An *in vitro* study of functions of embryonic membranes in the rat

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**SUMMARY**

Ten-day rat embryos have been cultivated *in vitro*, with different layers of the extra-embryonic membranes removed. The effects of presence or absence of each membrane on the morphology of the embryos, their histodifferentiation and their uptake of leucine into protein have been followed.

Explants with all membranes left intact failed to expand fully and to undergo axial rotation of the embryo, but nevertheless showed highest total protein and highest leucine uptake in biochemical estimations and in autoradiographs.

Explants with outer membranes removed and the visceral yolk sac left intact showed the most normal morphology and expansion of the extraembryonic cavities when compared with embryos removed from the uterus at 11.5 days' gestation, but they showed less protein and less leucine uptake than the first series.

Explants in which the visceral yolk sac was removed underwent little growth or development and had low total protein values and radioactivity counts. The amnion collapsed and the amniotic cavity disappeared. When the amnion was removed there was a greater incidence of death, as well as little or no development, and lower radioactivity counts than in the first two series.

It is concluded that the outer membranes and the visceral yolk sac play an important role in the transfer of small metabolites to the embryo, as well as in regulating the volume of the extraembryonic fluids.

**INTRODUCTION**

The structure and functions of mammalian embryonic membranes at early post-implantation stages have usually been studied with the embryo left *in situ* in the uterus, where the possibilities for experiment are very limited. However, besides descriptive histological and histochemical work, it has been possible to administer labelled proteins or other easily identifiable substances to the mother and to trace these through the membranes into the foetal tissues. Ultrastructural studies have yielded further information about mechanisms of transport of macromolecules through the membranes. Particular interest has been focused on the yolk sac in rabbits and rodents, since Brambell, Brierly, Halliday & Hemmings (1954) made their classic discovery that antibodies are transferred selectively across this layer in the rabbit. Wild (1970) has confirmed by fluorescent labelling methods that the endoderm cells of the visceral yolk sac are the

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main site of this protein selection. Electron-microscope observations using ferritin-labelled antibody (Slade, 1970) have shown, more precisely, that the uptake of protein into these cells is unselective, but that their transfer from the visceral endoderm into the adjacent cells is selective. Similar studies by Lambson (1966) on the cells of the visceral yolk sac in the rat indicate that there is an elaborately structured intracellular storage and transport system. Ferritin material remains in large storage vesicles in these endoderm cells until late in gestation, when the contents of the vesicles pass into adjacent mesenchyme and blood vessels, during the period when maternal antibodies are known to pass into the foetal circulation.

Besides storage and transfer of whole proteins, active proteolysis occurs in the yolk sac of the rat by means of ‘lyso-phagosomes’ (Beck, Lloyd & Griffiths, 1967). This may be presumed to be a nutritional function, analogous to that of the yolk sac in heavily yolked eggs. In birds (Borger & Peters, 1933) and in teleost fish (Van der Ghinst, 1935) the yolk sac is a site of rapid proteolysis.

Very much less is known in rodents about the function of the parietal yolk sac and of Reichert’s membrane, a non-cellular layer thought to be secreted by parietal endoderm cells (Wislocki & Padykula, 1953; Pierce, Midgley, Sri Ram & Feldman, 1962). Jollie (1968) pointed out that during the last third of gestation in the rat, some parietal endoderm is present in the sparse peripheral areas of the yolk-sac placenta. But here, gaps appear between the cells of both trophoblast and endoderm layers, leaving only Reichert’s membrane separating the maternal blood from the yolk-sac cavity. At these points, then, the properties of Reichert’s membrane determine what materials can pass from mother to foetus. The composition of this membrane is still uncertain however: Wislocki & Padykula (1953) found that it gave histochemical reactions indicative of a glycoprotein, possibly collagen, but subsequent ultrastructural studies have failed to confirm this or to throw other light on its structure. Since ferritin crosses Reichert’s membrane from mother to foetus in the rat, but thorotrast does not (Jollie, 1968), the membrane may play at least a passively selective role in the transfer of nutrients to the embryo.

Extensive work on late foetuses of other mammals (see review by Adolph, 1967) has suggested that a major role of the amniotic cells is the maintenance of fluid balance and regulation of the composition of the amniotic fluid. Its other assumed function is protection of the foetus against mechanical injury.

These previous investigations, either in utero or on exposed late foetuses, were not designed to follow the functions of individual membranes acting alone, or to analyse their mechanically protective roles. Furthermore, possible selective processes occurring within the maternal circulation or the uterine wall could not be ruled out. So in the work to be described here the in vitro culture method of New (1966) has been used, which has enabled the effects of removal of one or more layers of the embryonic membranes to be observed, and also the passage of radioactively labelled material from the medium into the embryo via those
Functions of rat embryonic membranes

membranes that were left intact. In this way a separate assessment of the roles of Reichert’s membrane, with its attached trophoblast and parietal yolk sac, of the visceral yolk sac and of the amnion, has been possible. No separate study was made of the allantois. The morphology and histological differentiation of the embryos has been observed, as well as their uptake of radioactive leucine into protein, during a 24 h culture period.

MATERIAL AND METHODS

Pregnant female Wistar rats, mated 10-5 days previously, were bled by heart puncture to obtain serum for the culture medium. They were killed, and the embryos were removed from the uterus in sterile Tyrode saline. The layout of the trophoblast, Reichert’s membrane, yolk sac and amnion round the embryos is depicted in Fig. 1 (a). In each experiment, some embryos (series (1)) were left with all these membranes intact. Embryos of series (2) were divested of Reichert’s membrane with its attached trophoblast cells and parietal yolk sac. Embryos of series (3) had the visceral yolk sac removed as well as the outer membranes, while in embryos of series (4) the amnion was also removed, leaving only the proximal roots of all the membranes, and also the stalk of the allantois, immediately adjacent to the embryonic body. Fig. 1 (b–d) illustrates the condition of the membranes in each series. Each embryo was then cultured in 0·5 ml of rat serum by New’s watchglass method (New, 1966) for 24 h. For the measurements of nutritional uptake, 0·05 ml (25 μCi) of L-leucine-4,5-³H (Radiochemical Centre, Amersham) was added to 5 ml serum (i.e. 5 μCi/ml).

At the end of the culture period, the embryos were examined in toto, then washed in five changes of isotonic saline (0·9 % NaCl) to remove traces of radioactive medium, and prepared either for biochemical estimations of total protein-bound radioactivity or for autoradiography to assess the distribution of tracer in the tissues. In all, 146 embryos were studied.

Biochemical estimations. After storage at -35 °C, each embryo was homogenized in 0·5 ml of cold 0·2 M phosphate buffer at pH 7; 0·1 ml of the homogenate was set aside for subsequent protein determination by the method of Lowry, Rosebrough, Farr & Randall (1951), and the rest was precipitated with trichloracetic acid (TCA) at a final concentration of 5 %. The precipitate was collected on a Millipore filter (pore size 0·45 μm) and dried at 70 °C overnight. Samples of the last washing fluid and of the TCA supernatant were also dried on Millipore filters. Each filter was then placed in a scintillation counting vial to which was added 15 ml of scintillation fluid (toluene 625 ml; 2-ethoxyethanol 375 ml; 2,5-diphenyloxazole (= PPO) 4 g; 1,4-bis-(5-phenyloxazolyl)-2 benzene (= POPOP) 0·1 g). Counts were made on a Beckmann LS-100 scintillation counter at an efficiency of 25–30 %. Background counts, from a vial containing a Millipore filter and fluid only, were made on each run.

Autoradiography. Embryos were fixed in Bouin’s fluid for 48 h, washed in tap
Fig. 1. Diagrams of transverse sections of the 10-day rat embryo, to show the membranes present in the different experimental series. (a) All membranes intact (series 1). (b) Reichert’s membrane with attached trophoblast and parietal yolk sac removed (series 2). (c) Visceral yolk sac also removed (series 3). (d) All membranes removed (series 4). A, Amnion; N, notochord; NT, neural tube; R, Reichert’s membrane; T, trophoblast; S, somites; TC, trophoblast cone; PYS, parietal yolk sac; VYS, visceral yolk sac (endoderm + mesoderm).

Water to remove excess fixative, then dehydrated in alcohols, cleared in methyl benzoate and embedded in paraffin wax (melting point 55 °C). From the ribbon of 5μm sections, successive groups of five sections were distributed sequentially on 5 slides, designated A, B, C, D and E for each embryo. Slides D and E were used for routine staining: D with nuclear fast red and E with haematoxylin and eosin. Slides A–C were de-waxed and then dipped in Ilford K 5 autoradiographic emulsion, diluted 1:1 with distilled water, at 40 °C in the dark room. They were stored in light-tight boxes at 2 °C for 4 days (slides A), 7 days (slides B) and 11 days (slides C), then developed in Kodak DF IB (7 min at 20 °C), fixed in Kodak Amfix, and subsequently stained with nuclear fast red. Grain
Fig. 2. (a) Embryo of series (1), within its membranes, still not rotated at end of culture period (× 40). (b) Transverse section through brain (on right of picture) and trunk region (left central) of a normal, semi-rotated embryo (× 50). (c) Normal embryo, with Reichert’s membrane and attached layers removed, having undergone rotation during culture (× 30). (d) Transverse section showing necrosis in neural tube (above) and brain tissue (below) of an embryo of series (3). (× 60).

Counts were made using an oil-immersion objective and an eyepiece grid, on five sections through the trunk region of each embryo, using slides of series A, since these had the clearest grains.

RESULTS

(a) General condition of the embryos

Comparisons of controls, lacking leucine in the medium with those in which the tracer was present, showed no differences in morphology or in histodifferentiation within each series. So evidently the presence of radioactive leucine did not affect the growth or differentiation of the embryos or their membranes. The normality of development differed between the four series of embryos however. Embryos of series (1), in which all membranes were left
Table 1. Biochemical data on uptake of tritiated leucine into embryos
(Each figure is the mean value for all embryos in the series ± s.e.)

<table>
<thead>
<tr>
<th></th>
<th>Series* (1)</th>
<th>Series (2)</th>
<th>Series (3)</th>
<th>Series (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Total radioactivity (counts/min/embryo)</td>
<td>134326 ± 15080</td>
<td>91752 ± 11610</td>
<td>55958 ± 8746</td>
<td>59787 ± 9882</td>
</tr>
<tr>
<td>B. Specific activity (counts/min/μg. protein)</td>
<td>750-1 ± 158-3</td>
<td>659-4 ± 133-0</td>
<td>758-2 ± 181-4</td>
<td>653-5 ± 112-0</td>
</tr>
<tr>
<td>C. Total protein (μg/embryo)</td>
<td>89-9 ± 20-4</td>
<td>54-9 ± 7-0</td>
<td>26-5 ± 5-6</td>
<td>38-5 ± 7-8</td>
</tr>
</tbody>
</table>

Results of analysis of variance within and between the four series:
(A) Total radioactivity: series (1) significantly higher than (2), (3) and/or (4). \( F = 8.09; P < 0.02 \).
(B) Specific activity: no significant differences: \( F = 0.144 \).
(C) Total protein: series (1) significantly higher than series (3) and/or (4): \( F = 6.268; P < 0.02 \).

* For description of series see Fig. 1.

intact, showed less expansion of the amniotic and excoelomic cavities than those of series (2) in which Reichert's membrane with attached trophoblast and parietal yolk sac had been removed (as is normally done in New's culture method). The majority of embryos in series (1) were also somewhat small and failed to undergo axial rotation, whereas those of series (2) had completed rotation after 24 h culture (cf. Fig. 2a, c). Series (1) embryos seemed to undergo normal differentiation in all other respects however: the heart was beating and a yolk sac circulation was present, and histologically the tissues appeared normal (Fig. 2b), with no difference from series (2) except the lack of rotation. In embryos of series (2) the most striking features at the end of the culture period were the great expansion of the amniotic cavity, usually to at least four times its original volume, and the larger size of the embryo and more vigorous heartbeats than in series (1).

By contrast with these two groups, embryos of series (3) and (4), with only the amnion or with no membranes present, were in poor condition at the end of culture. Inevitably they had suffered haemorrhage and were blanched, with no sign of blood circulation within the body. Of the 13 embryos in group (3), one embryo had retained a fairly normal 10-day appearance, but had not developed further: the others had all become stunted and opaque, with the amnion collapsed around them, and a faint heartbeat was the only indication of their survival. Thirty-two embryos of group (4) resembled these, but the other 12 in this group had apparently died during culture. In all embryos of groups (3) and (4) which were examined histologically, necrotic patches of tissue were apparent (cf. Fig. 2d).

(b) Tracer uptake: biochemical estimations

Twenty-four embryos of series (1), 20 of series (2), 10 of series (3) and 21 of series (4) were used individually for biochemical estimations. For each embryo,
Functions of rat embryonic membranes

Table 2. Uptake of tritiated leucine into different embryonic tissues, seen in autoradiographs

(Each figure is the mean of all counts on embryos in the series ± S.E. 
\( n = \) no. of embryos; 16–25 counts were carried out on each.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Series (1) ((n = 6))</th>
<th>Series (2) ((n = 10))</th>
<th>Series (4) ((n = 5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral yolk sac</td>
<td>30.73 ± 1.23</td>
<td>23.07 ± 1.99</td>
<td>—</td>
</tr>
<tr>
<td>Neural tube</td>
<td>33.28 ± 1.51</td>
<td>27.31 ± 1.88</td>
<td>28.95 ± 3.45</td>
</tr>
<tr>
<td>Somite mesoderm</td>
<td>29.13 ± 1.00</td>
<td>26.92 ± 1.55</td>
<td>29.58 ± 3.19</td>
</tr>
<tr>
<td>Gut endoderm</td>
<td>31.85 ± 0.84</td>
<td>28.66 ± 2.01</td>
<td>26.04 ± 1.34</td>
</tr>
</tbody>
</table>

Results of \( t \) tests between pairs of sets of data (series (2)):
- Neural tube significantly higher than yolk sac. \( t = 2.52; \ P < 0.05 \).
- Somite mesoderm significantly higher than yolk sac. \( t = 3.10; \ P < 0.02 \).
- Gut endoderm significantly higher than yolk sac. \( t = 2.32; \ P < 0.05 \).

Counts on all tissues taken together (series (1) and (2)):
- Series (1) significantly higher than series (2). \( t = 3.78; \ P < 0.01 \).

the total radioactivity in counts per minute was measured. Total protein and hence specific activity in counts/min/\( \mu \)g protein were also estimated. The means and standard errors for these data are set out in Table 1.

Looking first at the absolute activities (Table 1, A) it can be seen that series (3) and (4) do not differ significantly from each other, but that both have considerably lower counts than series (1) and (2). An analysis of variance within and between the four series shows that counts in series (1) are significantly higher than those of all other three series \( (F = 8.09, \ P < 0.02) \). The specific activities (Table 1, B) do not differ significantly between the four series however. This is due to the fact that total protein values were higher in series (1) than in series (2), and both of these groups had higher protein values per embryo than groups (3) or (4), as shown in Table 1, C. There was also considerable variability between experiments. The results of statistical tests on all these data are summarized at the foot of the table.

(c) Tracer uptake seen in autoradiographs

Because of the normal curvature of the rat embryo and the way in which the sections had been distributed between slides A to E (see Methods), only a few sections on each slide passed through a complete range of trunk tissues. Attention was therefore confined to neural tube, somite mesoderm, gut endoderm, and yolk sac when present. Either five or six grain counts were made on each of these tissues, in each of five sections on slides A. The means and standard errors for the counts on each tissue are set out in Table 2. There are no data for Reichert's membrane in series (1) because it was always very densely grained, making it impossible to obtain accurate counts.

First, comparing the grain counts in different tissues within each series of
embryos, there were no significant differences in series (1) or (4). In series (2), however, neural tube, somite mesoderm and gut endoderm each showed significantly higher counts than did the yolk sac endoderm. (Values for $t$ and $P$ are given at the foot of Table 2.) Next, comparing counts between the four experimental series, some significant differences were demonstrable between series (1) and (2). The counts from all the tissues, taken together, in series (1) are higher than those in series (2). In addition, the counts in neural tube and in yolk sac in series (1) are higher than in the corresponding tissues of series (2) embryos.

Owing to the poor condition of embryos in series (3) and (4), only a few of group (4) could be used for grain counts. These gave counts broadly comparable to series (1) and (2), however, as Table 2 shows.

**DISCUSSION**

From the results presented above, certain deductions may be made about the roles of the individual embryonic membranes in the rat. Considering first the amnion: it is clear that this membrane alone was not capable of regulating the fluid volume of the amniotic cavity *in vitro* under the conditions of these experiments. In embryos of series (3) where the amnion was the only membrane left intact, it had collapsed and the amniotic cavity had virtually disappeared. Another point about the amnion which is clear from the present results is that it does not function adequately as a nutritive organ. Embryos of series (3) had just as low tracer uptakes and as low protein contents as those of series (4) where no amnion was present, and both of these groups gave values far below those of series (1) and (2) (see Table 1, A and C). There is, however, some indication that the amnion has an important protective function, since there was a lower death-rate in series (3) than in series (4).

Turning now to the visceral yolk sac: it is clear first from the general morphology of the embryos after culture that this is the most important membrane for normal development. In its absence (series (3) and (4)), embryos were stunted and abnormal, whereas when it was present, without Reichert's membrane or the parietal yolk sac, normal expansion both of the embryo and its surrounding cavities took place and normal rotation of the axis occurred also. The biochemical and autoradiographic data indicate, however, that the nutritive functions of the visceral yolk sac are inadequate in the absence of the membranes external to it. Series (1) embryos in which all the membranes were present had significantly higher total protein values and absolute values for tracer uptake into protein than series (2), where only the visceral yolk sac was present (see Table 1, A and C). The higher grain counts in autoradiographs of series (1) embryos (Table 2) also indicate that the presence of the visceral yolk sac, Reichert's membrane and the trophoblast enhances uptake of amino acid into protein of the embryo. A further, striking feature in these autoradiographs was the dense graining in these outer membranes, suggesting that they had accumulated tracer either for
Functions of rat embryonic membranes

transmission to the embryo or for its own protein synthesis. Reichert's membrane is known to become thicker as development proceeds (Wislocki & Padykula, 1953), and as already mentioned, it appears to consist of glycoprotein. One possible reason for its dense graining may be that this protein is synthesized particularly rapidly during the 24 h from 10 to 11 days' gestation and that, being a stable structural material, it does not lose label by undergoing protein turnover, unlike the constantly changing intracellular proteins of the embryo. The membrane protein is moreover in a compact form, not dispersed and hydrated as are the cytoplasm proteins. The very attenuated, thin layer of trophoblast cells and parietal endoderm that covers part of Reichert's membrane (see Fig. 1a) has not been considered in the above discussion, since it was not a complete layer. The cells showed grains in autoradiographs, but no satisfactory counts could be done on them as they did not nearly fill a grid square and they had very little cytoplasm.

The present experiments were not designed to obtain evidence about the proteolytic activity of the visceral yolk sac, nor about transport of whole proteins through this membrane. Williams, Lloyd and Beck (1969) have, however, shown that proteolysis takes place in the yolk sac of the rat in vitro. A perhaps surprising feature of the present results (Table 2) is the relatively low grain counts in the yolk sac — lower still in the absence of Reichert's membrane. A possible explanation might be that most of the leucine is either passed on unchanged into the embryonic circulation or incorporated only transitorily into yolk-sac proteins, which are then rapidly broken down and the products transported to the embryo.

It should be noted that only protein-bound leucine is shown in the present results: free leucine was removed by the TCA treatment of the biochemical samples and by the histological processes prior to autoradiography. It follows that the higher grain counts in neural tube, axial mesoderm and gut in series (2) indicate a higher net protein synthesis rate in these tissues than in the yolk sac, which has lower counts. Conversely, the absence of significant differences in grain counts between the intraembryonic tissues of series (2) indicates that there were no significant differences in net protein synthesis rates in these tissues during the period of culture. Protein synthesis rates in neural tube and yolk sac of series (1) were, however, higher than those of the same tissues in series (2).

Returning now to the general functions of the embryonic membranes, we may conclude that, in the early rat embryo grown in vitro, the visceral yolk sac plays a major part in regulating the volume of extraembryonic fluids. The nutritive function of the yolk sac is supplemented in some way by the outer membranes, of which Reichert's membrane is itself undergoing protein synthesis. The structural properties of this membrane cause it to resist the expansion and rotation of the embryo in vitro, although protein synthesis and histological differentiation are not affected. Although this mechanical effect of the membrane has not been studied in utero, it may be suggested that the eventual size of the extraembryonic
cavities in the rat normally depends upon a balance between fluid inflow through
the yolk sac and amnion, tension in Reichert's membrane and the tonus of the
uterine muscles.

We are grateful to Mrs F. M. Parker for expert technical assistance and to Miss N.
Thomas for advice on biochemical methods. Expenses of some of the materials were met
by a grant from the Agricultural Research Council.

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(Manuscript received 25 October 1971)