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A histochemical and biochemical study of some aspects of placental function in the rat using maternal injection of horseradish peroxidase

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INTRODUCTION

It has long been accepted that certain extra-embryonic membranes utilize maternal tissues and secretions (the so-called embryotroph) for the nourishment of an early conceptus. Indeed before the establishment of a definitive placenta this method of supply is the only one available and its mechanism is therefore of some importance. The available evidence indicates that a process of phagocytosis is involved and suggests that embryotroph is digested *within* the cells of the extra-embryonic tissues (Beck & Lloyd, 1966), though some proteins resist breakdown and enter the embryo unchanged, at least in certain species (Brambell, 1954; Schechtman & Abraham, 1958; Mayersbach, 1958). The actual site of extra-embryonic digestion may well reflect the wide species variation seen in the morphology of the placenta and by the same token the cellular processes themselves could differ with the animal studied. Thus the demonstration of intracellular protein breakdown in one species does not necessarily exclude the possibility of a secretion of digestive enzymes by extra-embryonic membranes into the endometrium of another.

Intracellular digestive processes according to the lysosome concept of de Duve (1959, 1963) involve the ingestion of macromolecules in pinocytic or phagocytic vesicles termed phagosomes; these become secondarily associated with membrane-bound lysosomes containing hydrolytic enzymes and the resulting digestive vacuoles or lyso-phagosomes (Straus, 1963) allow intracellular digestion to take place without a general release of hydrolytic enzymes into the cell cytoplasm. Although many of the details are still obscure, the general features of these processes have been observed with both the light (Straus, 1964 *a*; Hirsch, 1962) and electron microscopes (Gordon, Miller & Bensch, 1965) and the reports of many workers using a wide variety of animal cells have testified to their ubiquity (Müller, Röhlich, Tóth & Törö, 1963; Novikoff, 1961; Cohn, Hirsch & Wiener, 1963; Seljelid, 1965).

Taking advantage of techniques described by Straus (1964*b*, *c*) we have studied embryotrophic nutrition in the rat. The plant enzyme horseradish peroxidase, a marker protein easily traceable histochemically, has been injected intravenously into pregnant animals and subsequent histochemical and biochemical investigations of the embryo and its membranes have traced the injected material to its point of association with the lysosomal enzyme acid phosphatase and have attempted to measure how much, if any, enters the embryonic tissues unchanged.

MATERIALS AND METHODS

(1) *Histochemistry*. Horseradish peroxidase (Sigma, type II) was injected intravenously into Wistar rats at timed stages of pregnancy and after varying periods conceptuses were removed under ether anaesthesia and fixed for a time depending upon the stage of pregnancy under investigation. At 8·5, 9·5, 10·5, 11·5 and 12·5 d conceptuses were removed from the uterus still encased within their decidual cells and fixed for 18–24 h before transfer to 30 % sucrose at 4 °C for storage. Additional series of 8·5 and 9·5 d egg cylinders (i.e. that portion of the conceptus which is invaginated into its own yolk-sac) were dissected free of decidua and most of their investing membranes and fixed for 1½ h only prior to storage in cold sucrose. 20·5 d foetuses, chorioallantoic placentae and yolk-sacs were separated and fixed independently for 18–24 h.

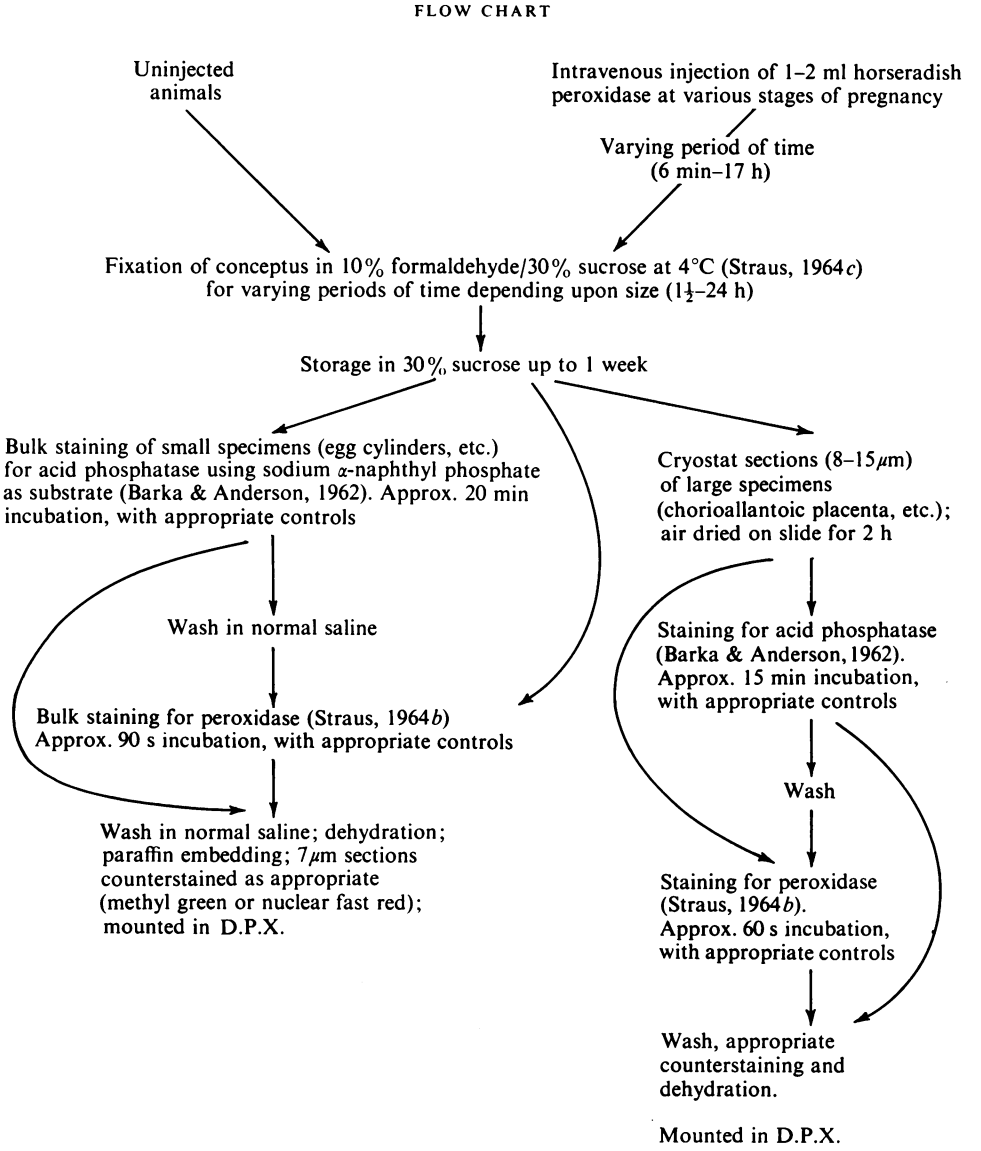
At 8·5 and 9·5 d whole egg cylinders were stained following removal of surrounding decidua; at 10·5 and 11·5 d separate small pieces of the visceral and parietal yolk-sac walls were used; at 12·5 d chorioallantoic placentae with pieces of attached yolk-sac wall were studied, while at 20·5 d chorioallantoic placentae as well as pieces of the villous and non-villous visceral yolk-sac lining were stained. The flow chart (see below) gives details of the fixation and staining methods employed.

In order to confirm the findings revealed by the above methods a few pregnant rats were injected subcutaneously with trypan blue to demonstrate phagosomes and some preparations were stained with PAS in order to show lysosomes (Novikoff, 1961).

(2) *Biochemistry*. One ml. of a 2 % aqueous solution of horseradish peroxidase (Sigma, Type II) was injected intravenously into Wistar rats at 11·5 and at 20·5 d of pregnancy. Individual embryos, foetuses, yolk-sacs and chorioallantoic placentae were removed about 6 h later from these animals as well as from uninjected controls and homogenized in water at 4 °C using a 'Tri-R' Teflon on glass homogenizer. A volume of 1 ml was used for all 11·5 d material, while 20·5 d material was prepared in about 10 vol. of water. Homogenates from 20·5 d tissues were clarified by centrifugation at 2 °C for 5 min at 600 ***g*** but this procedure was not followed for the 11·5 d preparations because of technical difficulties arising from the small quantities of tissue obtainable. Peroxidase was estimated by a method essentially that of Straus (1962) but modified in that buffer, tissue extract and N,N-dimethyl-*p*-phenylenediamine (Sigma Grade III) were pre-incubated for 1 h at 20 °C before addition of H_2O_2 and measurement of optical density increase. Only in this way could a small and constant reagent blank be obtained, as autoxidation of the diamine was found to proceed rapidly in the period immediately after mixture with the buffer and tissue extract. Peroxidase activity is expressed as the increase in optical density at 520 mμ/min/mg of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), bovine serum albumin being used as reference protein.

The acid phosphatase content of normal yolk-sac and chorioallantoic placenta was determined at 11·5 and at 20·5 d of gestation. Individual membranes were homogenized separately and the 20·5 d homogenates were clarified by centrifugation as above. Acid phosphatase was assayed at pH 4 using *p*-nitrophenyl phosphate as

substrate (Torriani, 1960) and pre-incubating the tissue homogenates for 10 min to inactivate microsomal phosphatases (Neil & Horner, 1964). The 20·5 d material was also assayed at pH 5 using β-glycerophosphate as substrate (Gianetto & de



Duve, 1955) in order to check the reliability of the more sensitive *p*-nitrophenyl phosphate method. Protein was again determined by the method of Lowry *et al.* (1951).

RESULTS

(*a*) *Histochemistry*

The details of implantation and placentation in the rat are well known and excellent summaries are available (Amoroso, 1952). No account of the gross morphology of the embryonic membranes will therefore be given. Acid phosphatase activity in the later stages of rat placentation has been described by Padykula (1958) and by Bulmer (1965), but neither of these authors have related their findings to the intracellular digestion of embryotroph.

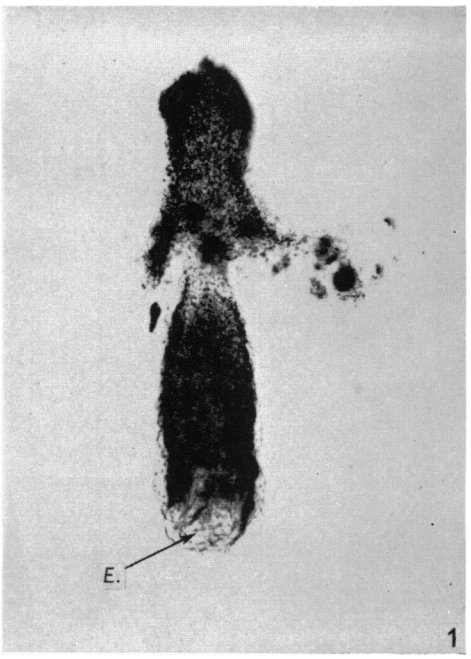
8·5 *days*

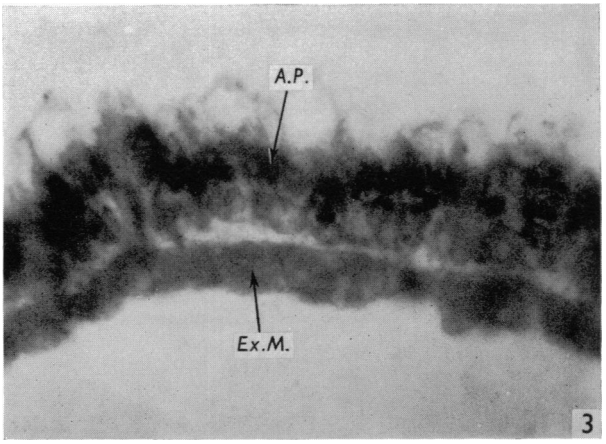
Bulk staining for peroxidase of a whole egg cylinder removed 6 h after maternal injection gave a strongly positive reaction at the abembryonic portion of the cylinder, but little evidence of activity at the embryonic pole (Fig. 1). Almost all the peroxidase was present either in the supranuclear parts of the cells of the visceral yolk sac epithelium or adsorbed on their surface. Very occasionally a cell of the embryonic endoderm or a trophoblastic giant cell gave a positive reaction, but the amount taken up by cells other than those of the visceral yolk-sac epithelium was very small; in some places the protein could be demonstrated in the cavity of the yolk-sac and adsorbed on Reichert's membrane. Close examination revealed that the activity of the ectoplacental cone was due principally to peroxidase present in the maternal blood sinuses. Figure 8 is a section through an egg cylinder at 8·5 d, stained for peroxidase following maternal injection. Though easily visible in the original preparation, the stain for peroxidase could not be reproduced in the preparation of the colour plate.

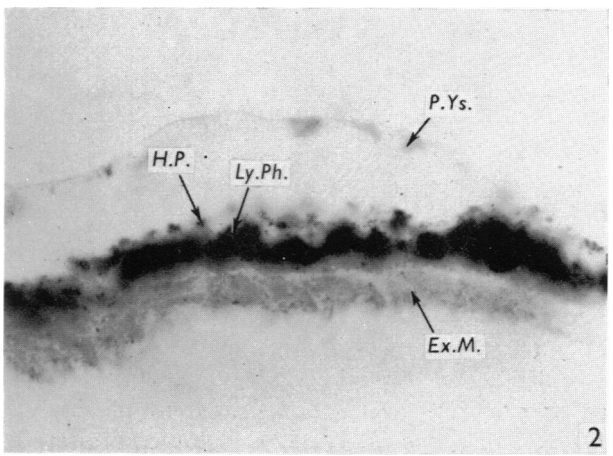
Supranuclear areas in the cells of the visceral yolk-sac epithelium gave a strongly positive reaction for acid phosphatase while the rest of the egg cylinder was negative or, in the case of the trophoblastic giant cells, the parietal layer of the yolk-sac epithelium and the occasional embryonic endodermal cell, only faintly positive. Little activity was apparent in the ectoplacental cone.

Double staining for peroxidase and acid phosphatase was performed 6 h after injection of the mother; it demonstrated clearly that the cells which had taken up peroxidase were rich in acid phosphatase. This was particularly true of the visceral layer of yolk-sac epithelium, although some suggestion of association between the ingested macromolecules and acid phosphatase was also seen in the embryonic endoderm and in trophoblastic giant cells. On high-power examination of the yolk-sac epithelium, peroxidase was observed adsorbed on to the plasma membrane of the cell apices and contained in apical vesicles or phagosomes. In deeper parts of the cell, though still in a supranuclear position, the phagosomes seemed to become secondarily associated with acid phosphatase contained within lysosomes and in the resultant lyso-phagosomes digestion of the peroxidase by proteolytic enzymes presumably takes place (Fig. 2).

Sections of implantation sites from animals injected with trypan blue at 7·5 d confirmed the phagocytic properties of the visceral yolk-sac epithelium, the dye being concentrated in supranuclear granules within these cells.







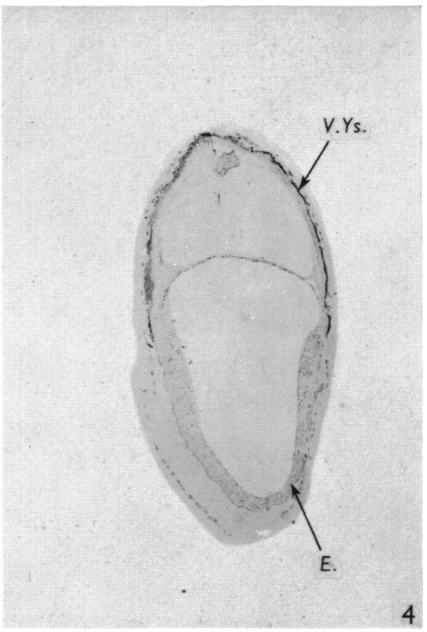
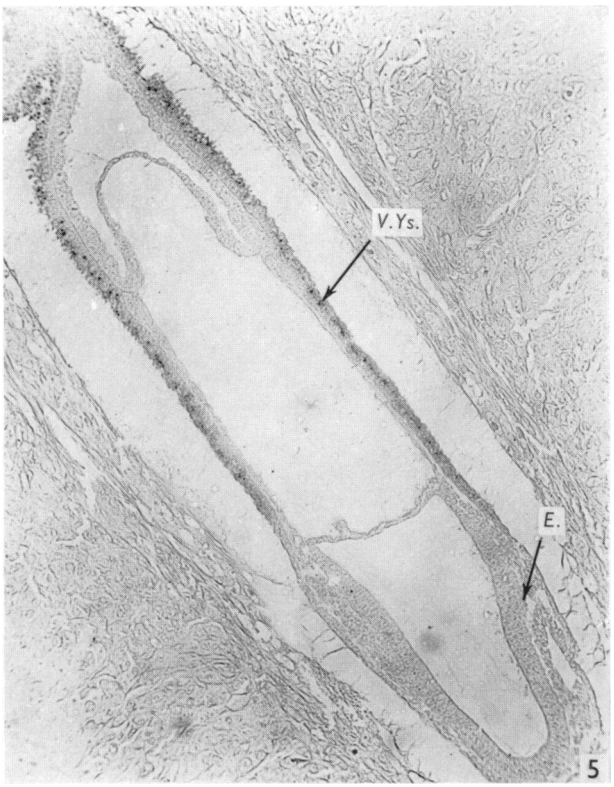


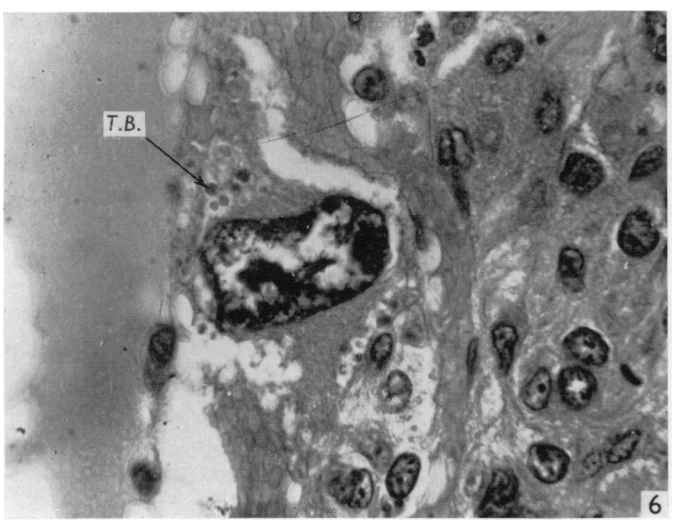
Fig. 1. Whole egg cylinder stained for peroxidase 6 h after injection of the enzyme into the mother at 8·5 d of pregnancy. No uptake has taken place at the embryonic pole (*E*) of the cylinder. (× 75.)

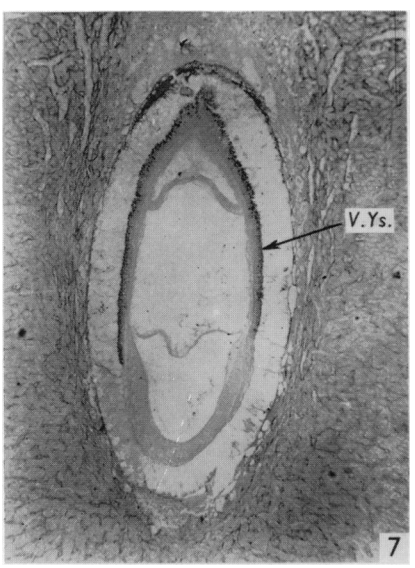
Fig. 2. Visceral yolk-sac epithelium at 8·5 d of gestation from a conceptus whose mother had been injected with horseradish peroxidase 6 h previously. Double staining for peroxidase and acid phosphatase has been performed. The injected peroxidase is seen as small granules in the apical region of the cells (*H.P.*); in the deeper parts of the cells fusion with lysosomes has occurred and digestive vacuoles or lyso-phagosomes have been formed (*Ly.Ph.*). No peroxidase is seen in either the extra-embryonic mesoderm (*Ex.M.*) or in the parietal yolk-sac epithelium (*P.Ys.*). (× 660.)

Fig. 3. High-power view of visceral yolk-sac epithelium at 9·5 d of gestation, stained for acid phosphatase. Acid phosphatase granules are clearly localized in the supranuclear parts of the epithelial cells (*A.P.*). The underlying extra-embryonic mesoderm (*Ex.M.*) is phosphatase-negative. (× 675.)

Fig. 4. Oblique section through egg cylinder at 9·5 d of gestation stained for horseradish peroxidase, from a mother injected with the enzyme 50 min previously. Peroxidase has been taken up by the cells of the visceral yolk-sac epithelium (*V.Ys.*) but not by the embryonic tissues (*E*). (× 75.)


V.Ys.
E.
5


T.B.
6


V.Ys.
7

9·5 *days*

Although no fundamental alteration in the distribution of acid phosphatase or in the phagocytic properties of the various tissues had occurred, some of the features indicated at 8·5 d (such as the localization of a positive acid phosphatase reaction in the visceral yolk-sac epithelium, Figs. 3 and 9) were more clearly seen at this stage. Serial removal of egg cylinders from an animal at intervals between 6 and 110 min after injection of peroxidase showed that a selective adsorption of the enzyme on to the surface of the visceral yolk-sac epithelium occurred even before removal of the first egg cylinder and was followed by phagocytosis and the gradual migration of the ingested material into deeper portions of the cell (Fig. 4).

Trypan blue injection 24 h before fixation confirmed the capacity of the visceral yolk-sac epithelium to concentrate the dye (Fig. 5). Large vacuoles at the cell apices were dyed a faint blue, whereas more deeply placed inclusions were much darker in colour and in turn gave way to deep blue supranuclear granules. When counterstained for acid phosphatase the latter were associated with a strongly positive reaction, whereas the apical vacuoles were phosphatase negative. Traces of trypan blue were also taken up by the trophoblastic giant cells (Fig. 6) and by an occasional embryonic endoderm cell, but here little concentration of the dye took place. The cells of the visceral yolk-sac endoderm contained vacuoles and had a free border which were distinctly PAS positive; these features were co-extensive with the ability to take up trypan blue and ended abruptly at the point of transition between extra-embryonic and embryonic endoderm (Fig. 7). At this point there also occurred a change from a tall columnar to a squamous epithelium.

10·5 *and* 11·5 *days*

Figures 10 and 11 illustrate the close correspondence in the visceral yolk-sac epithelium between the sites at which peroxidase is concentrated and those at which phosphatase is found. Double staining for peroxidase and phosphatase of material fixed 15 min after maternal injection showed most of the peroxidase lying in phagosomes *distal* to the acid phosphatase containing lysosomes. Later (60 min and longer after injection) considerable fusion of lysosomes and phagosomes had taken place but new phagosomes were continually being formed in the distal parts of the cell (Figs. 12, 13) probably because peroxidase remains in the serum for some hours after intravenous injection.

The parietal yolk-sac was, as at previous stages, distinctly if not very strongly phosphatase positive, but there was no evidence of peroxidase uptake by its cells.

Fig. 5. Longitudinal section through egg cylinder at 9·5 d of gestation from a mother injected trypan blue 24 h previously. The dye is specifically concentrated by the cells of the visceral yolk-sac epithelium (*V.Ys.*) and is absent from the embryonic tissues (*E*). (× 100.)

Fig. 6. Trophoblastic giant cell at 9·5 d of gestation from a rat injected with trypan blue 24 h before death. Trypan blue (*T.B.*) is seen in vacuoles in the cell cytoplasm. (×490).

Fig. 7. Longitudinal section through egg cylinder treated with trypan blue as in Fig. 5 but counterstained with PAS. The cells of the visceral yolk-sac epithelium (*V.Ys*) have a distinctly PAS positive free border which is co-extensive topographically with the area of trypan blue uptake. (×55.)

External to it the trophoblastic giant cells were also phosphatase-positive and occasionally showed uptake of peroxidase though this was negligible compared with the cells of the visceral yolk-sac epithelium (Fig. 18). While the embryonic blood cells both in control and experimental embryos gave a faint pseudo-positive reaction, it is clear that the injected enzyme had not traversed the yolk-sac epithelium for none was present in the mesenchyme immediately underlying this structure (Fig. 10).

12·5 *days*

At this stage a functional chorioallantoic placenta consisting of five layers has been established. Mesometrially the decidua basalis is seen next to which lies the penetration zone of trophoblastic giant cells with maternal blood spaces between them; there follows a spongy zone of cytotrophoblast penetrated by maternal blood vessels but not yet vascularized by allantoic vessels; a labyrinth of syncytiotrophoblast lying between maternal lacunae and containing a vascular core of allantoic mesoderm follows and finally a layer of allantoic mesoderm covers the embryonic aspect of the placenta. Six hours after injection of peroxidase the phosphatase-positive trophoblastic giant cells of the penetration zone had taken up traces of the enzyme but no uptake was demonstrable in any of the other placental layers, which, as might be expected, also contained less acid phosphatase than the penetration zone.

Peroxidase (Fig. 14) and acid phosphatase (Fig. 15) were clearly demonstrable in the visceral yolk-sac epithelium and the same relationship between lysosomes and phagosomes existed as at previous stages of development. Visceral yolk-sac examined 24 h after maternal trypan blue injection showed that the epithelium again concentrated the dye in supranuclear granules associated with a strongly positive reaction for acid phosphatase, while in the most apical regions of these cells the dye was seen in large faintly coloured vacuoles which were phosphatase-negative. No peroxidase was demonstrable in the embryonic tissues.

Fig. 8. Longitudinal section through egg cylinder at 8·5 d of gestation V.Ys., visceral yolk-sac epithelium; E, embryonic pole; T, trophoblastic giant cell; R, Reichert's membrane; E,C., ectoplacental cone. (× 70.)

Fig. 9. Longitudinal section through egg cylinder at 9·5 d of gestation stained for acid phosphatase. The enzyme reaction is well marked in the extra-embryonic endoderm of the visceral yolk-sac (V.Ys.) but little is seen in the cells of the embryonic pole. (E). (× 70.)

Fig. 10. Visceral layer of the yolk-sac stained for peroxidase from a mother who had been injected with the enzyme 70 min before death; 10·5 d of gestation. Peroxidase (H.P.) is confined to the yolk-sac epithelium; none is seen in the vitelline vessels (B.V.) of the extra-embryonic mesoderm. (× 390.)

Fig. 11. Visceral layer of the yolk-sac at 10·5 d of gestation, stained for acid phosphatase. The epithelial cells give a positive reaction (A.P.) in the same region in which they accumulated ingested horseradish peroxidase (cf. fig. 10.) (× 380.)

Fig. 12. Visceral layer of the yolk-sac at 11·5 d of gestation after maternal injection of horseradish peroxidase 90 min before fixation. Double staining for peroxidase and acid phosphatase has been carried out. The injected peroxidase (H.P.) is seen in the apical parts of the epithelial cells while the deeper parts contain digestive vacuoles (Ly.Ph.) giving a strongly positive phosphatase reaction which largely masks any peroxidase present in this region. (× 380.)

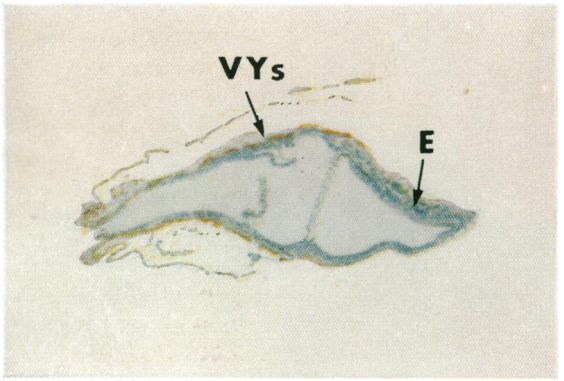


Fig. 9

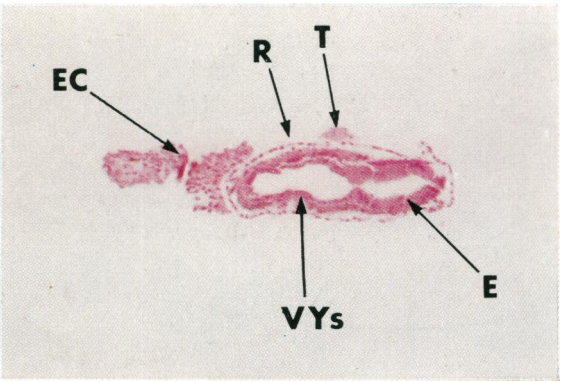


Fig. 8

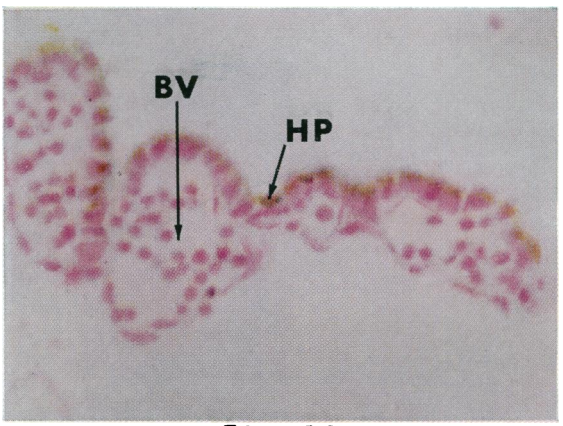


Fig. 10

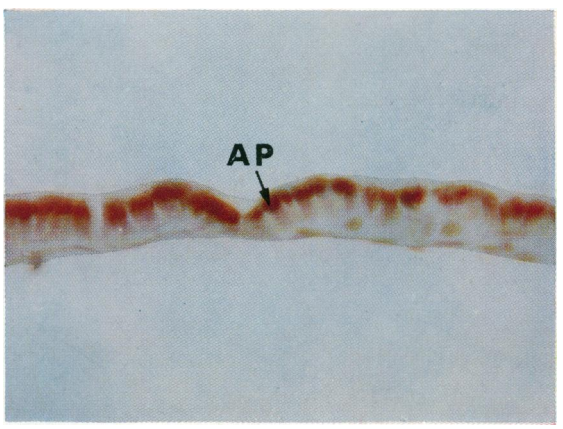


Fig. 11

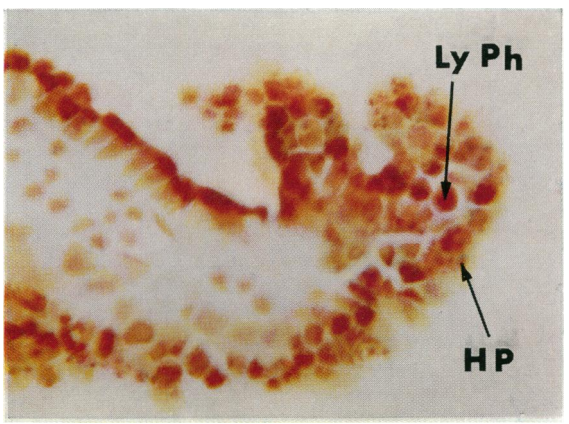


Fig. 12

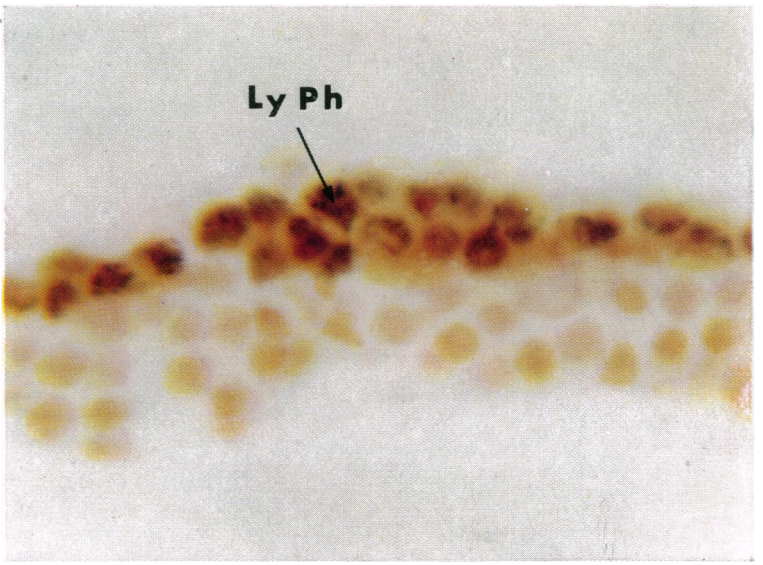


Fig. 13

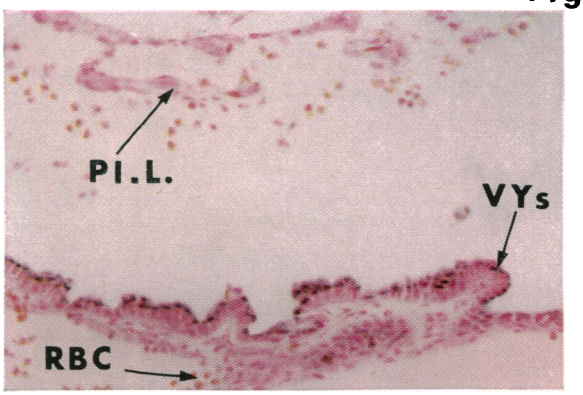


Fig. 14

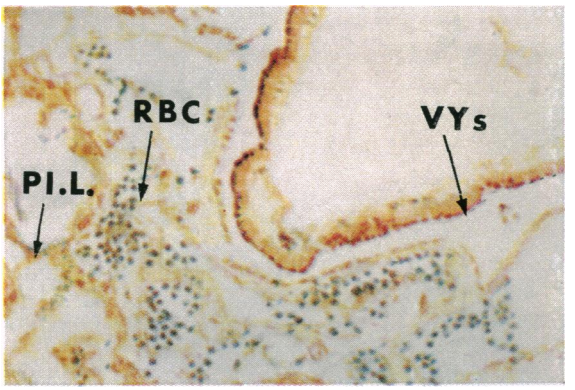


Fig. 15

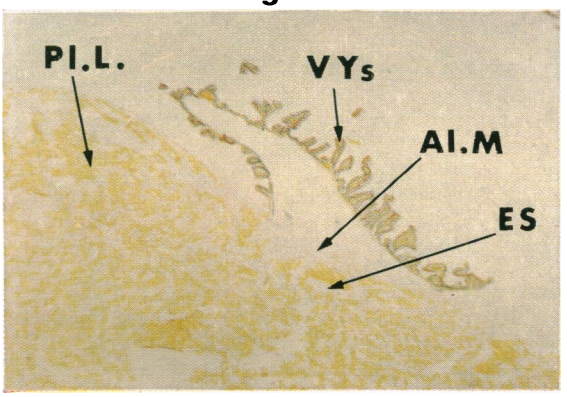


Fig. 16

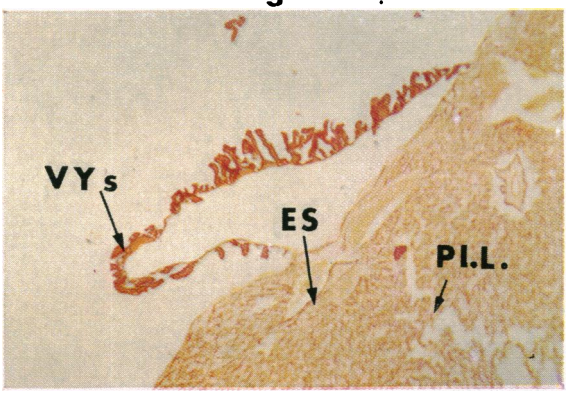


Fig. 17

20·5 *days*

Seventeen hours after injection peroxidase associated with acid phosphatase was present in lyso-phagosomes of the visceral yolk-sac epithelium; few free phagosomes were seen, presumably because of the long interval allowed between injection of peroxidase and fixation of the membrane. Sections through the chorioallantoic placenta 6 h after maternal peroxidase injection confirmed that there was no appreciable uptake of enzyme by the cells of the placental labyrinth, by the allantoic mesoderm or by the lining cells of the endodermal sinuses but, by contrast, a deep blue reaction was given by the nearby visceral yolk-sac endoderm (Fig. 16). As might be anticipated the latter was also strongly acid phosphatase-positive while only a faint reaction could be elicited from the placental labyrinth (Fig. 17). As at previous stages the trophoblastic giant cells of the penetration zone gave a moderate acid phosphatase reaction (Figs. 19, 20); this agrees with the findings of Deane, Rubin, Driks, Lobel & Leipsner (1962). Embryonic tissue gave a negative peroxidase reaction, and it is clear that no detectable amount of enzyme had crossed the placental barrier unchanged. Various embryonic tissues such as the dorsal root ganglion cells were strongly phosphatase-positive, but it is reasonable to assume that lysosomes in these situations are not concerned with the digestion of embryotroph.

(*b*) *Biochemistry*

Table 1 and Fig. 21 present the results of two typical experiments in which the levels of peroxidase activity in embryonic and placental material were determined 6 h after intravenous maternal injection. It will be seen that tissues from uninjected animals give a small positive assay figure; this agrees with Straus's (1958) observation that many tissues contain innate peroxidatic activity.

The results indicate clearly that at neither 11·5 nor 20·5 d is there any significant

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Fig. 13. Visceral layer of the yolk-sac at 10·5 d of gestation after maternal injection of horseradish peroxidase 70 min before fixation. Double staining for peroxidase and acid phosphatase has been performed. Digestive vacuoles or lyso-phagosomes (Ly.Ph.) are clearly seen in the epithelial cells. (× 690.)

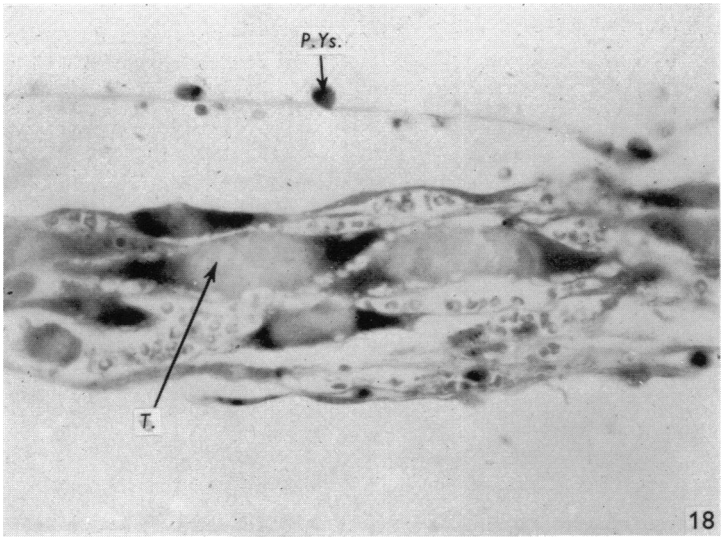
Fig. 14. Visceral yolk-sac (V.Ys.) and placental labyrinth (Pl.L.) at 12·5 d of pregnancy stained for peroxidase from a mother injected 6 h previously with the enzyme. Peroxidase is clearly seen in the yolk-sac epithelium but not in the underlying mesenchyme; the cells of the placental labyrinth are peroxidase-negative. Foetal blood cells (R.B.C.) give a pseudo-positive reaction both in this preparationand in control sections. (× 180.)

Fig. 15. Visceral yolk-sac (V.Ys.) and placental labyrinth (Pl.L) at 12·5 d of pregnancy from a mother injected 6 h previously with horseradish peroxidase; stained for both acid phosphatase and peroxidase. The supranuclear regions of the yolk-sac epithelium are strongly phosphatase-positive and this reaction to some extent masks the underlying peroxidase reaction. The cells of the labyrinth give only a weak reaction for acid phosphatase and none at all for peroxidase though the foetal blood cells (RBC) give a pseudo-positive peroxidase reaction. (× 140.)

Fig. 16. Visceral yolk-sac (V.Ys.) and placental labryinth (Pl.L.) at 20·5 d of pregnancy from a mother injected 6 h previously with horseradish peroxidase. Stained for peroxidase. The enzyme has been concentrated by the yolk-sac epithelium but no reaction is apparent in the underlying mesenchyme; no reaction is demonstrable in the allantoic mesoderm (Al.M.), the placental labyrinth or in the endodermal sinuses (E.S.). (× 18.)

Fig. 17. Visceral yolk-sac (V.Ys,) and placental labyrinth (Pl.L.) at 20·5 d of pregnancy stained for acid phosphatase. A strongly positive reaction is given by the yolk-sac epithelium and a moderately strong one by a nearby endodermal sinus (E.S.). The placental labyrinth gives only a weak reaction for acid phosphatase. (× 18.)

difference in peroxidase activity between the embryos of injected and control animals, indicating failure of the enzyme to cross the placental barrier in detectable amounts. In sharp contrast is the yolk-sac where in each case the activity in peroxidase-injected rats is approximately 100 times that of controls. While chorioallantoic



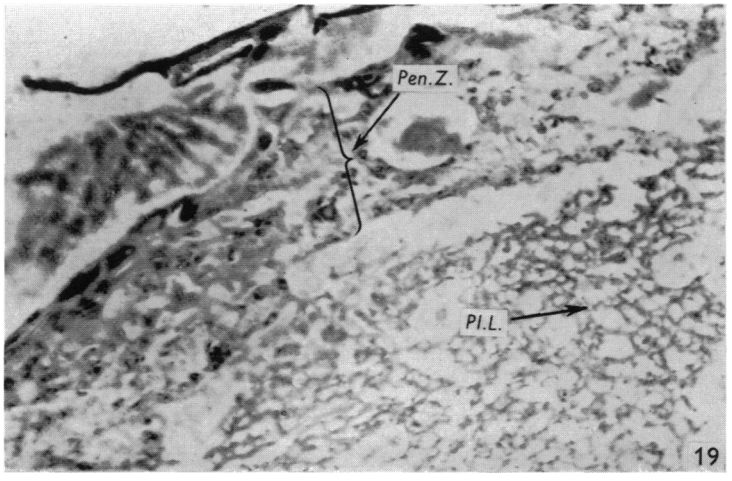


Fig. 18. Trophoblast and parietal yolk-sac epithelium at 11·5 d of gestation after maternal injection of horseradish peroxidase ½ h before fixation. Double staining for peroxidase and acid phosphatase has been performed. Neither the trophoblastic giant cells (*T.*) nor the cells of the parietal yolk-sac epithelium (*P. Ys.*) have taken up appreciable quantities of peroxidase but both are distinctly phosphatase-positive. (×450.)

Fig. 19. Mesometrial region of chorioallantoic placenta at 20·5 d of pregnancy. The superficial penetration zone (*Pen.Z.*) contains trophoblastic giant cells which are distinctly phosphatase-positive. Next to it the placental labyrinth (*Pl.L.*) gives only a weak reaction to the enzyme. At this stage of gestation the spongy cytotrophoblastic zone between the penetration zone and the placental labyrinth has disappeared. (×40.)

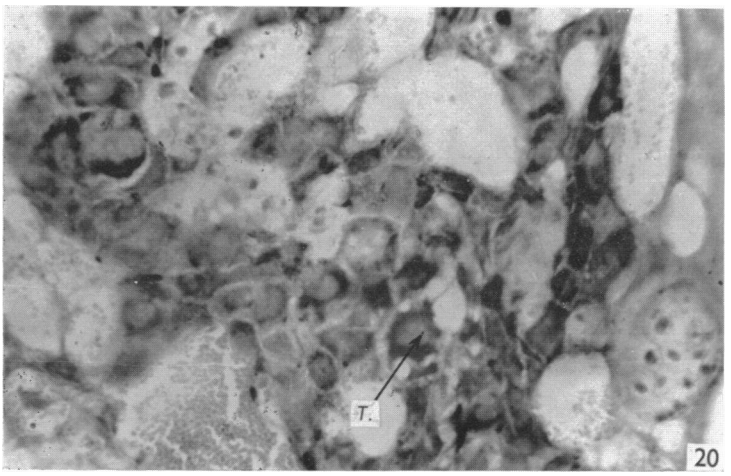


Fig. 20. Higher power view of penetration zone of chorioallantoic placenta at 20·5 d of pregnancystained for acid phosphatase. The cytoplasmic localisation of the enzyme in the trophoblastic giant cells (*T.*) is clear. (× 135.)

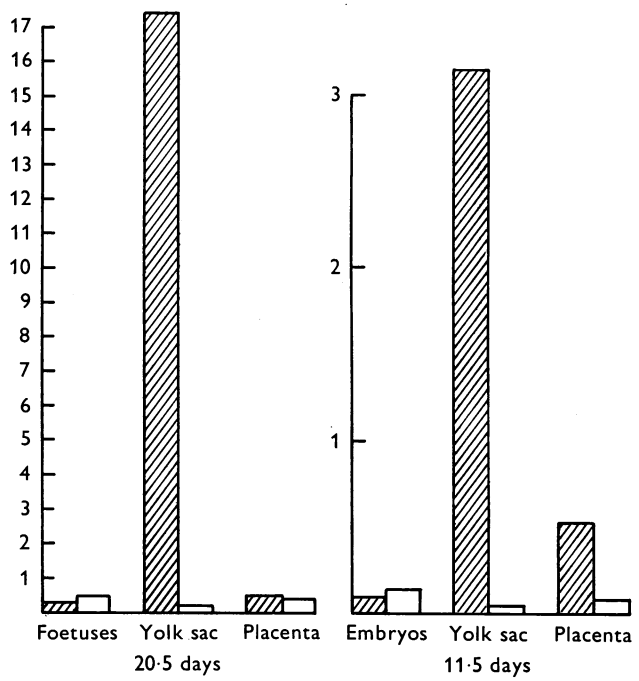


Fig. 21. Specific activities of peroxidase ($\triangle E_{520}$ per min per mg protein) in embryonic tissues and foetal membranes 6 h after maternal injection of horseradish peroxidase. ▨, Experimental; □, control.

placenta at 20·5 d shows no activity above that of controls, this tissue from the early embryos contains some additional enzyme; this probably reflects no more than contamination of chorioallantoic placenta with yolk-sac since it was not possible to separate the two completely at this stage of development.

Comparison of absolute values between the 11·5 and 20·5 d series is not meaningful, for in the latter the homogenates from each tissue were centrifuged prior to assay, a

Table 1. *Specific activities of peroxidase (ΔE_{520} per min/mg protein) in 11·5 d and 20·5 d rat conceptuses*

(Injected rats had received 20 mg horseradish peroxidase 6 h previously.)

	11·5 d		20·5 d	
	Injected	Uninjected	Injected	Uninjected
Embryos 1	0·10	0·19	0·23	0·29
2	0·04	0·09	0·17	0·38
3	0·09	0·13	0·42	0·33
4	0·08	0·14	0·24	0·77
Mean	0·08	0·14	0·27	0·44
Yolk-sacs 1	4·26	0·02	14·4	0·12
2	2·05	0·03	20·3	0·30
Mean	3·16	0·03	17·4	0·21
Chorioallantoic placentae 1	0·51	0·07	0·46	0·42
2	0·53	0·08	0·47	0·30
Mean	0·52	0·08	0·47	0·36

Table 2. *Specific activities of acid phosphatase in 11·5 d and 20·5 d foetal membranes*

(Activities are expressed as milli-micromoles of *p*-nitrophenol (nitrophenyl phosphate substrate) or of phosphate (glycerophosphate substrate) liberated per min per mg protein.)

		p-nitrophenyl phosphate substrate (pH 4)	*β*-glycero phosphate substrate (pH 5)
	20·5 days		
Rat 1 (10 conceptuses)	Yolk-sac	23·1 ± 6·0	40·5 ± 4·9
	Chorioallantoic placenta	12·2 ± 4·9	17·3 ± 4·6
	Ratio: $\frac{\text{Yolk-sac}}{\text{Chorioallantoic placenta}}$	1·9	2·3
Rat 2 (11 conceptuses)	Yolk-sac	25·1 ± 7·1	50·2 ± 11·4
	Chorioallantoic placenta	11·5 ± 5·3	21·4 ± 2·4
	Ratio: $\frac{\text{Yolk-sac}}{\text{Chorioallantoic placenta}}$	2·2	2·3
	11·5 days		
Rat 3 (6 yolk-sacs, 5 chorioallantoic placentae	Yolk-sac	38·5 ± 5·9	—
	Chorioallantoic placenta	19·4 ± 3·0	—
	Ratio: $\frac{\text{Yolk-sac}}{\text{Chorioallantoic placenta}}$	2·0	—

procedure which would considerably reduce the concentration of protein relative to peroxidase and lead to a general increase in specific activities.

Table 2 shows the acid phosphatase levels in yolk-sacs and chorioallantoic placentae obtained from rats at 20·5 and 11·5 d. At 20·5 d the mean level of enzyme activity in the yolk-sac was approximately twice that of the chorioallantoic placenta, using either β-glycerophosphate at pH 5 or *p*-nitrophenyl phosphate at pH 4 as substrate and a similar ratio was obtained at 11·5 d. Again only the 20·5 d material was clarified by centrifugation and *absolute* specific activities at the two stages of pregnancy are therefore not directly comparable. If it had been possible to treat all homogenates similarly, the specific activity of the 11·5 d material would have been higher than that indicated in Table 2. This does not, of course, affect the comparability of the *ratios* of activity between yolk-sac and chorioallantoic placenta.

DISCUSSION

Assays of homogenized yolk-sac, chorioallantoic placenta and embryo, prepared after maternal injection of horseradish peroxidase, demonstrate that unchanged enzyme does not cross the placental barrier in appreciable amounts. This investigation is not designed to indicate whether a similar barrier is operative for a wide variety of heterologous proteins, but Mayersbach (1958), on the basis of immunochemical tests and by means of fluorescence labelling, has shown that the whole spectrum of human as well as rat serum proteins enter the foetal circulation when injected intravenously into rats during the last third of pregnancy. Hemmings & Brambell (1961), in a discussion of protein transfer across foetal membranes, have reviewed the status of heterologous proteins in this respect.

Significant amounts of certain homologous proteins, such as circulating antibodies, are able to traverse the materno-foetal barrier in the rabbit (Brambell, Hemmings & Henderson, 1951) and recently Brambell (1963) has suggested a model to account for membrane selectivity. According to this, those proteins which cross the yolk-sac epithelium in rabbits and the gut epithelium in neonatal rats and mice do so by virtue of being attached to specific receptor sites within the cells which afford them passage; these sites protect their attached proteins from the intracellular degradation experienced by other ingested material, In the context of the present investigation such sites, if indeed they occur in the rat yolk-sac (which does not normally transmit significant quantities of antibody to the foetus), might be present in the walls of phagosomes. Alternatively these sites might be confined to special inclusion bodies which do not take up heterologous proteins (and are therefore not visualized by the peroxidase technique) and which do not fuse with the cell lysosomes.

The histochemical experiments described show that of all the foetal membranes only the visceral layer of the yolk-sac concentrates horseradish peroxidase. In order to reach it, before the 16th day, substances must traverse both Reichert's membrane and the continuous layer of trophoblastic cells on its external surface, though they may penetrate between the cells of the parietal yolk-sac epithelium which line the inner surface of the membrane. Indeed traces of both trypan blue and peroxidase have been seen in the trophoblastic giant cells but not in the parietal yolk-sac epithelium.

Within the visceral yolk-sac epithelium the now familiar pattern of intracellular digestion (Straus, 1963) is best illustrated by the horseradish peroxidase experiments. A short time after maternal injection this material is demonstrable in phagosomes at the cell apices. The lysosomes, indicated by a positive acid phosphatase reaction, although also supranuclear, are situated somewhat deeper in the cell substance. Some time later fusion of phagosomes and lysosomes occurs and the material present in the former is hydrolysed, its passage into the foetus being thereby prevented. Examination of the visceral yolk-sac from trypan blue-treated animals indicates that the apical vacuoles in the epithelial cells are pale blue; presumably within these vacuoles the dye is present together with albumin, for circulating dye is albumin-bound and is probably phagocytosed in combination with this protein. In deeper parts of the cell, though still in a supranuclear situation, the dye-albumin complex is brought into contact with lysosomal enzymes. Here the protein apparently undergoes digestion and the residual dye is then demonstrable is supranuclear clumps which are far denser than the apical vacuoles.

In functional terms the conclusions to be drawn from this investigation seem clear. Although levels of acid phosphatase activity are only twice those of the chorioallantoic placenta, the principal site for the digestion of embryotroph is undoubtedly the visceral layer of the yolk-sac epithelium. From here the products are presumably passed to the embryo either by diffusion or (later) by the vitelline vessels. During implantation and immediately thereafter, embryotroph is the only source of food available to the nidus but even after the formation of a true placenta, the functional capacity of the visceral yolk-sac remains undiminished and one can only speculate as to why this should be so. It may be that an important function of this organ is to allow certain proteins unhindered passage into the foetus and this may require the continued operation of a mechanism such as that postulated by Brambell (*vide supra*). Should this be so, then the essential nutritive function of the early yolk-sac may be superseded in later pregnancy by its function as a selective membrane which allows essential proteins to enter the foetus. To achieve this, the membrane would ingest all the proteins with which it is presented but would dispose of unwanted ones by intracellular digestion. Hemmings (1957), while studying protein selection in the yolk-sac splanchnopleur of the 24 d pregnant rabbit obtained results which would be consistent with this suggestion, and Lambson (1966) has presented electron microscopic evidence of ferritin transfer across the rat yolk-sac at the end of pregnancy.

SUMMARY

(1) Embryotrophic nutrition has been studied by the injection of an easily traceable protein (horseradish peroxidase) into rats at various stages of pregnancy. Histochemical observations have enabled the injected material to be followed into the foetal membranes and have demonstrated its association with lysosomal enzyme systems, while biochemical studies have demonstrated that none of it crosses the placental barrier unchanged.

(2) The visceral layer of the yolk-sac epithelium is the principal organ concerned with the digestion of embryotroph and, until the establishment of a chorioallantoic placenta, provides the only mechanism for embryonic nutrition.

(3) Even after the establishment of the true placenta, the digestive processes in the visceral yolk-sac epithelium continue. The reasons for this are not clear.

(4) No great powers of intracellular digestion have been demonstrated in the invasive trophoblast of the early implantation period or in the tissues developing from it.

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