikroskopische Untersuchung der Morphologie der Blastocysten mittels einer einfachen und raschen Methode, der sogenannten 'flat-mount'-Technik.

Diese histologische Technik wurde so angewandt, daß sie nicht nur rein qualitative, sondern auch quantitative Wertungen und Vergleiche von Kaninchen-Blastocysten durchzuführen erlaubte. Die Methode wurde zuerst an vielen Hunderten vollkommen normaler Blastocysten angewandt und daraufhin an Blastocysten, die man von behandelten Tieren erhielt. Auf diese Weise war es möglich die physiologische sowie die pathologische Beschaffenheit der Blastocysten zu erhellen. Die Untersuchung normaler Blastocysten unterstrich die Existenz einer bemerkenswerten Variabilität der Entwicklung, innerhalb und zwischen gleichaltrigen Würfen, sowie das Vorkommen von 5–10% nicht lebensfähigen Embryonen bei fruchtbaren Tieren. Die interessanten Eigenschaften, die bei Experimenten an Blastocysten von behandelten Tieren aufgedeckt wurden, sind folgende:

- die Geschwindigkeit, mit welcher exogene Wirkstoffe die freiliegenden Säugetier-Embryonen erreichen;
- die Verzögerung der Clearance im Blastocoel, im Vergleich mit jener in den peripheren mütterlichen Körperflüssigkeiten;
- die differenzierte Sensibilität morphologisch verschiedener Gebiete der Blastocysten auf Wirkstoffe, die vom Muttertier übermittelt werden;
- die individuelle Variabilität unter Embryonen desselben Wurfes im Grad der Reaktion auf Drogenwirkung;
- die Fähigkeit intrauteriner Regeneration nach minderen Schädigungen und, in der Folge, die Wiederaufnahme offenbar normalen Wachstums.

Einige der von uns untersuchten embryotropischen Substanzen besassen – sofern sie zur rechten Zeit und in geeigneten Mengen verabreicht wurden – die Fähigkeit der Verhinderung des antimesometrialen Anhaftens am Uterus, was physiologisch genau am 7. Trächtigkeitstag des Kaninchens erfolgt. Die Verhinderung des Anhaftens von Kaninchen-Blastocysten am Uterus am 7. Tage wurde als zusätzliches und nützliches Kriterium für das Testen embryonaler Empfindlichkeit auf gewisse, vom Muttertier übertragene Drogen, Antimetaboliten, und Hormone benutzt.

Résumé

Au cours de ces dernières années l'on a fait un usage courant de blastocystes de petits mammifères de laboratoire pour l'expérimentation. Pour nos

expériences, nous avons choisi le lapin, parce que, dans cette espèce, le blastocyste présente plusieurs avantages pour l'expérimentation, un des principaux étant la précision avec laquelle on peut déterminer l'âge des jeunes embryons, leur taille extraordinairement grande au stade du blastocyste, et leur grand nombre chez les animaux fertiles.

Nos travaux effectués à l'Agricultural Research Council Unit of Reproductive Physiology and Biochemistry, University of Cambridge, ont poursuivi deux buts principaux: 1. revue des données sur la *composition chimique du blastocyste*, et 2. étude de certains aspects des *relations mère-embryon*, y compris celle de l'endomètre et de sa sécrétion, et la pénétration dans le blastocyste de certains produits exogènes par l'intermédiaire de la mère. Les analyses chimiques des blastocystes de lapin ont révélé leur contenu en protéine, en amino-acides, en DNA, RNA, en certains coenzymes, en ions tels que Cl, Na et K, et en trois métabolites tels que glucose, bicarbonate et acide lactique, dont la teneur varie d'une manière caractéristique avec le développement du blastocyste.

L'étude de l'endométrium de lapin et du tissu placentaire a montré la présence dans ces tissus de l'anhydrase carbonique, enzyme contrôlé par voie hormonale, et que l'on a ensuite mis en évidence dans le tractus génital féminin non seulement de plusieurs mammifères, y compris l'opossum, mais aussi chez les oiseaux, les poissons et les reptiles vivipares. Tout dernièrement, l'on a mis en évidence la nature des acides nucléiques de la muqueuse de l'endomètre du lapin à l'aide de centrifugation fractionnée, et l'on a trouvé des composants caractéristiques pour les premiers stades de la gestation.

L'étude de l'apparition et de la nature de la sécrétion endométriale du lapin a montré deux types distincts de sécrétion, qui correspondent aux stades œstrogène et progestatif de la gestation. Un moyen simple de provoquer à volonté une sécrétion œstrogène suffisante pour l'expérimentation chimique, sans devoir ligaturer l'utérus, consiste dans l'injection intraveineuse d'œstrogène synthétique soluble dans l'eau, tels que le phosphate de polyœstrol, ou le phosphate de stilbœstrol. L'on a pu constater que plusieurs composants chimiques dans le liquide du blastocyste du lapin et dans la sécrétion de l'endométrium ont la même concentration, surtout les acides aminés, le glucose, le bicarbonate et le lactate.

Dans l'étude des relations mère-embryon, l'on a expérimenté 1. le passage et la concentration dans les blastocystes et le milieu utérin de sucres simples, d'ions marqués tels que P, S, K, Na, I, et 2. après administration à la lapine portante de différents produits médicamenteux, d'antimétabolites, de vitamines, d'hormones, etc., les modifications morphologiques dans le blastocyste, par une méthode simple et rapide, dite «flat-mount» technique. Cette technique histologique a été adaptée de telle sorte qu'elle permet une évaluation non seulement qualitative, mais aussi quantitative de blastocystes de lapin. Cette méthode a tout d'abord été appliquée à un grand nombre de blastocystes tout à fait normaux, ensuite à des blastocystes pré-

levés sur des lapines traitées. Il a été possible de cette façon de comprendre les aspects physiologiques et pathologiques du blastocyste. L'étude des blastocystes normaux a démontré l'existence de grandes différences de développements, parmi la même nichée et parmi des nichées du même âge, et la présence de 5–10% d'embryons non viables chez des lapines fertiles. Les expériences sur les blastocystes des animaux traités ont montré les résultats les plus caractéristiques suivants:

- la grande rapidité avec laquelle des produits exogènes pénètrent dans l'embryon de mammifère;
- le retard qu'il y a dans la clearance de l'espace blastocœlique pour le produit chimique, en regard du liquide maternel ambiant;
- la sensibilité différencielle de zones morphologiquement distinctes du blastocyste, vis-à-vis des agents transmis par l'organisme maternel;
- la variabilité individuelle des individus d'une même portée, quant à leur sensibilité aux produits chimiques;
- la faculté de réparation intrautérine lors de certaines lésions de faible importance, et la reprise d'une croissance apparemment normale.

Certaines des substances embryotropiques que nous avons étudiées, à condition qu'elles soient données au moment propice et à des doses convenables, sont capables d'inhiber la nidation utérine antimesométriale des blastocystes, qui habituellement prend place au 7e jour de la gravidité chez la lapine. Ce blocage de la nidation des blastocystes dans l'utérus au 7e jour a pu servir de critère additionnel utile pour tester la sensibilité de l'embryon à certains médicaments transmissibles par la mère, tels que certains antimétabolites et hormones.

Riassunto

Durante gli anni scorsi i blastocisti di diversi piccoli mammiferi da laboratorio furono adoperati in esteso a scopo di studi sperimentali. Per le nostre ricerche ci siamo serviti di conigli, dato che i blastocisti di questa specie offrono come oggetto di studio diversi vantaggi, specialmente per la precisione con cui si può stabilire l'età del giovane embrione, la grandezza dell'embrione allo stadio di blastociste ed il loro numero negli animali capaci di riprodursi.

Le nostre ricerche nell'Agricultural Research Council Unit of Reproductive Physiology and Biochemistry dell'università di Cambridge ebbero come scopo lo studio di due problemi: quello di raccogliere dati sulla *composizione chimica dei blastocisti*, e quello di spiegare alcuni aspetti riguardanti *i rapporti tra madre ed embrione* e che comprendono lo studio dell'endometrio e della sua secrezione, come pure lo studio della penetrazione di corpi estranei nei blastocisti attraverso la madre. L'analisi chimica dei blastocisti di coniglio ci fornì delle informazioni sul contenuto in proteine, aminoacidi, acido desossiribonucleinico e ribonucleinico, su certi coenzimi e ioni come il cloro, sodio e potassio come pure sul glucosio, bicarbonato ed acido lattico, tre metaboliti la cui quantità varia in maniera caratteristica con l'avanzare

dello sviluppo dei blastocisti. Le ricerche sull'endometrio e sul tessuto placentare dei conigli ci portò alla scoperta di un enzima a regolazione ormonale, l'anidrasi carbonica, la quale in seguito fu messa in evidenza, non solo negli organi di riproduzione femminili di molti mammiferi, l'opossum compreso, ma anche negli uccelli, nei pesci e nei rettili vivipari. Poco tempo fa si esaminarono mediante centrifugazione continua gli acidi nucleinici nell'endometrio dei conigli e si constatò che lo stadio iniziale della gravidanza è contraddistinto da un modello di acidi nucleinici di natura ben determinata e caratteristica.

Le ricerche sulla secrezione dell'endometrio dei conigli ci portò alla scoperta di due tipi differenti di secrezione, rappresentativi per lo stadio estrogeno e progestativo della gravidanza. Fu sviluppato un metodo semplice per provocare sperimentalmente nei conigli una secrezione di estrogeni in quantità sufficiente per degli esperimenti chimici. Ciò fu ottenuto mediante applicazione endovenosa di estrogeni sintetici idrosolubili quali il fosfato di «poliestriol» e di «stilboestrol». Nel liquido dei blastocisti dell'embrione di sei giorni e nel secreto dell'endometrio furono trovate uguali concentrazioni di sostanze, specie per quanto riguarda gli aminoacidi, il glucosio, il bicarbonato ed il lattato.

Le ricerche riguardanti i rapporti madre-embrione comprendono i seguenti esperimenti:

a) ricerche riguardanti il passaggio e la distribuzione di zuccheri e ioni marcati (fosforo, zolfo, potassio, sodio, iodio) nei blastocisti e nell'ambiente materno;

b) esperimenti fatti somministrando all'animale gravido sostanze con attività farmacologiche diverse come, prodotti del metabolismo, vitamine, ormoni ecc. dopo aver analizzato microscopicamente la morfologia dei blastocisti mediante un metodo semplice e rapido denominato «tecnica flat-mount».

Questa tecnica istologica fu adoperata in modo da permetterci valutazioni e paragoni sui blastocisti del coniglio che non erano di natura puramente qualitativa, ma anche di natura quantitativa. Il metodo fu sperimentato dapprima su centinaia di blastocisti assolutamente normali poi su dei blastocisti ottenuti da animali precedentemente trattati. In tal modo fu possibile di chiarire la natura fisiologica e patologica dei blastocisti. Le ricerche sui blastocisti normali chiarirono l'esistenza di una notevole variabilità dello sviluppo, sia nel seno di una medesima figliolata che fra due figliolate congeneri, come pure la presenza del 5–10% di embrioni non vitali negli animali fecondi. Le caratteristiche interessanti constatate durante gli esperimenti sui blastocisti di animali precedentemente trattati sono le seguenti:

- la velocità con la quale sostanze attive esogene raggiungono gli embrioni di mammifero;
- il fatto che la cosidetta «Clearence» del blastocele, in confronto a quella degli umori periferici dello organismo materno, è ritardata:

- la sensibilità differenziata di regioni morfologicamente differenti del blastociste quando sono sottomesse all'azione di sostanze attive trasmesse dallo animale madre;
- la variabilità individuale degli embrioni della stessa covata per quanto riguarda il loro grado di reazione all'azione delle droghe;
- la possibilità di rigenerazione intrauterina in seguito a piccole lesioni con la conseguente ripresa di uno sviluppo palesemente normale.

Alcune delle sostanze embriotropiche da noi analizzate mostrarono (applicate al momento giusto ed in quantità appropriata) la possibilità di impedire l'adesione antimesodermale all'utero, ciò che per il coniglio avviene fisiologicamente al settimo giorno di gravidanza. Tale impedimento dell'adesione dei blastocisti di coniglio all'utero durante il settimo giorno, fu adoperato come utile criterio complementare per il controllo della sensibilità embrionale rispetto a certe droghe, agli antimetaboliti ed agli ormoni trasmessi dall'animale madre.

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