THE INVESTIGATION OF SOME NUTRITIONAL REQUIREMENTS OF RAT EMBRYOS UNDERGOING ORGANOGENESIS IN VITRO

A THESIS PRESENTED BY

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ABSTRACT

Repeated culture of rat conceptuses of between $10\frac{1}{2}$ and $11\frac{1}{2}$ days' gestation in the same sample of rat serum showed that the serum has a finite ability to support embryonic growth and development. After four consecutive cultures of rat conceptuses the serum became exhausted and analysis of it showed that rat conceptuses utilized glucose and certain proteins present within the serum. It was also concluded that rat conceptuses secreted toxic dialysable and non-dialysable components into the medium during culture and these materials affected embryonic growth during subsequent culture of conceptuses in exhausted serum.

The nutritional value of free amino acids as a substitute for serum proteins was studied by the addition of leupeptin - a specific lysosomal enzyme inhibitor - to the culture serum. The effect of this was partially reversed if free amino acids were included in the leupeptin-containing serum. The inclusion of both essential and non-essential amino acids in the culture medium was found to be necessary for embryonic growth.

A model system for further studies of histiotrophic nutrition (the giant yolk sac) was devised by simple modification of New's culture technique to beyond the usual 48 hours thus allowing for maximum growth of the extra-embryonic membranes in vitro. After 7 extra days' culture in rat serum the giant yolk sac was 2 cm. in diameter and contained approximately 600 µl yolk sac fluid. The morphology, junctional permeability, histochemistry and endocytic activity of the giant yolk sac

showed this system was, in general, similar to the *in vivo* yolk sac of a comparable gestational age.

The giant yolk sac transported radio-labelled free amino acids which accumulated in the yolk sac fluid and analysis of this fluid showed it to contain 14 major proteins; some of these contained radio-labelled amino acids and were, therefore, synthesised by the giant yolk sac.

Finally, giant yolk sac fluid was able to improve the nutritional status of exhausted serum and, therefore, contained components which are vital to normal embryonic growth and development.

The major feature of the giant yolk sac system is its capacity to produce the products of histiotrophic nutrition in large quantities for biochemical analysis.

CHAPTER ONE

CHAPTER I

GENERAL INTRODUCTION

Embryo culture techniques, perfected by New et al. (1976 b), presently in use make it possible to grow rat embryos for 48 hours from the pre-somite head-fold stage *in vitro* at a rate almost identical to that seen *in vivo*. This thesis describes a series of experiments designed to examine certain important aspects of embryonic nutrition during organogenesis and a number of investigations, employing different techniques, were carried out.

Chapter III describes experiments in which rat serum (the culture medium used for growing rat embryos) was exhausted by repeated culture and regenerated in order to determine the relative importance of some of its constituents in maintaining embryonic development. In Chapter IV I describe how some of the catheptic enzymes of the visceral yolk sac vacuolar system were poisoned in order to assess the nutritional values of free amino acids added to the culture medium. Finally, in Chapter V, I describe a model system I have developed which is referred to as the "giant yolk sac" and which enables large quantities of the products of yolk sac transport and secretion to be collected, uncontaminated, by the culture medium. Key functional aspects of the model were verified and it was subsequently

used to study yolk sac amino acid transport and protein synthesis.

In this introduction a concise summary of the relevant period of embryonic development of the rat is presented and an account of histiotrophic nutrition in the mammal is given. The processes of endocytosis and intracellular digestion in the visceral layer of the yolk sac and their respective roles in embryonic nutrition are then considered and, finally, the culture techniques used throughout the study are evaluated.

Early embryology and extra-embryonic membrane development in the rat

In the rat multiparous gestation normally lasts 21 days with day zero being considered the time at which the oocyte is fertilised in the Fallopian tube. On the fourth day after fertilisation the early blastocyst stage embryo, having lost the zona pellucida, then enters the uterus and becomes implanted in a groove in the antimesometrial wall (Huber, 1915; Steven, 1975). The blastocyst (Figure 1(a)) consists of a layer of endoderm differentiating from the blastocoelic surface of the inner cell mass (Figure 1(b)). The way that development proceeds from here, however, is peculiar to the embryos of rodents and related species and underlies their suitability for culture.

The polar trophoblast grows to form the ectoplacental cone, the cells of which engulf maternal erythrocytes and later take part in forming the chorioallantoic placenta (Steven, 1975). The inner cell mass ectoderm is pushed into the blastocoele as the ectoplacental cone grows and the endoderm proliferates to cover the whole surface. A cavity - the primary embryonic cavity - then forms in the ectoderm, the bilaminar tube thus formed being referred to as the egg cylinder. In rodents and insectivores there develops an extra-embryonic membrane that is not present in other species. (It was first described by Reichert (1862) in the guinea pig and bears his name.) In the rat Reichert's membrane first appears at day six

of gestation as an amorphous secretion along the inner wall of the trophoblast (Tachi, Tachi & Linder, 1970). The parietal yolk sac is represented as a discontinuous layer of cells attached to the inner surface of Reichert's membrane, which may be considered as the basement membrane of that layer. The parietal and visceral yolk sac endoderms are continuous, enclosing the yolk sac cavity within them (Figure 1(c)). By the time of formation of the primitive streak during the sixth day after ovulation mesoderm is formed and migrates to lie between the endoderm and ectoderm in a manner similar to the formation of the human trilaminar disc. The mesoderm proliferates until ectoderm and endoderm are completely separated (Figure 1(d)), the only exceptions being around the neck of the egg cylinder and the region of the embryo proper.

A second cavity then develops in the substance of the mesoderm, forming a complete annulus in the wall of the egg cylinder (Figure 1(de)). The medial wall of this annulus, consisting of ectoderm and mesoderm, moves inward with the growth of the annulus until opposite sides meet and fuse. In this way the primary embryonic cavity is divided into two ectodermally-lined chambers, the ectoplacental and amniotic cavities. The region of apposed ectodermal cells proliferates towards both poles of the conceptus and the subsequent breakdown of this cord of ectoderm surrounded by mesoderm (Figure 1(f)) completes the formation of the mesoderm-lined exocoelom (day 9).

At this stage the egg cylinder consists of three chambers - the ectoplacental, the (extra-embryonic) coelomic and the amniotic cavities, separated by two membranes, the amnion and the chorion (Figure 1(g & h)). Reichert's membrane rests on the inner (embryonic) surface of the parietal endoderm, which is made up of relatively few cells; the gaps between these cells are large (Merker & Villegas, 1970). This membrane is, therefore, the only structure completely separating the egg cylinder from the maternal

blood in the early stage of gestation. A bud of mesoderm, the allantois, grows from the future hind gut region of the embryo towards the chorion at the abembryonic pole of the conceptus (Figure 1(g))and this will eventually fuse with the chorion and take part in the formation of the chorio-allantoic placenta (Figure1(h & i)). It can be seen that the embryo is "inverted", meaning that the ectoderm - which will form the animal's surface - lies within the concavity of the endoderm from which will develop the viscera.

The way in which this trilaminar embryo folds to bring the embryonic membranes into the adult configuration occurs as follows: the embryonic foregut and hindgut first appear as indentations on the endodermal side, marking the sites where the head and tail folds develop. The embryonic blastodisc pushes up into the amniotic cavity and, by means of the side folds, becomes a three-layered tube in the amniotic cavity. Ectoderm surrounds mesoderm and endoderm and the tube becomes convex ventrally along its length. This process is very similar to the way in which human embryos form the head and tail folds.

Meanwhile, the embryo itself begins to develop from the three germ layers, with the neural plate differentiating from the ectodermal layer. Table 1 summarises the principal points in the development of the embryo itself to day 12 of gestation. The embryo grows with its back pushed upward into the amniotic cavity and, as a result, the amnion changes from being a plane surface to a spheriod surrounding the embryo and in contact with it only at the vitello-intestinal duct on its ventral side.

Similarly, the form of the visceral yolk sac changes with the growth of the embryo and amnion from being a cylinder above to a sphere surrounding the amnion and the embryo. Hence, the original ends of the cylinder become less significant in size.

At this stage (day 11) the embryo has developed further and is convex dorsally (Figure 1 (j)); various other changes take place between days 9 and 11. The allantois reaches the chorion and the ectoplacental cavity becomes obliterated. Allantoic mesoderm covers the internal surface of the two-layered ectodermal disc, the other surface of which is in contact with the ectoplacental cone. Blood vessels form in the allantois and maternal vessles invade the ectoplacental cone. These changes result in the early formation of the chorio-allantoic placenta. Blood vessles develop in the vitelline mesoderm, which communicate with the embryonic vascular system. A vitelline circulation is established between day 11 and 12 of gestation and extends throughout the visceral yolk sac, but not the parietal yolk sac. Organogenesis is completed in the 48 hours during which turning takes place. The neural tube closes, the optic and otic vesicles become visible and there is development of the fore-limb buds and the branchial arches. At the 20 somite stage the heart begins to beat. Reviews by Nicholas (1942), Christie (1964), Edwards (1968) and Steven and Morriss (1975) have given detailed accounts of rat embryo development.

It must be remembered that, when experimenting with embryos, the exact time of mating is not known. There may indeed by slight variations between litters in the developmental stages reached at the time of explantation. Also, there are often individual differences between embryos of the same litter. Burlingame and Long (1939) found a somite number difference of as much as 6 between litter mates. When analysing embryonic development it is important, therefore to:

(a) look at a sufficiently large sample;

(b) look at the widest possible range of parameters within each embryo

in order to establish the extent of these variations.

Figure 1

Diagrammatic representation of the early development of the rat foetal membranes (Morriss, 1975).

Figure 1a

Blastocyst before implantation; day 4.

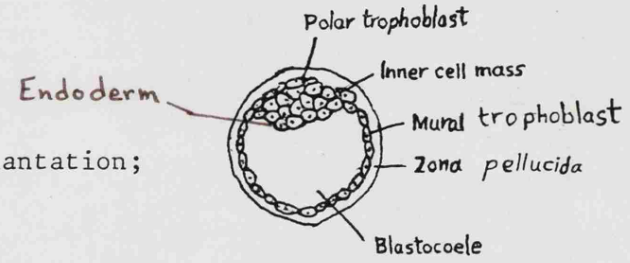


Figure 1b

Implanting blastocyst, showing the proliferation of polar trophoblast cells and differentiation of the inner cell mass into ectoderm and endoderm; day 5.

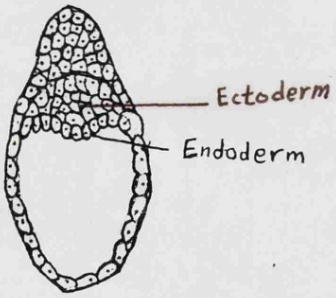


Figure 1c

Inversion of the germ layers and origin of Reichert's membrane; day 6.

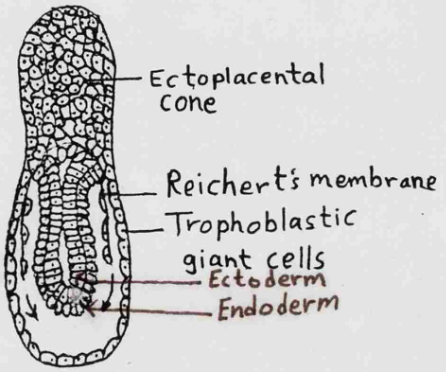
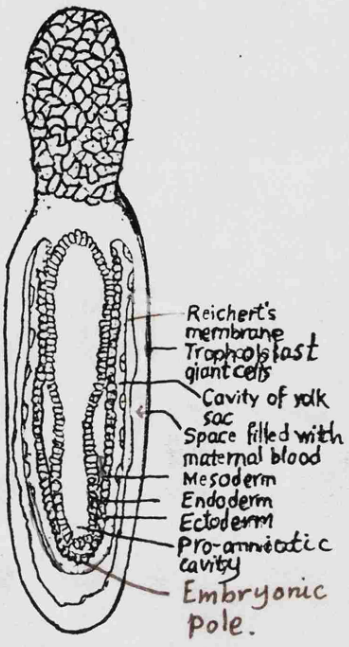


Figure 1d

Early appearance of mesoderm; Reichert's membrane now surrounds both embryonic and extraembryonic regions; day 6.



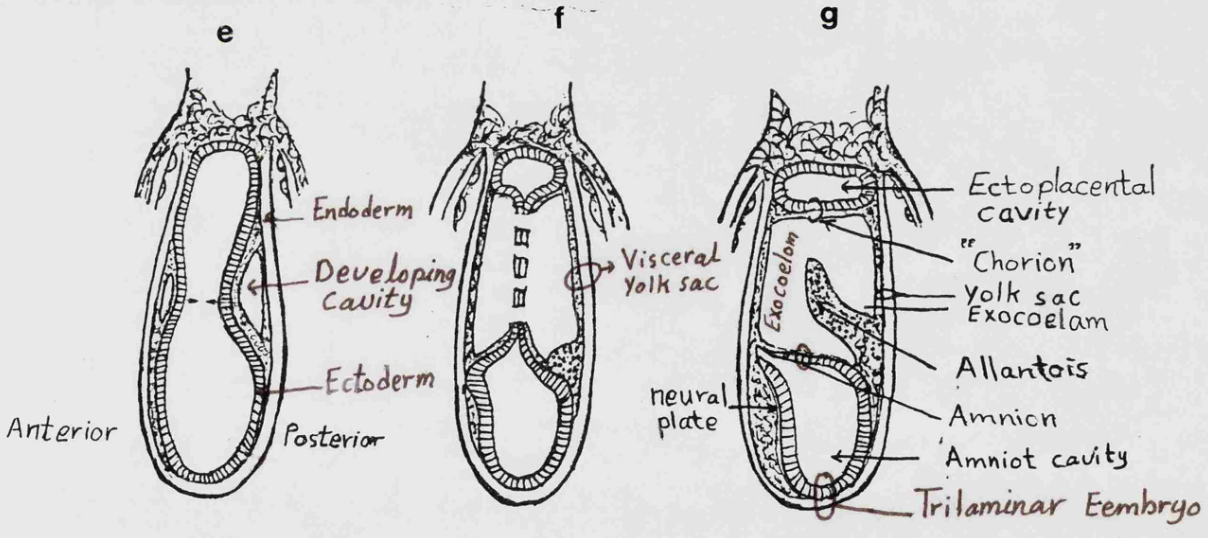


Figure 1 (e - g).

Showing the formation of the amnion, chorion and yolk sac; day 8 - 9.

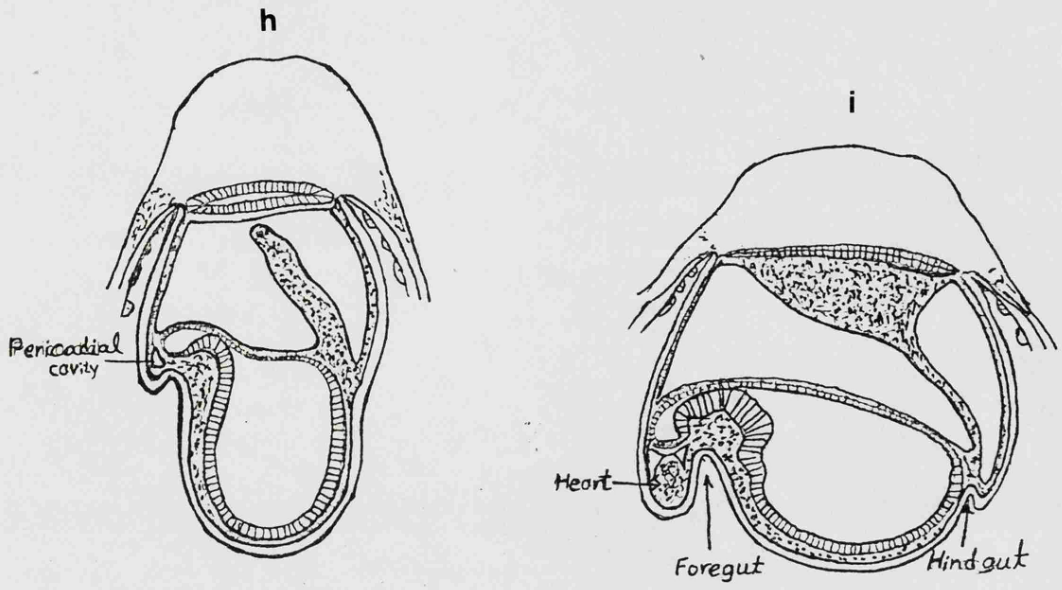


Figure 1 (h and i)

Showing fusion of the allantois with "chorion" and obliteration of the ectoplacental cavity, forming the allantoic placenta.

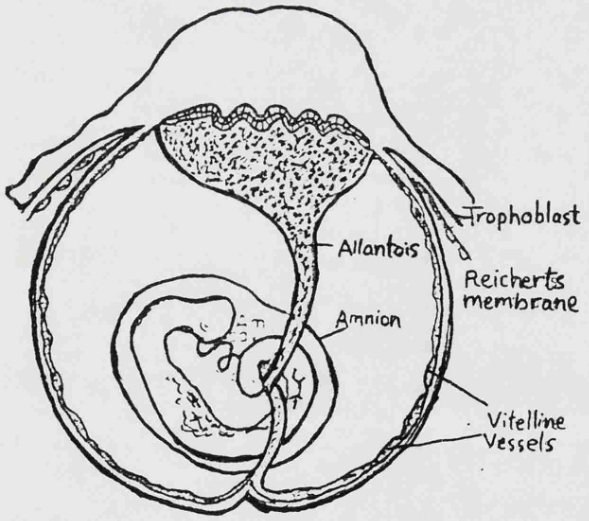


Figure 1 (j)

Further development of the allantoic placenta; day 11. The embryo is now convex dorsally, and the vitelline and allantoic vessels enter the abdomen at the umbilicus.

TABLE 1

Summarising the high points in the embryonic development of the rat between 9 and 12 days of gestation

Gestation age (days)	Somite Number	Main Commencing Features
9½-9¾	0-3	Formation of amnion and chorion, appearance of the allantoic bud at the posterior end of the embryo.
9¾-10	4-6	Neural plate appears and thickens. Neural fold rises. Appearance of head and tail folds and post-otic sulcus.
10-10½	7-9	Neural folds fused from the level of the second to the sixth somite. The embryo begins to turn towards a dorsally convex position. First Pharyngeal arch appears. The primitive infundibulum becomes obvious and deepens.
10½-10¾	10-14	Neural folds fused at diencephalic mesencephalic junction. Otic pit forms. Appearance of otic vesicles (Fig. 2a)
10¾-11¾	16-22	This stage begins with the closure of the anterior neuropore and rhombencephalic roof and the appearance of the anterior limb-bud; turning of the embryo is completed. The posterior neuropore and otic pit gradually close. (Fig. 2b).
11½-12	24-28	Posterior neuropore closes at the beginning of this stage. Heart divides into 4 chambers. Posterior limb-buds appear at the end of this stage. Optic vesicles invaginate to form the lens placode (Fig. 2c).

Figure 2

Diagramatic representation of early development of rat embryos (Christie, 1964). All., Allantois; A.n., Anterior neuropore; A.I.b., Anterior limb bud; Br.a., Branchial arch; Mx., Maxillary process; Otp., Otic pit; Ot.v., Otic vesicle; O.v., Optic vesicle; P.n., Posterior neuropore; V, Ventricle.

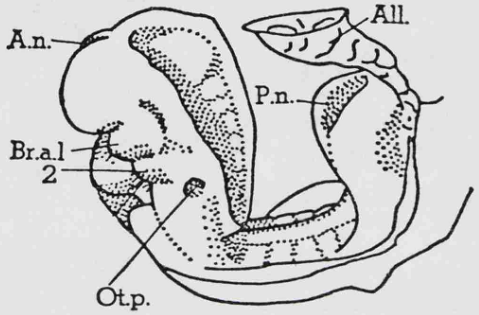
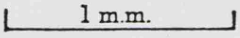


Figure 2a

Dorsal view of rat embryo between day $10\frac{1}{2}$ - $10\frac{3}{4}$ of gestation.



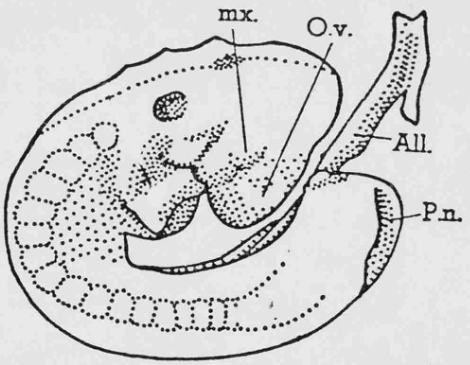


Figure 2b

Lateral view of rat embryo between $10\frac{3}{4}$ - $11\frac{1}{2}$ day of gestation.

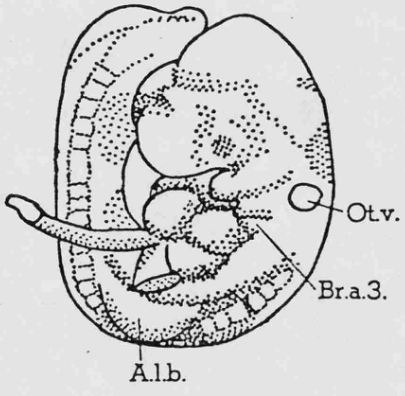


Figure 2c

Lateral view of rat embryo between $11\frac{1}{2}$ - 12 day of gestation.

Embryonic nutrition

In Eutherian mammals two types of nutrition - histiotrophic and haemotrophic - operate during development.

1. Histiotrophic nutrition - this results from the breakdown of maternal macromolecules (histiotroph) by the extra-embryonic membranes. The histiotroph may be derived from endometrial cells, glandular secretions and maternal blood or blood transudate (Amoroso, 1952). Its nature varies with each species but it is the only mode of nutrition available to a conceptus prior to the development of embryonic circulation and usually persists to some extent even after the establishment of circulation in the chorio-allantoic placenta. The histiotroph is usually broken down in the vacuolar system of cells of the extra-embryonic membranes (Beck et al., 1971). The soluble break-down products might pass through the fetal membranes to the embryo or, in special circumstances, whole macromolecules pass the placental barrier without the intervention of hydrolytic enzymes, e.g. IgG transfer (Bangham et al., 1958).

2. Haemotrophic nutrition - this consists mainly of an interchange of solutes between the maternal and fetal circulation and it begins when the fetal circulation perfuses the extra-embryonic membranes. Thus, at least a certain stage of organogenesis is required which involves the development of a beating embryonic heart. Theoretically, haemotrophic nutrition can exist when the embryonic and maternal blood streams come into close relationship to one another either in a chorio-vitelline or a chorio-allantoic circulation.

Haemotrophic transfer of solutes may occur by simple diffusion along a chemical or electro-chemical gradient. Sometimes (as in the case of glucose) diffusion is enhanced by chemical or physical factors so that it exceeds predictions estimated by molecular size, charge and lipid solubility (see Beck & Lloyd (1977) for a more detailed account of factors involved in histiotrophic and haemotrophic nutrition).

Embryonic nutrition in the rat

Implantation takes place at 5.5 days and the embryonic mass remains relatively quiescent whilst trophoblast invades the decidua and the walls of maternal capillaries; between 7.5 and 8.5 days of gestation gastrulation occurs. At this stage the embryonic disc probably derives its nutrition largely from the visceral layer of the extra-embryonic endoderm (the so-called 'inverted yolk sac placenta') which is able to degrade macromolecules by heterosis (this will be discussed later). The histiotroph comes from three sources:

(a) the maternal serum located in lacunae between trophoblastic cells;

(b) endometrial cells broken down by trophoblast;

(c) secretions of the uterine glands,

(Beck 1976, 1981).

At the 20 somites stage, when the embryonic head, tail and lateral folds are well developed, the allantoic bud fuses with the chorion to allow the inception of haemotrophic nutrition at about 11 or 12 days of gestation. On day 16 the parietal layer of the yolk sac, together with the overlying trophoblast, are ruptured leaving the visceral layer exposed to the uterine cavity where it is in an excellent position to pinocytoze and degrade uterine secretions (Everett, 1935; Bridgman, 1948). However, other authors suggest that rupture takes place after day 18 (Duval, 1891; Jensh et al., 1977).

In view of its dependence on histiotrophic nutrition during early organogenesis, rat development is a good system for the study of this type of nutrition.

The rat visceral yolk sac as the principal site of histiotrophic nutrition

Substantial evidence supporting the view that the visceral yolk sac is the principal site of histiotrophic nutrition between days 8.5 and 11

of gestation exists and is reviewed below.

The endodermal layer of the visceral yolk sac consists of columnar epithelial cells which are highly endocytic, especially during the mid- and late periods of gestation (Everett, 1935; Jollie & Triche, 1971; Williams et al., 1975, 1976; Gulamhusein et al., 1979; Gupta et al., 1979, 1981). At the egg cylinder stage digested materials released by the extra-embryonic tissues can find their way to the developing embryo by diffusion through a system of intercellular spaces which pervade the underlying mesenchyme (Beck & Lloyd, 1968): these intercellular spaces are in continuity with the extra-embryonic coelom (Figure 3 is a diagrammatic representation of this arrangement).

Later in gestation, when there is a beating heart, the vitelline blood vessels develop in the mesenchyme. The endoderm becomes lined with the visceral yolk sac basement membrane and the mesothelium lined with the serosal basement membrane. Both basement membranes become thickened near term and collagen fibres are laid down in them. Despite these changes in the structure of the visceral yolk sac the endoderm cells remain highly endocytic and there is little to suggest that their catabolic activities alter significantly as pregnancy proceeds (Beck & Lloyd, 1968).

Many workers have demonstrated the ability of the visceral yolk sac endoderm cells to endocytose materials at their apical plasma membrane surface. Markers which have been used to study endocytosis by light microscopy include trypan blue (Goldman, 1909; Wislocki, 1921; Everett, 1935; Beck et al., 1967 b), iron derived from digested maternal red blood cells (Wislocki et al., 1946), colloidal gold (Luse, 1957), fluorescent-labelled antibodies (Mayersbach, 1959; Larsen & Davies, 1962; Wild, 1970), radio-labelled albumin (Anderson, 1959) and ^{58}Co-labelled vitamin B_{12} (Padykula et al., 1966; Deren et al., 1966). Beck et al. (1967 b) demonstrated the uptake of both horseradish peroxidase and trypan blue by these

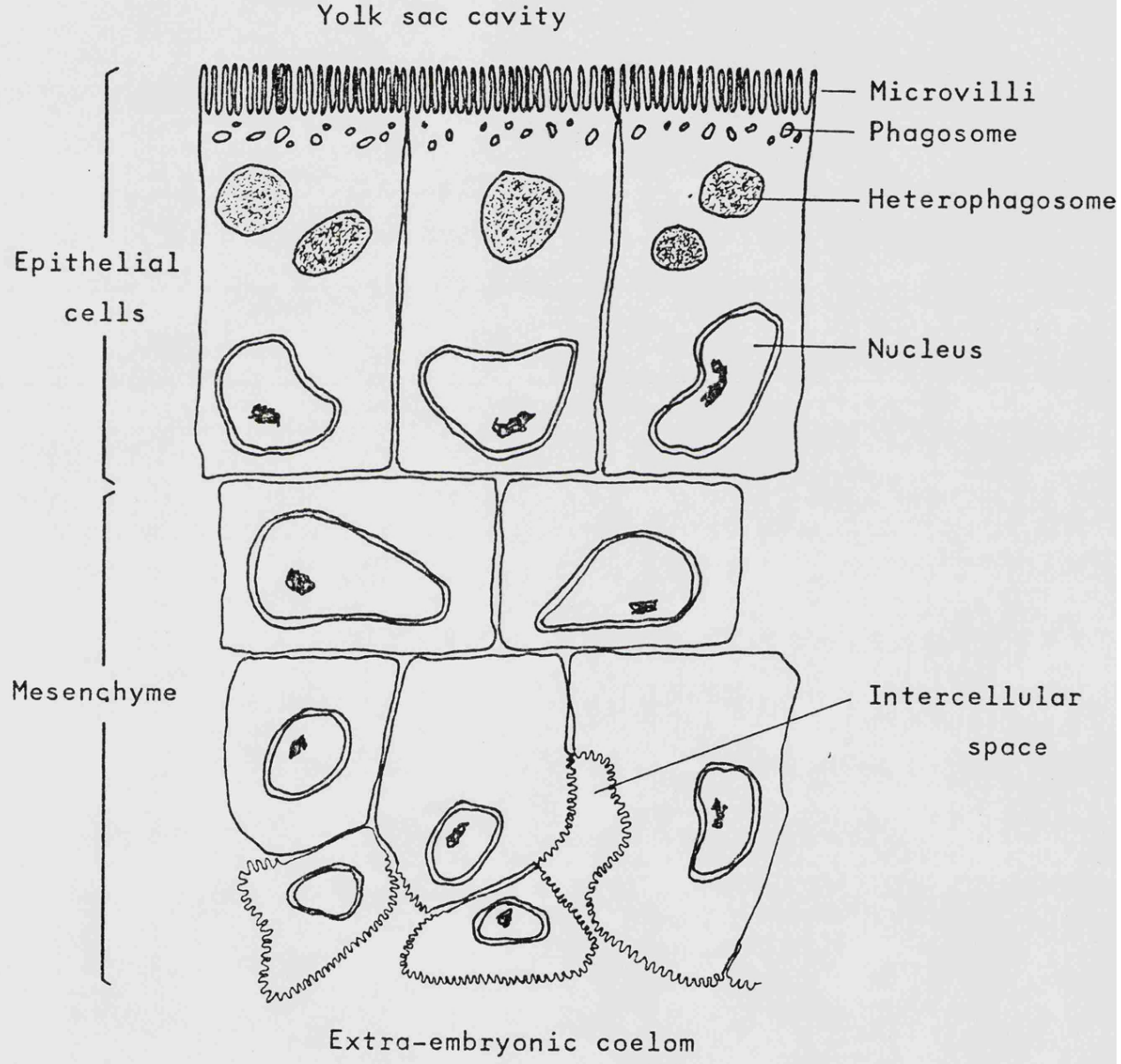


Figure 1

Diagram to show the arrangement of cell types in the 9½ day rat visceral yolk sac.

endoderm cells and found both markers localised in the same region of the cells.

The same phenomenon has also been studied by electron microscopy. Carpenter and Fern (1966) used thorotrast, Lambson (1966) and Slade (1969) used ferritin, Sharma and Peel (1979) used marker proteins - horseradish peroxidase - and rat serum proteins conjugated to fluorescein isothiocyanate; all found the markers distributed throughout the visceral yolk sac vacuolar system. Similar observations were made by Gupta et al. (1979; 1981) using cadmium-free ferritin and colloidal gold respectively. Krzyzowska-Gruca and Schiebler (1967) used ferritin and trypan blue and found both markers located in the same vesicles.

Considerable work has been done to show that the visceral yolk sac endoderm contains lysosomal enzymes (see below). The fusion of primary lysosomes with heterophagosomes provides the enzymes required for the digestion of endocytosed materials and this enables the visceral yolk sac to supply the embryo with simple molecules which can be used to build up its own macromolecules. Lysosomal enzymes which have been located in the visceral yolk sac by histochemical techniques include acid phosphatase (Wislocki et al., 1946; Johnson & Spinuzzi, 1966, 1968; Beck et al., 1967 b) and non-specific esterases (Johnson & Spinuzzi, 1966, 1968). Electron microscopical examination shows that acid phosphatase is localised in the primary lysosomes and the heterolysosomes but not in the endocytic vesicles or the canalicular system (Beck et al., 1967 b, 1971; Beck & Lloyd, 1968). The latter authors studied the location of maternally injected horseradish peroxidase in visceral yolk sac epithelial cells and, using light microscopy techniques, they demonstrated both this and acid phosphatase in the region close to the nucleus (i.e. heterolysosome region); however only horseradish peroxidase was evident near the cell apex (presumably composed primarily of the canalicular system and endocytic vesicles). This suggests that material endocytosed by the

visceral yolk sac endoderm cells is, in fact, digested by lysosomal enzymes within the endoderm cells.

Biochemical investigations confirm the histological findings. Lloyd et al. (1968) fractionated the homogenate of rat visceral yolk sac and found most of the acid phosphatase activity of the tissue was in the lysosomal fraction. Several workers have shown that tissues active in endocytosis contain lysosomes and can digest the material taken up (Ryser et al., 1962 b; Ryser, 1963; Ehrenreich & Cohn, 1967, 1968; Steinman & Cohn, 1972).

Fridhandler and Zipper (1964) were the first to show that the visceral yolk sac could digest material. In an <u>in vitro</u> study they measured the uptake of C^{14}-labelled rat neonatal haemoglobin and the release of C^{14}-labelled TCA-soluble digestion products. Schultz (1966) injected invertase into pregnant rats on days 11.5 and 12.5 of pregnancy and found much higher levels of invertasein the visceral yolk sac than in the embryo. These results imply that the invertase was being digested in the visceral yolk sac. Similarly, Beck et al. (1967 b) found that when horseradish peroxidase was injected into a pregnant rat the level of the marker enzyme in the visceral yolk sac rose to a peak following the injection but at no time did the embryo contain detectable amounts of enzyme. In an <u>in vitro</u> study Beck et al. (1968) cultured yolk sacs from rats which had been injected six hours previously with horseradish peroxidase and found that the level of the enzyme in the tissue decreased with time but that levels of peroxidase in the medium did not increase correspondingly. These observations imply that the yolk sac was breaking down the enzyme.

Williams et al. (1975 b) showed that ^{125}I-iodine labelled bovine serum albumin in culture medium was pinocytosed and intracellularly digested. The radio label was returned to the culture medium predominantly

as ^{125}I-iodotyrosine.

However, additional functions for the visceral yolk sac have been proposed. Brambell and co-workers (see Brambell (1970) for review) have shown that, in the rabbit, the visceral yolk sac is the main route of entry for antibodies to the fetus. In the rat, however, this route is less important and further entry is effected via the gut in neonatal life (Brambell & Halliday, 1956). It has also been shown that the yolk sac endoderm possesses mechanisms for the specific uptake and transport of maternal macromolecules by means of coated vesicles. These small organelles range in size from 50 to 200 nm diameter (Moxon, Wild & Slade, 1976) and appear to form at the apical surface. They are also found deep within the cytoplasm of the cell and fused with the basolateral surface on the embryonic side of the tight junction. Several authors have demonstrated the specific uptake of maternal macromolecules by coated vesicles - e.g. yolk proteins (Roth & Porter 1964); maternal IgG (Rodewald, 1973; Moxon & Wild, 1976; Wild, 1979; Liliberte et al., 1981; Huxham & Beck, 1981) and transferrin (Booth & Wilson, 1981). In each case the uptake has been directly linked to coated pits and vesicles. However, whilst these are common means of ligand entry into cells the eventual destination of these ligands varies. In many cell types much of the protein internalised into coated vesicles is degraded by the lysosomal system; but some so internalised somehow avoids fusion with the lysosomal system and effects transport of macromolecules across the endoderm (Huxham, 1982).

The yolk sac is also known to synthesise proteins including albumin, pre-albumin, alphafetoprotein, transferrin and embryo specific alpha-globulin in the chicken (Gitlin & Kitzes, 1967), in the human (Gitlin & Percelli, 1970), in mice (Janzen et al., 1982) and rats (Clark et al., 1982; Huxham, 1982). Other evidence as to the important nutritional role of the visceral yolk sac during organogenesis is provided by the production of growth-retarded and abnormal embryos resulting from cultures in media

containing anti-rat yolk sac antibody (New & Bernt, 1972; Freeman et al. 1982) and anti-rat placental IgG (Goetze et al., 1975).

Finally, Freeman et al. (1981) have described experiments that provide direct evidence that 9.5 to 11.5 day rat embryos obtain amino acids directly from proteins that are digested within the visceral yolk sac.

Evolution of culture techniques

For nearly a century now the ability to grow embryos in vitro has been continuously improved and the normal growth of an embryo can presently be studied in greater detail without having to overcome the additional problems of probing the mother. Since mammalian embryo culture eliminates the effects of variable maternal physiology highly controlled changes in the environment around the embryo can be made in order to study the influence of these changes on embryonic development (see Kochlar, 1975; Steele, 1975; New, 1978 and Beck, 1982, for further discussion on the application of embryo culture).

Although many attempts have been made to culture embryos during organogenesis from several species of experimental animals, the culture of mammalian embryos has only been successful in the last few years. In earlier attempts Waddington and Waterman (1933) cultured rabbit blastodics on plasma clots. However, success was very limited in that most embryos died but some did reach the 9-somite stage. This static watchglass type of culture proved to be very restricting to embryonic growth.

In 1938 Jolly and Lieure explanted rat embryos at a stage between the primitive streak and an embryo of a few somites with their visceral yolk sac intact. After culture in static homologous serum, using the watchglass technique, 37% of the embryos formed rhythmic heart beats and 9% developed a functioning vitelline circulation. Nicholas and Rudnick (1934, 1938) obtained similar results after culture of rat embryos in

heparinised rat plasma and embryo extract.

Attempts at mammalian embryo culture continued until the early 1960s with very little improvement (see reviews by New, 1973, 1978; Steele, 1975) but, in the last fifteen years, New and his co-workers at Cambridge have done much to improve these culture techniques. Initially they attempted culturing rat and mouse embryos at early somite stages on plasma clots on watchglasses, using variations in carbon dioxide and oxygen concentrations (New & Stein, 1964). Eventually the watchglass technique was used under favourable gas conditions, with homologous rat serum rather than plasma clots as a culture medium (New, 1966 b) - the latter giving reasonable results for 10 day old embryos.

The use of liquid media was thought to be more desirable because it is simpler to prepare and provides the mechanical support required for the enlarged yolk sac in addition to being more convenient for chemical analysis. Most in vitro studies of rat fetuses have depended on homologous serum as a culture medium, usually with the addition of antibiotics. Heterologous sera were found to support inferior growth and differentiation (New, 1966 b). New (1966 b, 1967) attempted to improve embryonic growth by increasing the amount of serum per embryo and by the renewal of serum during the culture period but the results, at best, showed only very slight improvements. In 1967 he designed a circulator system developed from an earlier model by Nicholas (1938). This apparatus allowed for the circulation and oxygenation of the medium to be maintained by a stream of bubbles injected from a cylinder containing a suitable gas mixture whilst the embryos remain stationary, thus allowing continuous observation of their development. The embryos may be anchored by means of the Reichert's membrane to a small strip of collagen-coated gauze attached to a piece of glass or stainless steel wire in the embryonic chamber (for details see New, 1971). Rat embryos can be cultured in this apparatus at any stage

for any two day period between the early egg-cylinder ($7\frac{1}{2}$ day) and 50 - 55 somites ($13\frac{1}{2}$ day) points. The 'circulator' technique allowed for more satisfactory development of embryos at all stages than did the watchglass cultures. Later New et al. (1973) devised a simple culture method involving rotating tubes and bottles. By this method the culture chambers are small cylindrical glass tubes or bottles in which part of the available volume is filled with liquid nutrient medium and the remainder with the required gas mixture. The bottles are laid horizontally on rollers, or attached to a rotating disc (Kochlar, 1975; Deuchar, 1976), and rotated at 30 - 60 rev/minute during incubation at 37^{o}C. This promotes oxygenation and assists respiration by keeping the explants gently swirling in the medium. The vessels can be gassed initially and at intervals during the culture or supplied with a continuous flow of gas throughout the culture period (New & Cockcroft, 1979).

In time certain improvements were made to the rat serum in its use as a culture medium. Steele (1972) established that the method of preparation of serum was critical to the development of the rat egg-cylinders in culture. For example, as had previously been observed by New and Daniel (1969), he showed that if blood was allowed to clot and permitted to stand overnight prior to centrifugation and separation the heart primordia of embryos grown in this medium usually failed to fuse and development was subsequently retarded. Conversely, if the blood was centrifuged immediately after collection the embryos grown in this serum had a single heart and development was greatly improved. Steele and New (1974) thus showed that head fold stage rat embryos develop better in immediately centrifuged (i.c.) serum than in delayed centrifuged (d.c.) serum. The harmful properties of d.c. serum are probably the products of normal blood clotting rather than the sodium potassium (Steele, 1972) or calcium (Steele & New, 1974) ion concentrations of the medium. Klein et al. (1978) have demonstrated by gel electrophoresis that a protein difference between the two sera could

be related to the growth supporting capacity. Fresh d.c. serum differs from fresh i.c. serum by the reduced concentration of this same protein and by the absence of two others with molecular weights higher than 2000 k daltons.

Heat treatment (heat-inactivation) is a standard method for inactivating complement in serum and it has been shown that both d.c. and i.c. rat sera contain similar amounts of complement which is almost entirely destroyed by heating (Steele & New, 1974). Heat inactivation of rat serum at 56^{o}C for 30 minutes improves its capacity to support embryonic growth (Steele & New, 1974; New at al., 1976 a). In addition, total protein synthesis by the embryo is increased and the frequency of abnormalities of heart, neural tube, allantois and shape are reduced. The beneficial effects of pre-heating are independent of those of immediate centrifugation. Maximum growth of the embryos is, therefore, obtained when the serum undergoes both treatments (i.e. heat-inactivation and immediate centrifugation). The effects on embryonic growth of serum heat-inactivation may be concerned more with nutrition than antigenicity. Any of the enzymes or other proteins in the serum could be altered by the heat treatment and the possibility has not yet been excluded that the effects seen in culture result from changes other than, or in addition to, the inactivation of complement.

Gas mixture in the culture chamber must serve to meet the oxygen demand of the embryo and control the pH of the serum; it is necessary to include 5% CO_2 in the atmosphere to control the pH of the medium - the remainder of the gas mixture is O_2 and N_2. Several factors must be considered regarding the percentage of oxygen in the mixture since this plays a central role in the metabolic processes associated with utilization of nutrients for cellular function and the availability of an adequate supply in tissues promotes energy metabolism and enhances cell division. The glycolytic pathway of energy metabolism is functional in rat embryos and the need for

O_2 progressively increases with further differentiation and growth (Shepard et al., 1970; Tanimura & Shepard, 1970; Neubert et al. 1971 a, b). The increasing O_2 concentration required as a consequence of growth is accompanied by a well-documented shift in the metabolic pathways used by the embryo, taking place at about 10½ days. By various means it has been shown that an early glycolysis-dominated metabolism later gives way to an energy metabolism dominated by the pentose shunt and then the Kreb's cycle and electron transport (Shepard et al., 1970).

There is also a shift from anaerobic to aerobic respiration and that this coincides roughly with the beginning of chorio-allantoic placenta function suggests that the early embryonic environment *in vivo* is too anaerobic to allow the more efficient aerobic respiration of Kreb's cycle. The slow movement of blood around the conceptuses *in vivo* (Merker & Villegas, 1970) tends to support this conclusion but, whether or not this is the reason, *in vitro* - where the allantoic placenta does not function - the O_2 concentration can usefully be raised from 20% to 40% around the time when the placenta would function *in vivo* (New et al. 1976 b; Sanyal, 1979).

The high concentration of oxygen necessary for growth from 10½ to 11½ days is harmful to the 9½ day egg cylinder and 5% O_2 is all that is required from 9½ to 10½ days (New et al. 1976 a). Beyond the 12th day progressively higher concentrations are needed and eventually hyperbaric oxygen (up to 2 atmospheres) is required (New et al. 1970 a, b). These workers (1976 a) effected further improvement in the development of head fold rat embryos in culture by culturing the 9½ day rat embryos in heat-inactivated homologous i.c. serum using the roller bottle system and by gassing them initially with 5% O_2, 5% CO_2 and 90% N_2, then 20% O_2, 5% CO_2 and 75% N_2 after 24 hours. Eventually New et al. (1976 b) achieved comparable growth and differentiation of rat embryos cultured between 9½ and 11½ days' gestation to that of litter mates left to grow *in vivo* by using the above

method plus an additional gassing with 40% O_2, 5% CO_2 and 55% N_2 after 32 hours' culture. The method is described in detail in Chapter II.

Since the primary aim of this work is a study of embryotrophins it is necessary to summarise relevant work carried out with different culture media.

It has been shown that homologous i.c. heat-inactivated serum is the medium in which 9½day old rat embryos can grow for 48 hours equally as well as litter mates left to grow in vivo, and also that development of somite stage rat embryos was normal in autologous serum, serum from other pregnant female rats, from non-pregnant females, from males or from rats of different strains (New, 1966 b; 1967). However, Buckley et al. (1978) have shown that minor anomalies of axial rotation may be somewhat more frequent in embryos that have been exposed at the egg cylinder stage to male serum as compared with those exposed to autologous serum. In contrast, heterologous sera are often harmful. Rabbit serum, for example, killed rat embryos within 2 hours (New, 1966 b); it became less harmful if pre-heated to 56°C but remained inferior to homologous serum as a culture medium. Commercially prepared samples of calf, horse and sheep sera were also very inferior to rat serum but Tanimura and Shepard (1970) found human serum to be an acceptable culture medium and Chatot et al. (1980) reported the successful culture of rat embryos on human serum supplemented with glucose. Recently Reti et al. (1982) showed that rat embryos cultured in 100% human serum are not as well developed as embryos cultured in whole rat serum and only supplementation of the human serum with 10% rat serum resulted in the restoration of the nutritional status of the human serum. Indeed, with this supplement embryos in fact are slightly superior after culture to those grown in pure rat serum.

No chemically defined medium which can substitute for whole serum has yet been reported. Some of the standard tissue culture media have been

tested for their capacity to support embryonic growth but the results have been very poor. It seems essential that at least part of the culture medium should be serum. In mixtures of serum and chemically defined media (Weymouth's medium, M 199) differentiation of embryos may proceed as far as in whole serum but protein synthesis is usually significantly reduced (New, 1973). However, other rodent embryos grown under different conditions may benefit from dilution of the serum. Givelber and Dipaolo (1968) found whole homologous serum (unheated) unsatisfactory for hamster embryos and obtained better results by diluting it with McCoy's medium. Clarkson et al. (1969) showed that mouse embryos developed better in Weymouth's medium with 20% rat serum than in whole or 10% serum. Cockroft (1973; 1974) cultured rat fetuses of $12\frac{1}{2}$ and $13\frac{1}{2}$ days' gestation with the yolk sac and amnion opened and obtained better development in Tyrode's solution plus 25% rat serum than in whole serum. Daniels (1971) recommends the dilution of rabbit serum for growing rabbit embryos from the primitive streak stage onwards.

In 1976 Gunberg initiated a more systematic approach to the variation of some components in serum individually. He dialysed rat serum against balanced salt solution to give a medium consisting only of the macromolecular components of the serum with a few salts of sodium, potassium, calcium and megnesium. Various possible energy-yielding compounds were then added to this medium for testing and it was found that $10\frac{1}{2}$ day rat embryos cultured in the dialysed serum alone failed to thrive but, when glucose was added at a concentration of 0.9 mg/ml, development was enhanced. When mannose, fructose or pyruvate were substituted for glucose development was poor.

Later, in 1979, Cockroft cultured $9\frac{1}{2}$ day old embryos for 48 hours in rat serum extensively dialysed against balanced salt solution. He achieved optimum growth and differentiation virtually identical with that obtained in whole serum with the addition of glucose and certain vitamins - panto-

thenic acid, riboflavin, i-inositol and folic acid.

Klein et al. (1978) cultured more than one embryo per ml/serum in an attempt to identify the proteins taken up by rat embryos; gel electrophoresis of the used serum showed a protein with a molecular weight of 125 k daltons which was depleted from it.

CHAPTER TWO

CHAPTER II

GENERAL METHODOLOGY

A. CHOICE OF ANIMALS

Commercially obtained Wistar rats (supplied by Anglia Laboratory Animals) were used and groups of 6-10 rodents per cage were housed in light tight rooms at 21°C. They were given 14 hours of artificial light and 10 hours of darkness per day and fed on balanced rat chow and water *ad libidum*.

B. MATING OF ANIMALS AND THE TIMING OF PREGNANCY

Females were introduced into single mating cages during the afternoon (at 16.00 hours) and were checked the following morning for the presence of vaginal plugs. Rats were considered to be pregnant on the day a vaginal plug was found, the embryo therefore being regarded as half a day old at 12 noon.

C. PREPARATION OF SERUM FOR CULTURE

The culture serum was obtained from Wistar rats (without regard to sex or age) by the following method: after being anaesthetised with diethyl-ether each rat was laid on the bench and a small beaker containing a pad of ether-soaked cotton wool was placed over its head to maintain the condition. Care was taken not to administer a terminal overdose before the blood was aspirated. The abdominal wall was cleansed by 70% alcohol and a midline longitudinal incision was made, the intestines

being displaced to the left. In order to expose the dorsal aorta, a mid-line opening in the peritoneum covering the posterior body wall was made. Blood was withdrawn from the aorta exposed at its point of bifurcation with the iliac vessels using a sterile 10 ml. syringe and a 19G needle. Immediately following collection, the blood was centrifuged (before clotting could occur) at 1000 x G for 10 minutes to precipitate the blood cells and allowed to establish a fibrin clot in the supernatant plasma.

After allowing half an hour for complete formation, the clot was squeezed to remove the serum it contained and the blood recentrifuged (1000 x G for 10 minutes) (Steel, 1972; Steel & New, 1974). Clear serum (about half the volume of the original blood) from 20-60 rats was pooled, antibiotics in the form of Penicillin (100 units/ml of serum) and Streptomycin (100 mg/ml of serum) were added and the serum was stored at $-20^{o}C$ until required. The addition of antibiotics aimed to prevent the growth of bacteria in this highly nutritive medium.

Before being used as a culture medium the serum was always heat-inactivated by placing it in a water-bath at $56^{o}C$ for 30 minutes (New et al., 1976a).

D. CULTURE METHOD

Pregnant rats were sacrificed by diethyl-ether overdose on day 10 or 11 and the ventral body wall was cleansed with 70% alcohol. Using clean instruments a longitudinal cut, extending from the perineum to the xyphoid process and two transverse cuts medially on either side exposing the entire abdominal cavity were made. At this stage of pregnancy embryos were implanted in the uterine wall and each had become completely surrounded by proliferating decidual tissue which blocked the uterine lumen and stretched the uterine muscle. A transverse cut below the bifurcation of the uterus separated the uterus from the mesentary and it was washed

several times in Hanks' solution before it was transferred to a sterile petri-dish again containing Hanks' solution. If an acid reaction was obtained, (indicated by the liquid turning from orange to yellow) then the uterus or conceptuses were transferred to fresh dishes of Hanks' solution. From this stage onwards sterile precautions were taken throughout and the remainder of the procedure was carried out under a dissecting microscope. With the aid of two pairs of watchmaker's forceps the decidual masses containing the conceptuses were exposed by opening the anti-mesometrial side of the uterus.

The pear-shaped decidual masses were dissected out, care being taken not to squeeze or puncture the conceptuses, and the decidua opened. The blunt (non-embryonic) end was pierced and held and with the outer forceps a meridional cut was made between the arms of the first forceps. The decidua opened into two halves, one of which contained the conceptus. (Figures 1 and 2). This part was halved again by another meridional cut, leaving the conceptus attached to one quarter of the decidua from which it could be easily detached.

After removal each conceptus was transferred to a fresh dish of Hanks' solution using a sterile Pasteur pipette. The Reichert's membrane was opened, this being best seen at the pole opposite the ectoplacental cone where it stood clear of the embryo. It was grasped at this point with sharp watchmaker's forceps and opened to the level of the ectoplacental cone (New & Stein, 1964).

The 9.5 or 10.5 day conceptuses (Figures 3 and 4) were transferred to cylindrical 30 or 60 ml capacity culture bottles - usually 3 conceptuses in the smaller and 6 in the larger capacity bottles. The volume of culture medium used was 1 ml per conceptus and the bottles were incubated for 48 hours in a roller incubator (New, Coppola & Terry, 1973) (Figure 5). The bottles were gassed initially with 5% O_2, 5% CO_2 and 90% N_2,

Figure 1

Half of the decidual mass at 9½ days of gestation, bearing the conceptus. (Mag x 20).

Figure 2

Half of the decidual mass at 10½ days of gestation bearing the conceptus. (Mag x 12.5).

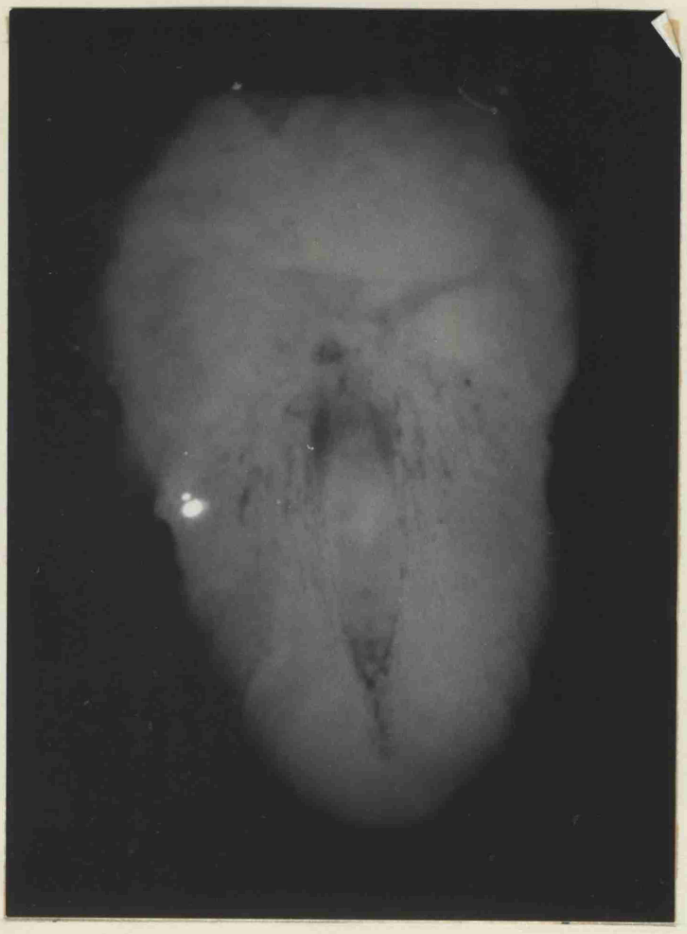


Figure 1.

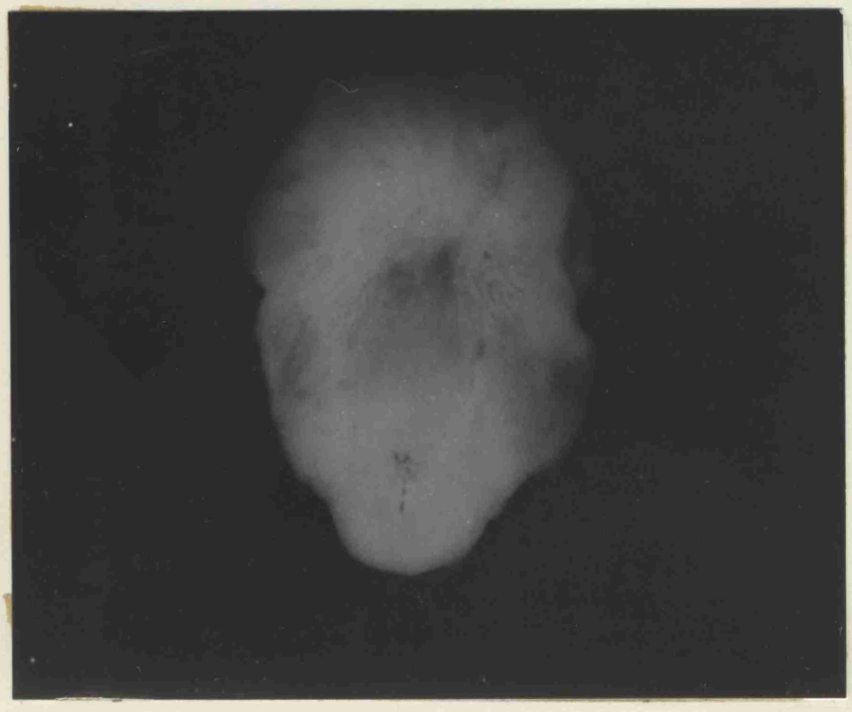


Figure 2.

Figure 3

A rat conceptus at 9½ days with the Reichert's membrane removed. (Mag x 36).

Figure 4

A rat conceptus at 10½ days with the Reichert's membrane removed. (Mag x 25).

Figure 5

Rollar incubator used for culture of rat conceptuses.

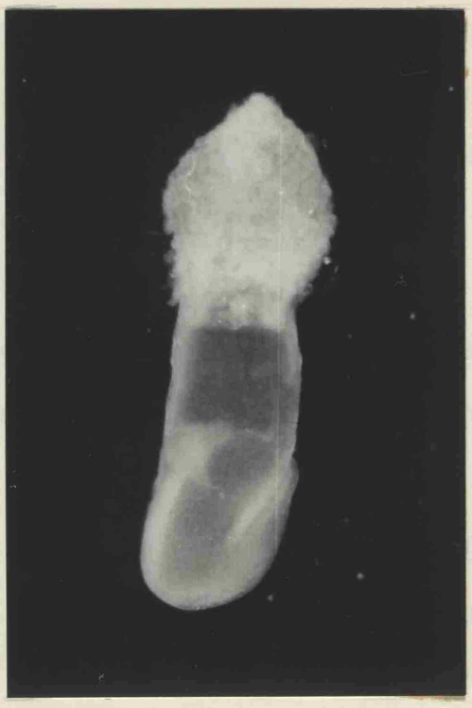


Figure 3.

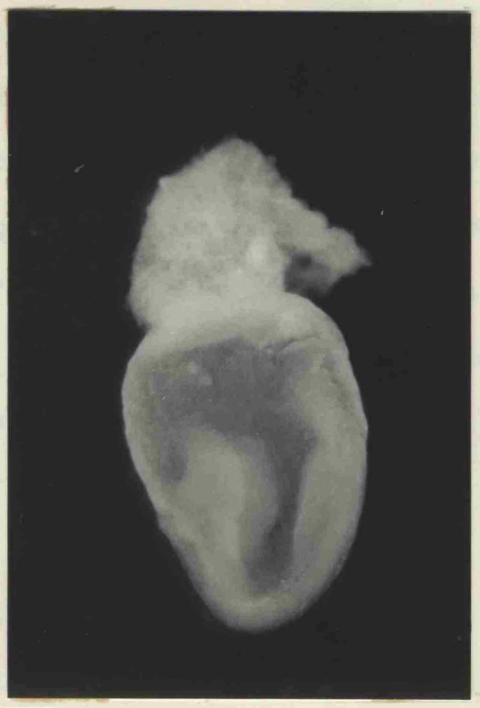


Figure 4.

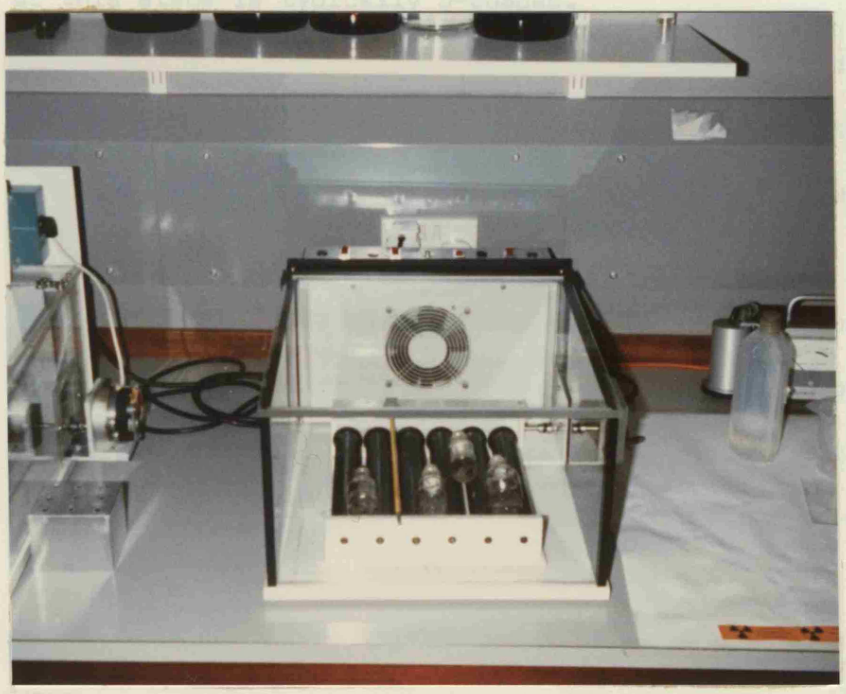


Figure 5.

followed by 20% O_2, 5% CO_2 and 75% N_2 and 40% O_2, 5% CO_2 and 55% N_2 at 22-24 hours and 38 hours respectively (New, Coppola & Cockroft, 1976a). The bottles containing embryos explanted at 10.5 days were incubated for 24 hours; and were gassed initially with 20% O_2, 5% CO_2 and 75% N_2 followed by 40% O_2, 5% CO_2 and 55% N_2 after 18 hours. Gassing was carried out by means of a steady flow from the tip of a glass pipette held over the surface of the serum in the culture bottles for 1.5 minute periods. At the end of the culture period the conceptuses were removed from their containers using a large bore pipette for immediate examination.

E. ASSESSMENT OF EMBRYONIC GROWTH

At the end of the culture period 11.5 day-old conceptuses (Figure 6) were examined, fresh and without fixation, and the following parameters were determined using a dissecting microscope:

1. The presence of heart beats, as seen through the visceral yolk sac: Only those embryos with vigorous, rhythmic heart movements were counted as having a heart beat. The heart at this stage is typically S-shaped.
2. The presence of a vitelline circulation: Only those conceptuses showing vigorous movement of blood cells within the yolk sac capillary system were considered to show normal characteristics.
3. Yolk sac diameter: The widest diameter of the yolk sac was measured using a graduated micrometer in the microscope.
4. Fused allantois: This was examined after opening of the yolk sac. The allantois should extend from the hind end of the embryo and be fused with the chorion.
5. Turning of the embryo: By 11.5 days the embryo should have completed its rotation to the fetal position with the ventral surface concave, with its caudal end on the

right side of the head (Figure 7).

6. Closure of the neural tube: The neural tube should be closed along its entire length. This includes the anterior and posterior neuropores and the rhombencephalic roof.

7. The presence of normal optic vesicles: These should be well developed on both sides of the head.

8. The presence of normal otic vesicles: These should appear round on both sides of the rhombencephalon.

9. The presence of fore limb buds: These should appear as prominent, lateral thickenings on both sides of the embryo at the same level at, or just below, the level of the heart.

10. Somites: The number of somite pairs was counted and their shape checked.

11. Crown/rump length: This was measured using an eyepiece graticule from the highest point of the head to the rump (extremely retarded embryos were not included in this measurement since crown and rump was ill-defined).

    N.B: At this stage in the assessment, embryos are either fixed for further examination and photography or washed in Hanks' solution and stored at $-20^{o}C$ for the subsequent measurement of protein content.

12. Protein content of the embryo: This was achieved using a modification of the original method of Lowry et al (1951) outlined in Appendix A.

Figure 6

Rat conceptus at 11½ days. The embryo is clearly visible through the visceral yolk sac. The visceral yolk sac bears an extensive network of blood vessels. (Mag x 20).

Figure 7

Lateral view of an 11½ day old normal rat embryo grown *in vitro*. (Mag x 22.5).

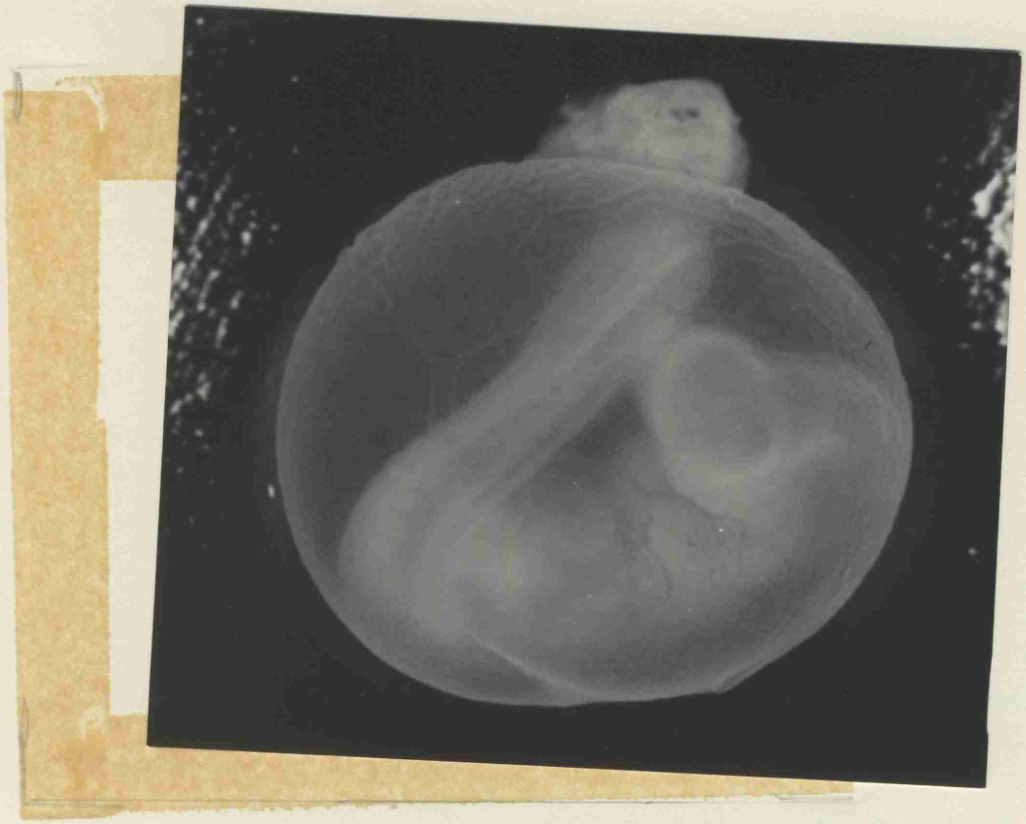


Figure 6.

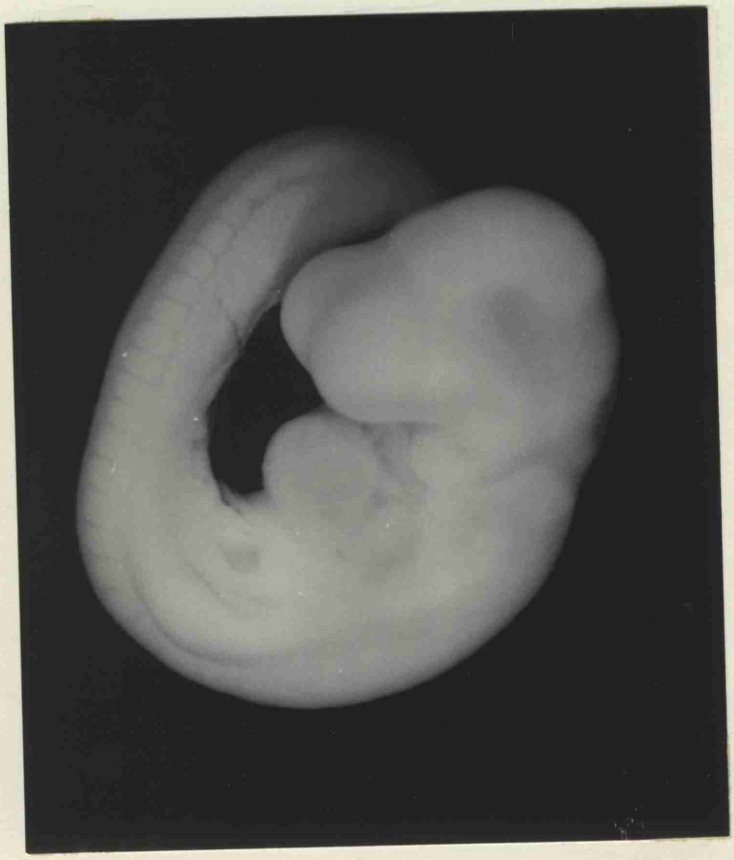


Figure 7.

CHAPTER THREE

CHAPTER III

SECTION I

SERUM EXHAUSTION

INTRODUCTION

Improvements in culture techniques have made it possible to achieve growth and differentiation of the pre-somite head-fold stage rat embryo *in vitro*, at a rate almost identical to that seen *in vivo* over a 48-hour period (New et al., 1976b).

Growth of embryos has been possible in media containing whole rat serum, although some dilution with a simple balanced salt solution is feasible without adverse effects (Cockroft & Coppola, 1977).

To exhaust components of the serum Klein et al (1978) cultured either four or nine embryos per 2 mls. of serum. They found that growth and development of embryos cultured four per 2 mls of serum was considerably greater than those cultured as nine embryos per 2 mls of serum.

In this section the nutrient requirements of rat embryos *in vitro* during a 24-hour period for the 6-9 somites stage to the 24-26 somites stage were investigated. In order to achieve exhausion, serum was repeatedly used for culture of embryos between days 10.5 and 11.5, using one embryo per one ml of serum. The serum was considered exhausted when all embryos cultured in it showed no significant signs of growth.

Progressive changes of glucose level, pH and osmolarity in the

serum were also recorded. Factors other than these are investigated in the subsequent two sections.

MATERIALS AND METHODS

Ten and a half days old conceptuses were obtained from pregnant Wistar rats at noon of day 11 of gestation (see Chapter II, D). Immediately centrifuged, pooled serum was collected from 20-30 rats (see Chapter II, C) and was heat-inactivated at 56^{o}C for 30 minutes prior to use. The conceptuses were cultured one per ml of serum.

In some experiments described in this chapter two types of references were used as controls. First, to control for growth and differentiation of conceptuses cultured in experimental media, embryos from the same litter were cultured in fresh, heat-inactivated (H.I.) serum. The second reference was to control for the changes that might have taken place in the serum during the processes of exhaustion. For this fresh H.I. serum was treated identically to the exhausted serum in terms of repeated gassing, incubation, freezing and thawing except that no embryos were cultured in it - this was called control serum.

A. In order to exhaust the nutrient materials in serum, conceptuses were cultured repeatedly in the same sample of serum by the method described in (Chapter II, D). Samples of fresh, heat-inactivated serum from the same pool were used as control serum. After four incubations without embryos control serum was tested for its suitability to support embryonic growth. At the end of the 24-hour incubation period conceptuses were transferred from the serum and assessed for the degree of growth and development (see Chapter II, E). The used serum was then transferred to a sterile container and stored at -20^{o}C until required for another culture. In the initial serum exhaustion experiment the following possible changes which could have occurred in serum as a result of repeated gassing, incubation, freezing and thawing were monitored after each culture. The following values obtained were then compared to those of fresh serum:

1. Osmolarity (see Appendix B);
2. pH. This was estimated immediately after the serum was used.

3. Glucose level. This was carried out using the Boehringer glucose oxidase colourimetric standard assay kit (Boehringer Mannheim GmbH Diagnostica).

B. In an attempt to restore the growth supporting properties of exhausted serum, 1.5 mg/ml D-glucose and 10 ul/ml Eagle's Minimum Essential Medium (MEM) Vitamin concentrate (Flow Laboratories (See Appendix C)) were added, the osmolarity was corrected to 300 milliosmoles/kgH_2O with sterile distilled water. Two controls were run - fresh H.I. serum and fresh H.I. serum which was incubated, gassed, frozen and thawed five times.

Ten and a half days old conceptuses were cultured in the above media for 24 hours as before and, at the end of this time, the embryos were examined and assessed for the degree of growth and development as in Chapter II, E.

RESULTS

A. GROWTH OF EMBRYOS IN REPEATEDLY USED SERUM

Embryos cultured in serum used for the first time (fresh H.I. serum) were all normal, exhibiting features similar to those of the 11.5 day old *in vivo* embryo (Table 1).

Embryos cultured in serum used once differed only slightly in all criteria to those embryos cultured in fresh H.I. serum. Although 94% of the embryos showed morphological features similar to those of the first group, the mean values for yolk sac diameter, somite numbers, crown-rump length were all significantly reduced ($p < 0.002$, Student's 't' test) with the exception of the protein content ($p < 0.01$, Student's 't' test) (Table 1 and Figure 1). Further use of the serum resulted in a drastic decrease in its ability to support embryonic gorwth. Only 8% of embryos cultured in serum used twice grew normally. A common defect was non-closure of the neural tube at the rhombencephalon. Only 17% of the embryos were completely turned (i.e. of normal body form), the remainder were posteriorly convex. The mean somite number was reduced by an average of seven when compared to that of embryos cultured in fresh H.I. serum, the mean crown-rump length was reduced by 1.5 mm and protein content was reduced by 68% when compared to those cultured in fresh H.I. serum.

Embryos cultured in serum used three times showed no signs of growth (Table 1 and Figure 1). All embryos were anteriorly convex and the neural tube was open in the cranial and caudal areas.

pH Changes

The study of pH values of control serum showed a decline in these after each incubation (Table 2), that in the experimental serum being more marked. The pH values of serum in which embryos were cultured three and four times were significantly less than the pH values from serum which was

repeatedly frozen and thawed ($p < 0.05$ Student's 't' test, Table 2).

Changes in Osmolarity

The osmolarity increased with each incubation in both experimental and control sera. The increase was significant at $p < 0.02$, $p < 0.001$ and $p < 0.05$, $p < 0.002$ and $p < 0.001$ respectively using Student's 't' test (Table 3). Comparison of the osmolarities of experimental and control sera after each incubation showed no significant difference using the same test as above (Table 3).

Changes in Glucose Levels

The initial level of glucose in serum was 1.77 mg/ml. Table 4 shows the gradual depletion of glucose in experimental serum with each use. By comparison, glucose levels were found to be increased with every incubation in the serum repeatedly frozen and thawed (Table 4).

Validation of Serum Repeatedly Frozen and Thawed

The serum is treated in the same way as the exhausted serum (i.e. gassing, incubation, freezing and thawing) except that no conceptuses were cultured in it. In order to validate the capacity of the control serum to support embryo growth and differentiation - at the first attempt, conceptuses were cultured in control serum without the correction of osmolarity, and, in this, only 25% of the embryos showed normal morphological features (Table 5); at the second attempt, the osmolarity of the control serum was corrected to that of the fresh H.I. serum ($\simeq$ 300 millios/kg). In this serum all embryos grew normally and all parameters assessed were similar to those of embryos cultured in fresh H.I. serum with the exception of the yolk sac diameter which was significantly reduced ($p < 0.002$, using Student's 't' test, Table 5).

B.

Embryos cultured in both fresh H.I. serum and repeated frozen and

thawed serum with corrected osmolarity grew normally and the results are summarised in Table 6.

Conceptuses Cultured in Exhausted Serum Supplemented With Glucose and Vitamins

Examination of embryos cultured in this medium revealed that 69% had abnormal morphological features, 29% had normal morphological features but had significantly reduced values for yolk sac diameter, somite number, crown-rump length and protein content when compared to embryos cultured in fresh H.I. serum (Table 6). Turning was completed in 33% of the embryos but still the majority were posteriorly convex. Seventy one percent showed a neural tube defect which manifested as non-closure of the rhombencephalon and the posterior neuropore. Table 6 summarises the results.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in (mm) ± standard error	Mean Somite Number ± standard error	Mean Crown-Rump Length (mm) ± standard error	Mean Protein Value (ug/embryo) ± standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Fresh H.I. Serum	36	36 (100)	36 (100)	36 (100)	34 (94)	34 (94)	34 (94)	36 (100)	36 (100)	4.316 ±0.055	26.41 ±0.284	3.522 ±0.043	212.21 ± 5.7
Serum Used Once	36	36 (100)	33 (91)	36 (100)	34 (94)	35 (97)	34 (94)	36 (100)	36 (100)	3.988 ±0.066**	25.62 ±0.029**	3.3611 ±0.04**	193.77 ± 4.33***
Serum Used Twice	36	36 (100)	6 (17)	35 (97)	5 (14)	6 (17)	3 (8)	28 (78)	29 (80)	3.82 ±0.108*	19.3 ±0.87*	1.966 ±0.097*	68.011 ± 3.28*
Serum Used Three Times	36	24 (67)	0 -	22 (61)	0 -	0 -	0 -	22 (61)	24 (67)	3.038 ±0.14*	11.6 ±0.58*	1.68 ±0.041*	48.79 ± 2.64*

TABLE 1 Growth and development of 10.5 days rat embryos, cultured for 24 hours in fresh heat-inactivated serum, used once, serum used twice and serum used three times.

Figures in paranthesis denote percentage number of embryos in the group.

Using Student's 't' test;

* Significant at $p < 0.001$ when compared with fresh H.I. serum.
** Significant at $p < 0.002$ when compared with fresh H.I. serum.
*** Significant at $p < 0.01$ when compared with fresh H.I. serum.

Figure 1

Comparison of growth of embryos cultured in fresh serum and in serum used repeatedly.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Protein content.

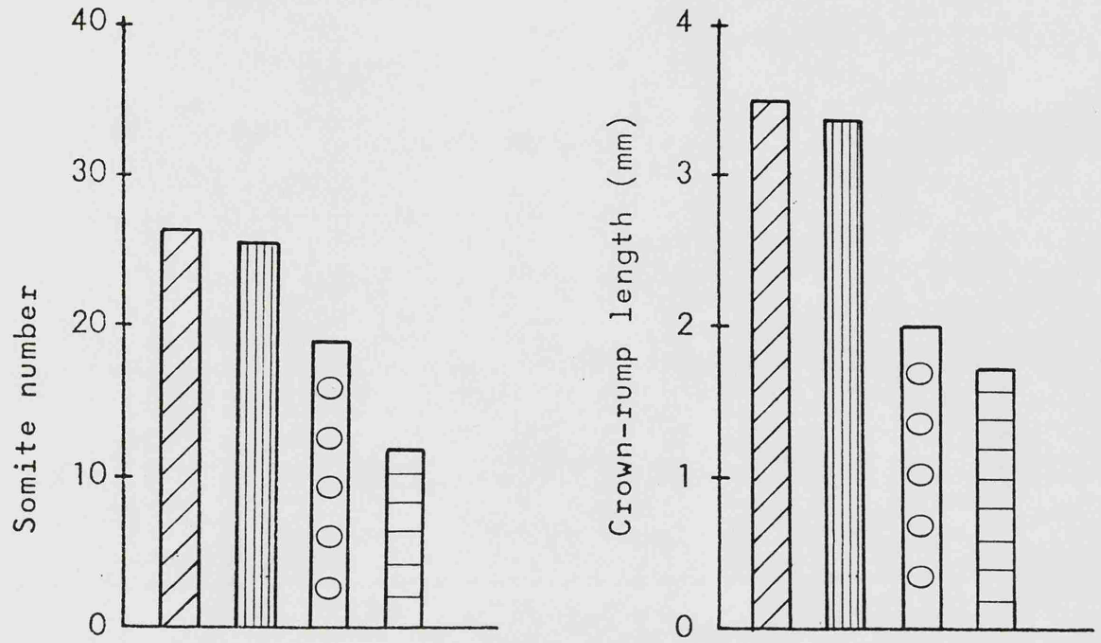
Each rectangle in the histograms represent the mean. For details of numbers of embryos and standard errors of means, see Table 1.


Fresh Heat-Inactivated Serum.


Serum Used Once.


Serum Used Twice.


Serum Used Three Times.


Somite number
40
30
20
10
0
A
Crown-rump length (mm)
4
3
2
1
0
B

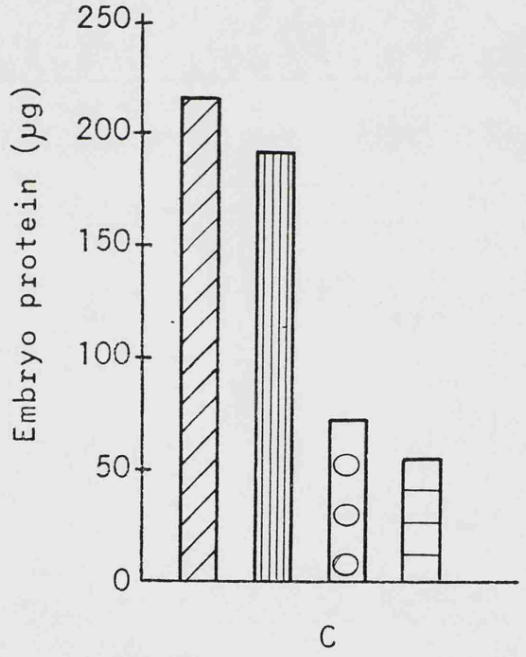

Embryo protein (µg)
250
200
150
100
50
0
C

TABLE 2

pH changes in serum used for repeated culture of embryos (experimental serum) and on serum incubated repeatedly without embryos prior to testing (control serum) measured immediately after culture.

	Experimental Serum pH	Control Serum pH
Fresh H.I. Serum	7.854 ±0.0638 n=12	7.887 ±0.0818 n=8
First Incubation	7.3166 ±0.0401 n=12 p <0.01	7.28 ±0.032 n=8 p <0.01
Second Incubation	7.1683 ±0.0476 n=12 p <0.01	7.287 ±0.054 n=8 p <0.01
Third Incubation	7.118* ±0.0426 n=8 p <0.01	7.275 ±0.0631 n=8 p <0.01
Fourth Incubation	6.973* ±0.061 n=8 p <0.001	7.16 ±0.0648 n=8 p <0.001

Using Student 't' test;

* significant at $p < 0.05$ when pH values of experimental serum were compared with corresponding control serum values.

TABLE 3

Osmolarity changes in serum used for repeated culture of embryos (experimental serum) and in serum incubated repeatedly without embryos prior to testing (control serum).

	Osmolarity millios/kg in Experimental Serum	Osmolarity millios/kg in Control Serum
Fresh H.I. Serum	295.833 ± 5.2655 n=12	297.5 ± 4.8419 n=8
First Incubation	315 ** ± 4.6548 n=12	309.375* ± 3.897 n=8
Second Incubation	327.333**** ± 3.628 n=12	321.875*** ± 3.897 n=8
Third Incubation	333.333**** ± 3.136 n=12	331.875**** ± 3.529 n=8
Fourth Incubation	354 **** ± 6.304 n=12	350.625**** ± 5.6215 n=8

The osmolarity of both experimental and control sera increased with each incubation.

Using Student 't' test;

* significant at $p < 0.05$ when compared with FHIS
** significant at $p < 0.02$ when compared with FHIS
*** significant at $p < 0.002$ when compared with FHIS
**** significant at $p < 0.001$ when compared with FHIS

Comparison of the osmolarities of experimental and control sera, after each incubation showed no significant difference.

TABLE 4

Glucose levels in serum used for repeated culture of embryos (experimental serum) and serum incubated repeatedly without embryos prior to testing (control serum).

	Glucose Level mg/ml in Experimental Serum	Glucose Level mg/ml in Control Serum
Fresh H.I. Serum	1.7752 ±0.0673 n=10	1.7752 ±0.0673 n=8
First Incubation	0.857 ±0.0956 n=10	1.864 ±0.0574 n=8
Second Incubation	0.263 ±0.0438 n=10	2.0037 ±0.0712 n=8
Third Incubation	0.10 ±0.0343 n=8	2.187 ±0.079 n=8
Fourth Incubation	0.06 ±0.02 n=8	2.415 ±0.0858 n=8

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in (mm) ± standard error	Mean Somite Number ± standard error	Mean Crown-Rump Length (mm) ± standard error	Mean Protein Value (ug/embryo) ± standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Fresh H.I. Serum	24	24 (100)	24 (100)	24 (100)	23 (96)	24 (100)	24 (100)	24 (100)	24 (100)	4.216 ±0.04	26.4 ±0.07	3.46 ±0.03	185.78 ± 6.33
Control Serum Osmolarity Corrected	18	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	4.09 ±0.036**	26.3 ±0.125	3.4 ±0.05	176.31 ±10.33
Control Serum Osmolarity Not Corrected	12	12 (100)	3 (25)	12 (100)	3 (25)	4 (33)	4 (33)	12 (100)	12 (100)	3.13 ±0.31**	21.6 ±0.5*	2.43 ±0.11*	122. ± 4.6*

TABLE 5 Growth and development of 10.5 days rat embryos cultured for 24 hours in fresh heat-inactivated serum and serum previously incubated without embryos four times (control serum) with and without correction of osmolarity.

Figures in parantheses denote the percentages number of embryos in each group.

Using Student 't' test;

* Significant at $p < 0.001$ when compared with the control group.
** Significant at $p < 0.002$ when compared with the control group.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in (mm) ± standard error	Mean Somite Number ± standard error	Mean Crown-Rump Length (mm) ± standard error	Mean Protein Value (ug/embryo) ± standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Fresh H.I. Serum	18	18 (100)	18 (100)	18 (100)	18 (100)	17 (94)	18 (100)	18 (100)	18 (100)	4.18 ±0.04	26.1 ±0.31	3.48 ±0.06	214.5 ± 5.33
Exhausted Serum + Glucose + Vit.	21	21 (100)	6 (29)	20 (95)	6 (29)	7 (33)	8 (38)	21 (100)	21 (100)	2.73 ±0.08*	21.5 ±0.22*	2.32 ±0.05*	94.3 ± 5.11*
Control Serum Osmolarity Corrected	18	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	4.1 ±0.06	26.36 ±0.43	3.53 ±0.06	208.6 ± 7.2

TABLE 6 Growth and development of 10.5 days rat embryos cultured for 24 hours in fresh H.I. serum, exhausted serum supplemented with glucose and vitamins and serum incubated repeatedly without embryos with its osmolarity corrected (control serum).

Figures in paranthesis denote the percentage number of embryos in the group.

Using Student's 't' test;

* Signficant at $p < 0.001$ when compared with control group.

DISCUSSION

The results suggested that repeated incubation, gassing, freezing and thawing of serum does affect the growth of rat embryos. This was attributed to the increase in osmolarity due to water evalporation from serum during incubation. However, the above serum restored its capability for supporting normal embryonic growth after correction of its osmolarity. In the present study the glucose level in the control serum increased after each incubation. This could also be due to water evaporation during incubation. Contrary to this there was rapid depletion of glucose by the embryos from the culture serum, this suggests that anaerobic glycolysis and the pentose-phosphate shunt are the major energy generating processes in these embryos (Shepard et al., 1969, 1970). Studies of the pathways of energy metabolism in rat embryos in vitro by Aksu et al. (1968), Shepard et al. (1969, 1970) and Tanimura and Shepard (1970) showed a very high rate of glucose consumption by the embryos and their membranes between days 10 and 11 in vitro and about 90% of the glucose consumed at this stage was found to be converted to lactic acid. Spielmann et al. (1973) found that 11-day embryos were more affected than 12-day embryos by an inhibitor of glycolysis. In rat embryos Tanimura and Shepard (1970) found the pentose-phosphate shunt to be most active at 10.5 days.

It can thus be concluded that glucose is an essential requirement for energy metabolism in embryos during early organogenesis (Gunberg, 1976; Cockroft, 1979; Sanyal & Wiebke, 1979: Sanyal, 1980). However, supplementation of exhausted serum with glucose and vitamins did bring about a little improvement in embryonic growth which suggested that abnormal growth in exhausted serum was not simply due to depletion of glucose.

Two possibilities could be put forward:

1. Accumulation of toxic material in the serum. This could be the result of accumulation of by-products from the metabolic activities of the growing embryos and/or their membranes. In this context Tanimura and Shepard (1970) found that 90% of the glucose utilized by the rat embryos and their membranes was converted to lactic acid. Sanyal (1980) found an increase in the level of urea, uric acid and creatinine in the serum used for culturing 10.5 days embryos for 24 hours, which he believed was due to metabolic processes of the embryo and other tissues of the conceptus.
2. Depletion of a nutrient component other than glucose from the used serum.

Although only a limited quantity of protein is processed by the cultured conceptuses relative to the large amount of protein present in the serum, it is possible that by repeated culture one or more of the specific serum macromolecules may become depleted. It has been shown that the visceral yolk sac endoderm of rat conceptuses in vitro transports specific serum macromolecules intact to the yolk sac fluid and, presumably, to the fetal circulation (Huxham & Beck, 1981). In addition to the transport of IgG shown by the above authors there are many other serum constituents which may be required by the embryo which could similarly be obtained by transport across the yolk sac. Such molecules could be any of the known protein growth factors present in the serum, for example Nerve Growth Factor (NGF), Multiplication Stimulating Activity (MSA), Cationic Growth Factors (CGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF). Although these growth factors differ physically and chemically they share a common biological function in that all of them (with the exception of NGF) stimulate DNA synthesis and protein synthesis in vitro. More specifically, FGF is known to stimulate carbohydrate, amino acid and nucleotide transport (Rudland

et al., 1974) and NGF is known to stimulate the transport of glucose (Liuzzi et al., 1968) and nucleotides (Angeletti et al., 1965). Clearly, in the absence of one or more of these growth factors from the exhausted serum, the biochemical processes of the rat embryos are likely to be seriously deficient. Experiments involving the addition of one or a combination of these growth factors to exhausted serum could demonstrate which of the factors are required to restore the suitability of the exhausted serum to support embryonic growth.

CHAPTER III

SECTION 2

A STUDY OF THE FACTORS IN SERUM AFFECTING EMBRYONIC GROWTH

INTRODUCTION

The supplementation of the exhausted serum with glucose and vitamins did not bring about an improvement in the nutritional suitabilities of the serum to support normal embryonic growth (see Chapter III, Section 1). Two possibilities were considered: a) accumulation of toxic materials and (b) deficiency of nutritional components. In this section the above possibilities were investigated further.

A. Accumulation of Toxic Material

To investigate this, exhausted serum was dialysed extensively against glucose-free balanced salt solution, using 24 Å pore size visking tubing. It was then supplemented with glucose and the eight vitamins present in MEM and subsequently tested for its suitability to support embryonic growth.

B. Deficiency of Nutritional Components

The possible depletion of one or more of the serum proteins by the growing embryos was considered. Samples of fresh heat-inactivated serum, serum which was repeatedly incubated without embryos (control serum)and exhausted dialysed serum used once (which had failed to support normal embryonic growth) were analysed by electrophoresis on gradient polyacrylamide gels.

MATERIALS AND METHODS

A pool of immediately centrifuged serum was prepared from 40 rats (see Chapter II, Section C). It was always heat-inactivated prior to use. An aliquot of pooled serum was stored at -20°C untreated.

A second aliquot was exhausted (see Chapter III, section 1) and a sample of this serum was retained. The remainder was dialysed against balanced salts solution (BSS) for 3 days (Appendix E).

A third aliquot of the pool was dialysed against BSS as above.

Ten and a half day old conceptuses were obtained from ten Wistar rats (Chapter II, D) and placed in culture bottles containing:

1. Fresh, heat-inactivated serum (Fresh H.I. serum);
2. Fresh H.I. serum dialysed and supplemented with 1.5 mg/ml D-glucose and the eight (MEM) vitamins (Appendix C);
3. Exhausted serum supplemented with D-glucose and vitamins as in 2, with the osmolarity corrected to 300 milliosmol/kg H_2O with sterile distilled water and the eight (MEM) vitamins;
4. Exhausted dialysed serum, supplemented with D-glucose and the eight (MEM) vitamins as in 2.

At the end of the culture period (24 hours) the conceptuses were examined and assessed for the degree of embryonic growth (Chapter II, E).

Analysis of Serum Proteins

In order to detect proteins used by the embryos during repeated culture in the same serum, the following samples were analysed by polyacrylamide gel electrophoresis:

1. Fresh H.I. serum;

2. Exhausted dialysed serum which had been tested after dialysis and failed to support embryonic growth;
3. Fresh H.I. serum treated identically to the exhausted sample in terms of gassing, incubation, freezing and thawing.

The electrophoresis was carried out, using 5 ul samples on gradient polyacrylamide gels type PAA 4-30% and 2-16% (Pharmacia Fine Chemicals) (Appendix G).

RESULTS

All embryos cultured in fresh H.I. serum and fresh H.I. serum dialysed against BSS after supplementation with glucose and vitamins, grew normally and exhibited features identical to those of 11.5 day embryos grown *in vivo*. Table 1 summarises the results.

Conceptuses Cultured in Exhausted Serum Supplemented with Glucose and Vitamins

Embryos cultured in this medium were retarded; the results as summarised in Table 1 and Figure 1 are in agreement with those in Chapter III, Section 1.

Conceptuses Cultured in Exhausted Dialysed Serum Supplemented with Glucose and Vitamins

Embryonic growth was retarded in this medium when compared to the growth in the control serum but was superior to that found in exhausted undialysed serum in all parameters assessed (Table 1 and Figure 1).

Analysis of Serum Proteins by Polyacrylamide Gel Electrophoresis

A - using 4.30% gradient polyacrylamide gels the electrophoretic pattern of fresh heat-inactivated serum showed the presence of 20 major protein bands (Figure 2); the average molecular weights of these bands are given in Table 2. The intensity of band staining was dependent upon the quantity of protein present and, accordingly, the degree of staining was classified - very slight (+), faint (++), moderate (+++) and dark (++++). Of the twenty bands in the fresh heat-inactivated serum 4 were darkly stained, 8 were moderately stained, 7 were faintly stained and 1 was slighted stained. The electrophoretic pattern of the control serum (Figure 2) showed the presence of 22 protein bands, 19 of which had similar molecular weights to bands in fresh H.I. serum. The differences between the fresh H.I. serum and the control serum electrophoretic pattern are:

1. the absence of a band with a molecular weight of 62k daltons in the control serum;
2. three new protein bands in the control serum, with molecular weights of 440k, 360k and 200k daltons;
3. the band with an average molecular weight of 1,300k daltons was more lightly stained (++) in the control serum, compared to that in the fresh H.I. serum pattern (+++).

Electrophoresis of exhausted dialysed serum showed 17 protein bands (Figure 2) and the average molecular weights of these bands are shown in Table 2. Comparison of the electrophoretic pattern of exhausted dialysed serum with that of fresh H.I. serum shows the following changes:

1. four protein bands with molecular weights of 800k, 540k, 230k and 56k daltons were missing (Figure 2);
2. a new band of molecular weight of 440k daltons was detected in the exhausted dialysed serum;
3. one band in the range of 520k daltons was more intensely stained in the exhausted serum;
4. two bands in the range of molecular weight 1,300k and 62k daltons were less intensely stained (++) than in fresh H.I. serum.

Comparison of the electrophoretic pattern of exhausted dialysed serum with that of the control serum showed the following changes:

1. five protein bands with molecular weights of 800k, 540k, 360k, 230k and 200k daltons were missing (Figure 2);
2. two bands of molecular weight 1,300k and 62k daltons respectively were less intensely stained than in the control serum.

B - using a 2-16% gradient polyacrylamide gel the electrophoretic patterns obtained complemented the results obtained using a 4-30% gradient polyacrylamide gel. Using the 2-16% gel ten bands were resolved in the control serum, 8 in the fresh H.I. serum and 7 in the exhausted dialysed serum. The results are summarised in Figure 3 and Table 3.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in (mm) ± standard error	Mean Somite Number ± standard error	Mean Crown-Rump Length (mm) ± standard error	Mean Protein Value (ug/embryo) ± standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Fresh H.I. Serum	24	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	4.238 ±0.053	26.55 ±0.231	3.638 ±0.0719	211.85 ± 6.355
Fresh H.I. Serum Dialysed + glucose + vit	18	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	4.2 ±0.08	26.3 ±0.33	3.59 ±0.055	204.1 ± 4.42
Exhausted Serum + glucose + vit	20	17 (85)	8 (40)	18 (90)	7 (35)	7 (35)	6 (30)	19 (95)	20 (100)	2.95 *±0.099	20.33 *±0.396	2.26 *±0.049	92.633 *± 4.197
Exhausted Dialysed Serum + Glucose + Vitamins	28	27 (96)	27 (96)	27 (96)	13 (46)	9 (32)	13 (46)	28 (100)	28 (100)	3.27 *±0.065	23.94 *±0.66	2.83 *±0.067	135.56 *± 3.84

TABLE 1 Growth and development of 10.5 day rat embryos cultured for 24 hours in fresh H.I. serum, fresh dialysed H.I. serum supplemented with glucose and vitamins, exhausted serum supplemented with glucose and vitamins and exhausted dialysed serum supplemented with glucose and vitamins.

Figures in paranthesis denote the percentage number of embryos in each group.

Using Student's 't' test;
* Significant at $p < 0.001$ when compared with fresh H.I. serum.

Figure 1

Comparison of growth of embryos cultured in fresh serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Protein content.

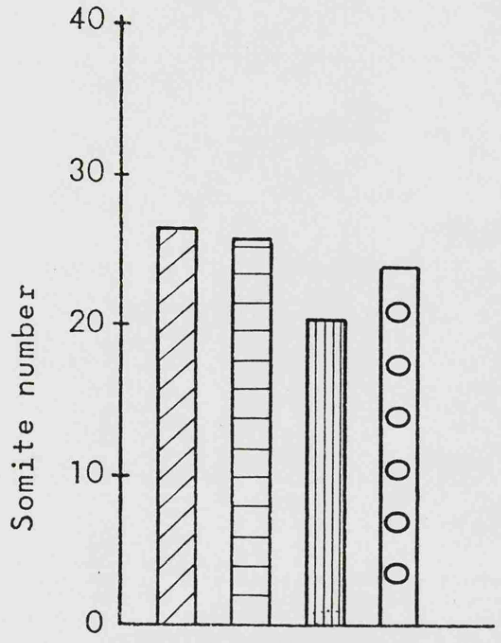
Each rectangle in the histogram represents the mean; for details of numbers of embryos and standard errors see Table 1.

Fresh Heat-Inactivated Serum.

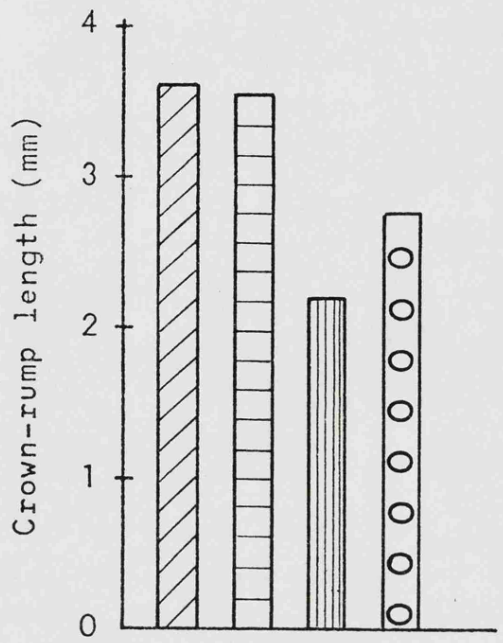
Fresh Heat-Inactivated Dialysed Serum plus Glucose and Vitamins.

Exhausted Serum plus Glucose and Vitamins.

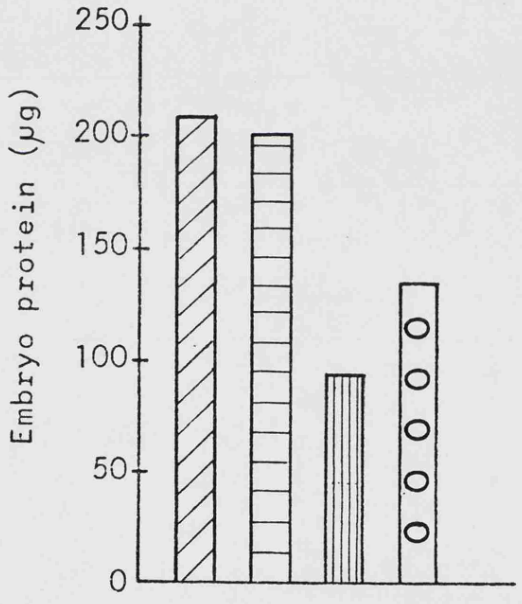
Exhausted Dialysed Serum plus Glucose and Vitamins.


Somite number
40
30
20
10
0

A


Crown-rump length (mm)
4
3
2
1
0

B


Embryo protein (µg)
250
200
150
100
50
0

C

TABLE 2

Average molecular weight distribution of proteins from fresh H.I. serum repeatedly incubated without embryos (control serum), fresh H.I. serum and exhausted dialysed serum used once. Separation on 4-30% gradient, polyacrylamide gel at pH 8.3.
Degree of staining (+) very slight, (++) faint, (+++) moderate and (++++) dark.

	Control Serum		Fresh H.I. Serum		Exhausted Dialysed Serum	
1	1,300	+++	1,300	++++	1,300	++
2	880	++++	880	++++	880	++++
3	800	++	800	++	-	-
4	540	++	540	++	-	-
5	520	+++	520	+	520	+++
6	460	++	460	++	460	++
7	440	++	-		440	++
8	360	++	-		-	-
9	350	+++	350	+++	350	+++
10	250	++++	250	++++	250	++++
11	230	++	230	++	-	-
12	200	++	-		-	-
13	180	++	180	++	180	++
14	160	++	160	++	160	++
15	140	+++	140	+++	140	+++
16	130	+++	130	+++	130	+++
17	120	+++	120	+++	120	+++
18	110	+++	110	+++	110	+++
19	94	+++	94	+++	94	+++
20	78	+++	78	+++	78	+++
21	68	++++	68	++++	68	++++
22	62	+++	62	+++	62	+
23			56	++	-	-

Figure 2

Polyacrylamide-gel electrophoretogram (gradient 4/30%) at pH 8.3.

A - Exhausted dialysed serum used once
B - Molecular weight marker proteins
C - Fresh heat-inactivated serum
D - Fresh heat-inactivated serum repeatedly incubated without embryos (control serum).

Figure 3

Polyacrylamide-gel electrophoretogram (gradient 2/16%) at pH 8.3.

A - Exhausted dialysed serum used once
B - Molecular weight marker proteins
C - Fresh heat-inactivated serum
D - Fresh heat-inactivated serum repeatedly incubated without embryos (control serum).

Figure 2.

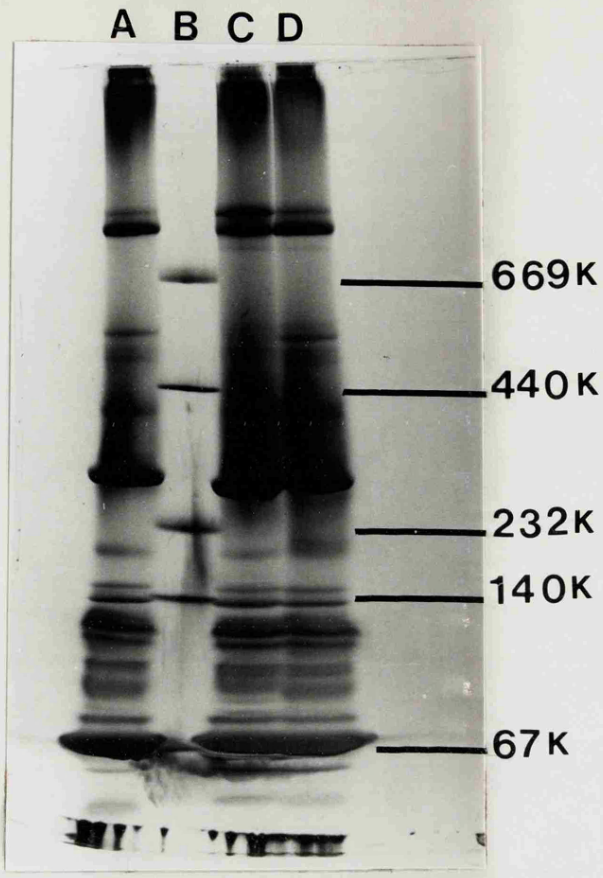


Figure 3.

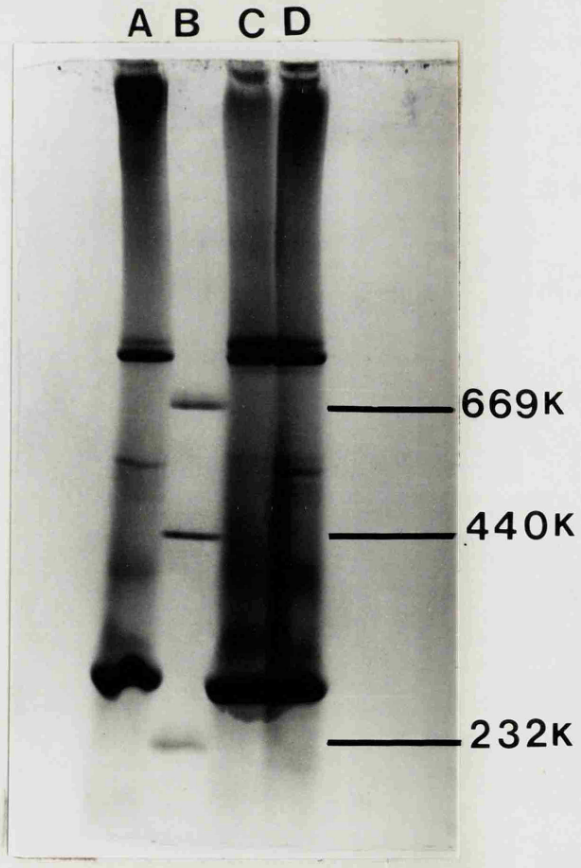


TABLE 3

Average molecular weight distribution of proteins from fresh H.I. Serum repeatedly incubated without embryos (control serum), fresh H.I. serum and exhausted dialysed serum used once. Separated on 2-16% gradient polyacrylamide gel, at pH 8.3.
Degree of staining (+) very slight, (++) faint, (+++) Moderate and (++++) dark.

	Control Serum		Fresh H.I. Serum		Exhausted Dialysed Serum	
1	1,300	++++	1,300	++++	1,300	++
2	880	++++	880	++++	880	++++
3	800	++	800	++	-	-
4	540	++	540	++	-	-
5	520	+++	520	+	520	+++
6	460	++	460	++	460	++
7	440	++	-	-	440	++
8	360	++	-	-	-	-
9	350	+++	350	+++	350	+++
10	250	++++	250	++++	250	++++

DISCUSSION

Culture of conceptuses in dialysed fresh H.I. serum supplemented with glucose and vitamins resulted in the growth of embryos similar to those obtained by Cockroft (1979).

The partial improvement which took place in the growth and differentiation of embryos cultured in dialysed exhausted serum could be due to the clearance from the medium of small molecular weight toxic materials (dialysis by visking tubing clears the serum of substances up to 12,000-14,000 daltons). Tanimure and Shepard (1970) and Robkin and Cockroft (1978) reported accumulation of lactic acid in culture medium used for rat embryos during organogenesis. Sanyal (1980) found an accumulation of urea, uric acid and creatinine in the culture serum.

Failure of the exhausted serum to support normal differentiation and growth suggests either the presence of non-dialysable large molecular weight toxic material or a depletion of nutrient materials from the medium or a combination of both factors.

The changes in the electrophoretic pattern of the serum repeatedly incubated without embryos (control serum) (viz: a decrease in the staining intensity of a band of M.W. 1,300k daltons, and the appearance of three new protein bands of M.W. 440k, 360k and 200k daltons) could be the dissociation of oligomeric proteins into protein monomers as a result of repeated incubation, gassing, freezing and thawing. In spite of these changes, however, embryos cultured in control serum grew and differentiated normally (Chapter III, Section 1, Table 6), indicating that the changes seen in the control serum were not vital for normal embryonic growth.

The changes in the electrophoretic pattern of the exhausted serum, when compared to that of the fresh heat-inactivated serum, (viz: the absence of three protein bands of M.W. 800k, 540k and 230k daltons, and decrease in the intensity of staining of two bands at 1,300k and 62k

daltons could only have resulted from the presence of conceptuses in the serum, since none of these changes was present in the control sera. One explanation could be the consumption of these proteins by the growing conceptus. Several authors (Beck et al., 1967; New & Brent, 1972; Payne & Deuchar, 1972; Goetze et al., 1975; Sharma & Peel, 1979; Freeman et al., 1981) have produced evidence supporting the hypothesis that the yolk sac performs a nutritional role by endocytosis of macromolecules followed by intracellular digestion in secondary lysosomes and passage of the digested products to the developing embryo.

Although embryos and their membranes process very little protein during the culture period compared to the quantity of protein in serum, they may be selective in their needs. Consistent with this explanation for the changes of electrophoretic pattern is our finding that the proteins which disappeared from the exhausted serum were all present in small quantities in the fresh serum (faintly stained ++); similarly, the two bands which had reduced staining intensity (++) initially showed a greater degree of staining (++++ and +++). The changes in the pattern of exhausted serum could be the dissociation of the oligomeric proteins by substances produced by the conceptuses and excreted into the culture medium. For example, Sanyal (1980) has reported the accumulation of urea in the culture serum of rat embryos during organogenesis.

Since protein molecules present in nanogram amounts in serum would not be detected by Coomassie Brilliant Blue Staining, it is unlikely that serum growth factors (as listed in Chapter III, Section 1) would be detected on the electrophoretograms.

CHAPTER III

SECTION 3

IMPROVEMENT OF EXHAUSTED SERUM FOR THE GROWTH OF EMBRYOS

INTRODUCTION

In Chapter III Section 2, analysis of serum proteins by electrophoresis suggested depletion of certain proteins from the exhausted serum. However, the relative improvement in embryonic growth noted in exhausted serum after dialysis against BSS suggested that there was accumulation of toxic material in the medium during culture. At this stage both the depletion of certain nutrients and the accumulation of toxic materials might be responsible for the failure of the exhausted serum to support embryonic growth.

In this section a set of experiments was designed to, firstly, determine the minimum quantity of fresh serum diluted in a chemically defined medium required to sustain embryonic growth and, secondly, differentiate between the importance of the depleted nutrients from the exhausted serum and the effects of toxic materials accumulated in it during repeated culture. Such serum and exhausted dialysed serum were each supplemented with the minimum quantity of fresh serum required to support normal embryonic growth.

MATERIALS AND METHODS

Ten and a half day old conceptuses were obtained from 20 Wistar rats and cultured (Chapter II, Section D) in pooled immediately centrifuged serum (each from 25-30 rats) as before (Chapter II, Section C).

Experiment 1 - In order to establish the maximum serum dilution required to support normal growth of 10.5 day old embryos over a period of 24 hours, serial dilutions of serum were prepared with sterile BSS (Appendix D) containing antibiotics (Appendix F) and supplemented with 1.5 mg/ml D-glucose with the 8 (MEM) vitamins (Appendix C). The following cultures were performed:

1. Fresh heat-inactivated serum - 100%;
2. Fresh H.I. serum - 80% - + BSS - 20% - supplemented with glucose and vitamins;
3. Fresh H.I. serum - 60% - + BSS - 40% - supplemented with glucose and vitamins;
4. Fresh H.I. serum - 40% - + BSS - 60% - supplemented with glucose and vitamins;
5. Fresh serum - 20% - + BSS - 80% - supplemented with glucose and vitamins;
6. Fresh H.I. serum - 10% - + BSS - 90% - supplemented with glucose and vitamins.

At the end of the culture period embryonic growth was assessed for the degree of normal development (Chapter II, Section E).

Experiment 2 - Serum was exhausted as before (i.e. used four times), a sample was retained and the remainder dialysed against BSS for a period of four days (Appendix E). Exhausted sera (dialysed and undialysed) were supplemented with 1.5 mg/ml D-glucose and the 8 (MEM) vitamins (Appendix C).

Conceptuses were cultured for 24 hours (Chapter II, Section D) as

follows:

1. Fresh heat-inactivated serum;
2. Exhausted, dialysed serum supplemented with glucose and vitamins;
3. Medium - consisting of 80% exhausted, dialysed serum supplemented with glucose and vitamins - + 20% fresh H.I. serum;
4. Medium - consisting of 80% exhausted, dialysed serum - + 20% BSS supplemented with 1.5 mg/ml glucose and 10 ul/ml of the 8 (MEM) vitamins. (This medium was to control for the affect of 20% dilution in medium 3 above).
5. Medium - consisting of 80% exhausted serum supplemented with glucose and vitamins - + 20% fresh H.I. serum.

At the end of the culture period embryo growth was assessed for the degree of normal embryonic development (Chapter II, Section E).

RESULTS

Experiment 1

In this experiment embryos were cultured in 80%, 60%, 40% and 20% serum diluted with BSS, glucose and vitamins and, in each instance, the embryos grew normally showing morphological features similar to those in fresh H.I. serum (Table 1). A single exception was found in the medium consisting of 20% serum and 80% BSS where two parameters were significantly affected - namely the yolk sac diameter at $p < 0.01$ (Student's 't' test) and the protein content, which was reduced by 10% when compared to that of the fresh H.I. serum group $p < 0.05$ (Student's 't' test) (Table 1).

Embryos cultured in medium consisting of 10% serum and 90% BSS were all abnormal and all parameters were significantly reduced at $p < 0.001$ (Student's 't' test) (Table 1).

Experiment 2

Embryos grown in exhausted, dialysed serum supplemented with glucose and vitamins, showed evidence of abnormalities and growth retardation. The abnormalities (Table 2 and Figure 1) were similar to those previously seen (Chapter III, Section 2).

Embryos cultured in exhausted, dialysed serum supplemented with 20% fresh H.I. serum revealed a marked improvement in the capability of the medium to support growth. About 90% of the embryos so cultured showed normal morphological features. However, the mean values of somite number, crown-rump length and protein content were less than for those grown in the fresh H.I. serum (Table 2 and Figure 1).

The growth details of embryos cultured in exhausted dialysed serum diluted with 20% BSS (as summarised in Figure 2) are similar to those obtained when 100% exhausted, dialysed serum supplemented with glucose and vitamins (Table 2 and Figure 1) was used.

On the other hand, the supplementation of exhausted, undialysed serum

with 20% fresh heat-inactivated serum did not improve embryonic growth; all embryos cultured in this medium were not normal (Table 2 and Figure 1).

	Number of Embryos	Number of Embryos Showing:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter in (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
Fresh Heat-Inactivated Serum	20	20 (100)	20 (100)	20 (100)	20 (100)	19 (95)	20 (100)	20 (100)	20 (100)	4.2 ± 0.081	26.1 ±0.38	3.49 ±0.06	208.2 ± 8.42
Fresh H.I. Serum 80% + 20% BSS	18	18 (100)	18 (100)	18 (100)	17 (94)	18 (100)	18 (100)	18 (100)	18 (100)	4.35 ± 0.09	26.83 ±0.27	3.55 ±0.072	222.08 ±11.01
Fresh H.I. Serum 60% + 40% BSS	18	18 (100)	17 (94)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	3.988 ± 0.116	26 ±0.161	3.423 ±0.044	199.24 ± 5.174
Fresh H.I. Serum 40% + 60% BSS	18	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	4.08 ± 0.081	25.66 ±0.396	3.4 ±0.082	189.42 ± 7.84
Fresh H.I. Serum 20% + 80% BSS	26	26 (100)	26 (100)	26 (100)	25 (96)	25 (96)	26 (100)	26 (100)	26 (100)	3.86 * ± 0.106	25.86 ±0.34	3.36 ±0.073	186.9 ** ± 8.72
Fresh H.I. Serum 10% + 90% BSS	22	18 (82)	12 (55)	22 (100)	11 (50)	8 (36)	3 (14)	18 (82)	21 (95)	3.44 *** ± 0.091	20.72 *** ±0.724	2.295 *** ±0.086	114.86 *** ± 3.521

Table 1 Growth and development of 10.5 day rat embryo cultured for 24 hours in fresh H.I. serum and in different dilutions of fresh H.I. serum with BSS and supplemented with glucose and vitamins.
Figures in paranthesis denote the percentage number of embryos in the group.
Using Student's 't' test;
* Significant at $p < 0.01$ when compared with growth in fresh H.I. serum
** Significant at $p < 0.05$ when compared with growth in fresh H.I. serum
*** Significant at $p < 0.001$ when compared with growth in fresh H.I. serum

Figure 1

Comparison of embryonic growth after culture in fresh serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Protein content.

Each rectangle in the histogram represents the mean value. For details of numbers of embryos and standard errors see Table 2.

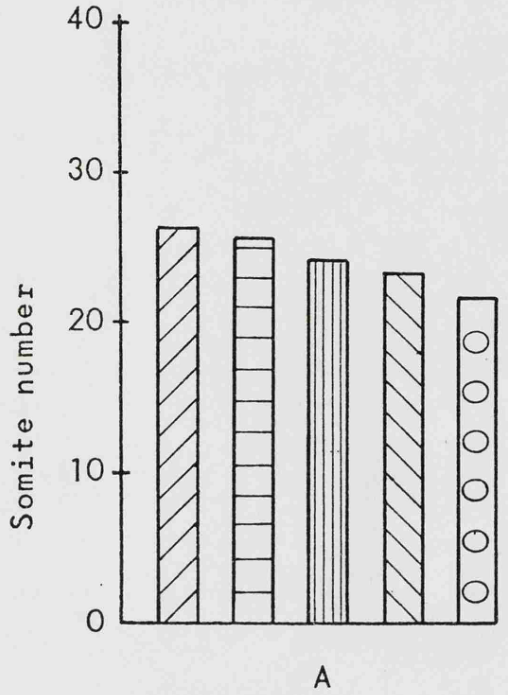
 Fresh Heat-Inactivated Serum.

 Exhausted Dialysed Serum plus Glucose and Vitamins.

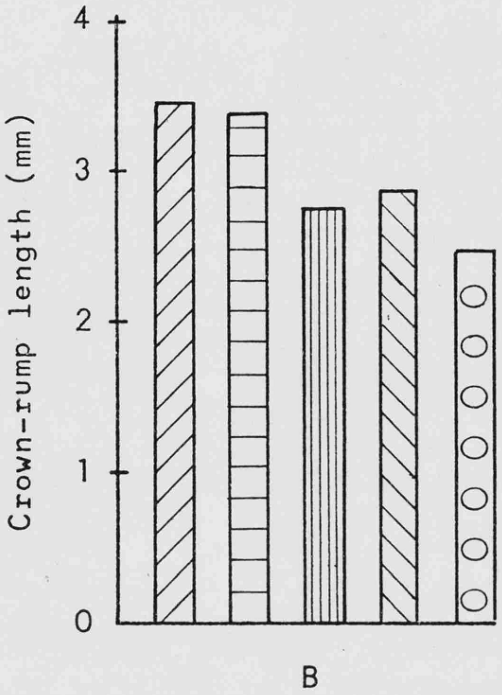
 Exhausted Dialysed Serum 80% plus Fresh H.I. Serum 20%.

 Exhausted Dialysed Serum 80% plus BSS 20%.

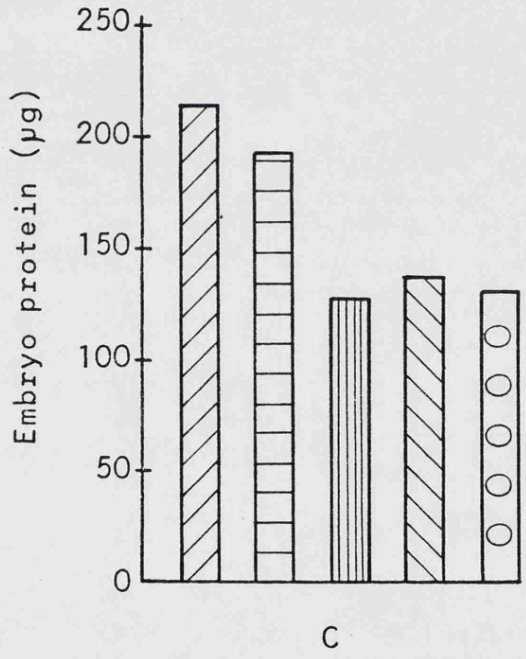
 Exhausted Serum 80% plus Fresh H.I. Serum 20%.


Somite number
40
30
20
10
0

A


Crown-rump length (mm)
4
3
2
1
0

B


Embryo protein (µg)
250
200
150
100
50
0

C

	Number of Embryos	Number of Embryos Showing:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter in (mm) ± standard error	Mean Somite Number ± standard error	Mean Crown-Rump Length (mm) ± standard error	Mean Protein Value (ug/embryo) ± standard error
Fresh H.I. Serum	20	20 (100)	18 (90)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	4.06 ±0.054	26.61 ±0.23	3.55 ±0.05	215 ± 4.45
Exhausted dialysed Serum + glucose and vitamins	12	12 (100)	5 (42)	12 (100)	5 (42)	5 (42)	6 (50)	12 (100)	12 (100)	3.3 **** ±0.12	22.1 **** ±0.53	2.53 **** ±0.08	129.73 **** ± 7.35
Exhausted dialysed serum 80% + 20% Fresh H.I. serum	23	23 (100)	21 (91)	23 (100)	22 (96)	21 (91)	20 (87)	23 (100)	23 (100)	3.9 * ±0.079	25.94 ** ±0.27	3.38 *** ±0.051	193.105 **** ± 3.85
Exhausted dialysed serum 80% + 20% BSS	18	18 (100)	8 (44)	18 (100)	12 (67)	11 (61)	8 (44)	18 (100)	18 (100)	3.405 **** ±0.093	25.55 **** ±0.258	2.683 **** ±0.069	128.71 **** ± 3.441
Exhausted Serum 80% + 20% Fresh H.I. Serum	12	12 (100)	9 (75)	12 (100)	10 (83)	10 (83)	7 (58)	12 (100)	12 (100)	3.43 **** ±0.49	23.58 **** ±0.36	2.93 **** ±0.03	134.61 **** ± 8.33

Table 2 Growth and development of 10.5 day rat embryos cultured for 24 hours in Fresh and Exhausted Serum.
Figures in parantheses denote the percentage number of embryos in each group.
Using Student's 't' test;
* Significant at $p < 0.1$ when compared with growth in fresh H.I. serum.
** Significant at $p < 0.05$ when compared with growth in fresh H.I. serum.
*** Significant at $p < 0.02$ when compared with growth in fresh H.I. serum.
**** Significant at $p < 0.001$ when compared with growth in fresh H.I. serum.

DISCUSSION

In the preliminary experiment we found that a medium consisting of 20% fresh heat-inactivated serum and 80% BSS supplemented with glucose and vitamins, was capable of providing at least the minimum nutrients required by 10.5 day old embryos for normal growth and differentiation for 24 hours. The embryos synthesised, however, 10% less protein than those cultured in 100% fresh heat-inactivated serum.

Previous authors (New, 1973; Cockroft, 1973; 1974 and 1976) attempting to culture rat embryos during organogenesis in 25% serum diluted in Tyrode's solution have shown that, with such diluted serum, the protein contents of embryos and in some cases the somite numbers, were significantly less than in their control groups. The results presented in this section show that the growth and differentiation of embryos was superior to embryos cultured in comparable dilutions of serum by the above authors. This could be attributed to the difference between dilution of serum with BSS supplemented with glucose and vitamins (which are essential requirements for embryonic growth in vitro (Cockroft, 1979; Sanyal & Wiebeke, 1979; Sanyal, 1980)) and dilution of serum with Tyrode's only.

In the final experiment in this section the supplementation of the exhausted, dialysed serum with 20% fresh heat-inactivated serum resulted in a marked improvement in the capability of the medium (further supplemented with glucose and vitamins) to support embryonic growth; 90% of the embryos were normal.

These results would appear to indicate that the two possibilities (Chapter III, Sections 1 and 2) - i.e. depletion of nutrient components and the accumulation of toxic micro and macromolecular materials - are operating simultaneously. First, the improvement in embryonic growth could only have taken place as a result of nutrients provided by the 20% fresh heat-inactived serum when added to the exhausted dialysed serum.

When a similar amount of BSS was added to the exhausted medium growth was poor and similar to that obtained in 100% exhausted dialysed serum. Second, failure to achieve 100% normal growth (identical to that resulting from the use of fresh heat-inactivated serum) is most probably due to the accumulation of undialysable toxic macromolecules present in the exhausted medium. Further evidence of the effect of toxic material accumulation in exhausted serum is shown by the retarded growth of embryos cultured in undialysed serum supplemented only with 20% fresh H.I. serum.

CHAPTER FOUR

CHAPTER IV

SECTION 1

INTRODUCTION

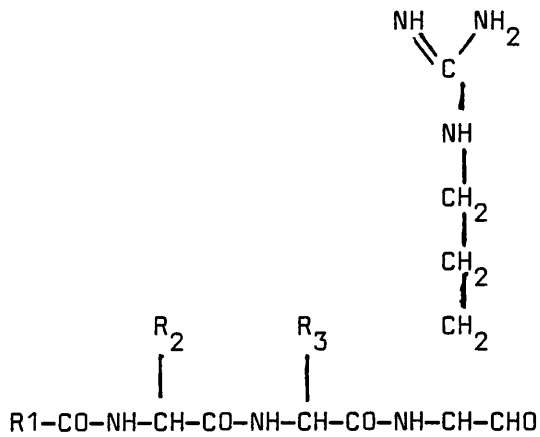
INTRODUCTION

1. The Chemistry and Preparation of Leupeptin

Leupeptin is the name given to a group of tetrapeptides, produced by several strains of actinomyces. It was first discovered by Aoyagi, et al. (1969a), during their screening of actinomycetes culture filtrates for activity to inhibit plasmin and trypsin.

Leupeptins, include acetyl-or propionyl-L-leucyl-L-leucyl-DL-argininal (leupeptins Pr-LL and Ac-LL) and their analogues which contain L-Isoleucine or L-valine instead of L-leucine (Kondo, et al., 1969).

Since leupeptins can contain various analogues, such as isoleucine and valine instead of 2 molecules of leucine, the general formula of leupeptins given by Kondo, et al., 1969 is as follows:



R_1, R_2, R_3, Alkyl Radicals

Leupeptins in general have a molecular weight of about 550, depending upon the alkyl radicals.

The most pruified leupeptins, Pr-hydrochloride and Ac-hydrochloride, are in the form of a white powder, have no ultraviolet absorption and are soluble in water (Kondo et al., 1969).

Aoyagi et al. (1969 a) have described a lengthy method for the preparation of leupeptin from actinomycetes. However, leupeptin Ac-LL and Pr-LL can be chemically synthesised by the oxidation of the corresponding argininal derivatives, as described by Kawamura et al. (1969).

2. Biological Activities of Leupeptin

Leupeptins are powerful and specific protease inhibitors. They inhibit proteolysis by plasmin, trypsin and papain and inhibit the thrombokinase reaction and coagulation of blood from man and rabbit but not blood from rat and dog, (Aoyagi et al., 1969 a, b; Maeda et al., 1971). Leupeptins do not inhibit proteolysis by -chymotrypsin (Aoyagi et al., 1969 b).

Leupeptins have been found to be specific inhibitors for some of the lysosomal peptidehydrolases, inhibiting cathepsin B (Ikezawa et al., 1971; Evans et al., 1978), cathepsin H (Kireschke et al., 1976 and 1977) and cathepsin L (Kireschke et al., 1977). Leupeptins do not influence cathepsin A (Ikezawa et al., 1972), cause partial inactivation of cathepsin C only after prolonged incubation (Huisman et al., 1974) and have no effect on cathepsin D. Leupeptins can be absorbed orally and at least about 25% is excreted in urine. Oral administration of leupeptin exhibited an anti-inflammatory effect on odaema (Aoyagi et al., 1969 b).

When injected into animals leupeptins are excreted in the urine without being hydrolysed. Thus suggests a direct effect of leupeptins in

vivo (Aoyagi & Umezawa, 1975).

Leupeptin ointment, when applied immediately to burns, suppresses pain and blister formation, which may be due to inhibition of kinin formation.

Furthermore, leupeptin inhibits the phytohemagglutinin (PHA) stimulated DNA synthesis in guinea-pig blood (Saito, et al., 1972, 1973).

3. Inhibition of the lysosomal Pathway of Proteolysis by Leupeptin

The low k dis. values for Leupeptin-Cathepsin B (2×10^{-8} mol/litre) and for Leupeptin-Cathepsin L (3.7×10^{-8} mol/litre) (Kirschke, et al., 1977) demonstrates the remarkable affinity between enzyme and inhibitor; so strong, in fact, that binding is practically irreversible (although strictly speaking competitive).

Huisman, et al., 1974 have used the microbiol inhibitors pepstatin, leupeptin, antipain to investigate the specific roles of various cathepsins in lysosomal digestion of serum albumin. Their results suggested that these lysosomal cathepsins act in a synergistic way to degrade proteins, and that "cathepsin B or another leupeptin sensitive thiol proteinase is essential" for protein breakdown to proceed. The following (Figure 1) shows the time course of albumin degradation by lysosomal extracts treated with inhibitors of various lysosomal enzymes.

The application of leupeptin to rat hepatocytes in culture (Hopgood, et al., 1977) was found to cause an inhibition of overall protein turnover. Leupeptin is presumed to enter into endocytic vacuoles and reach the enzymes in the same way as ingested proteins. Recently, it has been shown using the same tissue that C^{14} release from prelabelled proteins (as a measure of protein breakdown) was reduced by the action of leupeptin added to the culture medium (Seglan, et al., 1979)

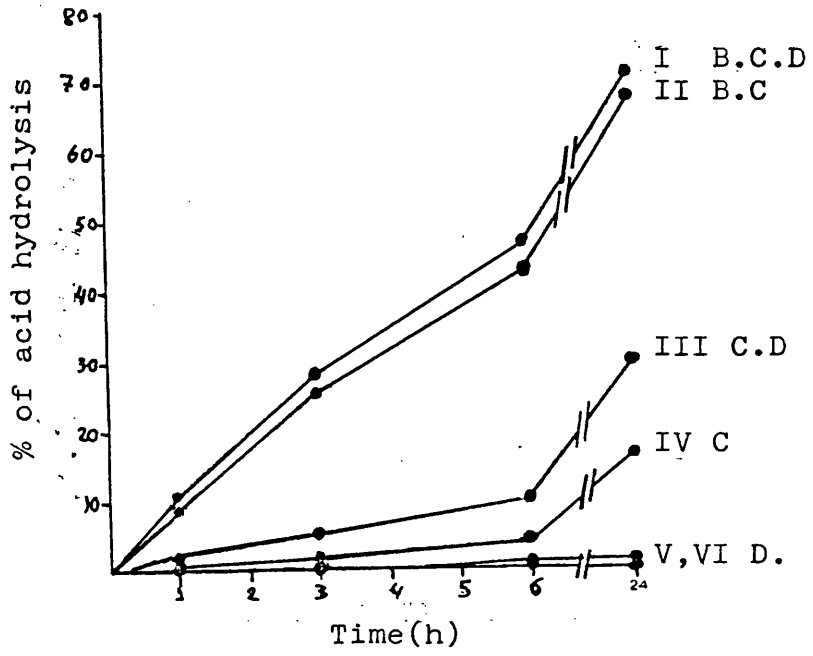


Figure 1

Degradation of native bovine serum albumin by lysosomal extracts at pH 5.0. I, no inhibitor present; II, 4.8 uM Pepstain; III, 33 uM Leupeptin, 22 uM Antipain, or both; IV, 4.8 uM Pepstain and 33 uM Leupeptin or 22 uM Antipain or both; V, 10mM Monoiodoacetate; VI, 10mM monoiodoacetate and 4.8 uM Pepstain. The cathepsins shown on the right hand of the figure indicate enzymes known to be active under the incubation conditions (Huisman, et al., 1974).

Moreover, this effect was only slightly enhanced to that of a known lysosomal inhibitor, NH_4Cl. Thus, leupeptin has been shown to be a fairly specific inhibitor of the lysosomal pathway of protein degradation. In good agreement with the tight binding between leupeptin and cathepsin *in vitro* (Kirschke et al., 1977), leupeptin action on hepatocytes was found to be essentially irreversible.

4. Inhibition of Histiotrophic Nutrition of Rat Embryos In Vitro By Leupeptin

In vivo the early embryos receive their nutritional requirements for growth by histiotrophic nutrition. This process, together with the structures and mechanisms responsible, has been described in Chapter I.

A transudate of maternal serum (histiotroph) is pinocytosed by the visceral yolk sac endoderm and after fusion of these vesicles with primary lysosome the protein is degraded to amino acids which can then be used to supply the embryo with raw materials for protein sysnthesis (Freeman et al., 1981).

This process is quite distinct from haematotrophic nutrition, where small molecules are transferred between the fetal and maternal circulation unaltered. Rat embryos cultured from $9\frac{1}{2}$ - $11\frac{1}{2}$ days using New's culture methods (1976) are known to utilise the proteins in histiotroph (serum) in order to build up their own proteins, after having first broken down the maternal biopolymers in the lysosomal system of the visceral yolk sac endoderm. Embryos cultured between days $9\frac{1}{2}$ and $11\frac{1}{2}$ are identical to those grown to $11\frac{1}{2}$ days *in vivo* in every respect measured (Cockroft, 1976; New *et al.*, 1976).

Recently, morphological studies of the endodermal cells of visceral yolk sac obtained *in vitro* after 48 hours of culture, have shown that both the volume and surface area of the vacular compartments of the cells give

measurements which are identical to those from specimens obtained from the *in vivo* situation (Gupta, et al., 1982). It seems likely, therefore, that the cultured conceptuses are functioning in a similar way to those grown *in vivo*.

More recently Beck and Lowy (1982) have studied the effect of the addition of different leupeptin doses to the culture serum of cultured rat embryos. They found that leupeptin caused growth retardation and abnormalities of embryonic development. Moreover, their electron microscopical examination of yolk sac specimens from leupeptin treated conceptuses showed distinct morphological changes akin to lysosomal storage disease. The effect of leupeptin was found to be dose dependent. They believe that the leupeptin effect is due to inhibition of protein breakdown in the visceral yolk sac epithelium, which deprives the cultured rat embryo of amino acids and causes both growth retardation and malformation.

In summary, leupeptin has been shown to be an inhibitor of several important lysosomal proteinases (cathepsin B, H and L) and has also been shown to inhibit the lysosomal pathway of protein degradation in various tissues, notably the rat hepatocyte (Hopgood, et al., 1977). Moreover, the presence of leupeptin in the culture medium impairs the growth of $9\frac{1}{2}$ days old rat embryos cultured for 48 hours (Beck and Lowry, 1982).

The work described in this chapter aims to investigate the possibility of reversing the embryopathic effect of leupeptin on rat embryos growing *in vitro* between $10\frac{1}{2}$ to $11\frac{1}{2}$ days, by supplementing the culture serum containing leupeptin with amino acids. In this way the embryo could be supplied with the necessary amino acids otherwise unobtainable from the leupeptin inhibited breakdown of maternal serum.

CHAPTER IV

SECTION 2

EFFECT OF LEUPEPTIN ON RAT CONCEPTUSES IN VITRO

AIM

In this section a set of experiments were designed to study the effect of increasing leupeptin doses (1,2,4 and 8 ug/ml culture serum) on $10\frac{1}{2}$ day old rat embryos cultured for 24 hours in order to establish the extent of embryonic retardation and malformation produced by each dose.

MATERIALS AND METHODS

Leupeptin Ac-leu-leu-L-Argininal 1/2 $H_2SO_4 . H_2O$ powder was obtained from Peptide Institute, Inc. Osaka, Japan. It was stored in a sealed container at 4^oC in the dark. Leupeptin solution was prepared not more than 3 days before use, 4 mg/10 mls of sterilised balanced salt solution (BSS) (see Appendix D). A 10 mls aliquot of the sterile BSS alone was placed in a similar tube, and both were sealed and stored at 4^oC and placed in the dark. Measured quantities of the solution were withdrawn using 5-50 microlitre micropipette fitted with a sterile tip.

Pools of immediately centrifuged serum, each from 5-20 rats, were prepared as in Chapter II, C. Serum was always heat-inactivated before use.

The required amount of Leupeptin was added to the serum in the culture bottles. In all cases a quantity of sterile BSS was added into the control vessel in order to equalise the volume in each culture bottle. In this way any dilution of the serum, and any possible contamination resulting from the addition of leupeptin solution was controlled. The pH and osmolarity of the treated serum were checked and found to be within the normal range of untreated serum.

Ten and a half day old conceptuses were obtained from 19 Wistar rats (see Chapter II, C). Conceptuses from each mother were divided into 5 groups. Six series of culture were made using 136 conceptuses. The conceptuses were cultured for 24 hours (see Chapter, II, D) in the following media.

1- Control serum (Fresh, H.I. serum). This was to control the growth and differentiation of embryos cultured in experimental media.

2- Serum containing 1 ug/ml leupeptin.

3- Serum containing 2 ug/ml leupeptin.

4- Serum containing 4 ug/ml leupeptin.

5- Serum containing 8 ug/ml leupeptin.

At the end of the culture period embryonic growth was assessed as described in Chapter II, E.

RESULTS

1. Conceptuses 10½ days old Cultured in Fresh Heat-Inactivated Serum

Embryos in the control groups grew normally (Figure 2). Table 1 and Figure 1 summarise all the results. Occasionally, the control embryos did show abnormal turning or neural tube defects, but this could have been caused by damage during the explantation procedure. A total of 102 embryos were cultured in the presence of three different doses of leupeptin.

2. Conceptuses 10½ days old Cultured in Serum Containing 1 ug/ml Leupeptin

All embryos grow normally in this medium; results are summarised in Table 1 and Figure 1. They show no significant difference (using Student's 't' test) between all the individual parameters assessed in this group of embryos and the control embryos.

3. Conceptuses 10½ days old Cultured in Serum Containing 2 ug/ml Leupeptin

The presence of 2 ug/ml leupeptin in the culture medium produced some retardation in the growth and abnormality of differentiation of embryos when compared with embryos grown in control serum. The parameters which were significantly affected, (Student's 't' test) were as follows: the mean values of yolk sac diameter, which was reduced by 8.25%, the somite number, which was on average 3 somites fewer, the crown-rump length, which was reduced by 8.66%, the protein content which was reduced by 11.42% and the percentage of embryos which failed to turn completely which was 13%. In most cases the embryos were dorsally convex, but the tail was lying to the left of the head. Neural tube defects were present in 10% of embryos, the defect was manifested as a non-closure of the rhombencephalic region and/or the posterior neuropore (Figure 3).

4. Conceptuses 10½ days old Cultured in Serum Containing 4 ug/ml Leupeptin.

All the parameters used to assess the growth of conceptuses in serum containing 4 ug/ml leupeptin were affected, they were significantly inferior ($p < 0.001$ using Student's 't' test) to those of embryos exposed

to 2 ug/ml leupeptin. The results are summarised in Table 1.

Comparison of growth and differentiation of embryos in this group and that of the control embryos shows a marked reduction of the mean values of yolk sac diameter by 45%, a reduction of the somite number by 40.5%, the crown-rump length was reduced by 33.8% and the protein content was reduced by 55% (see Figure 1).

The percentage of normally turned embryos was drastically affected, only 29% had completed the inversion. Neural tube defects were present in 77% of the embryos, manifested as non-closure of the cranial and/or caudal part, Figure 4. The majority of the embryos showed poorly developed optic vesicles - only 35% had normal optic vesicles; likewise fore-limb bud formation was affected and only 32% of the embryos showed a paddle shaped fore-limb bud.

5. Conceptuses 10½ day old Cultured in Serum Containing 8 ug/ml Leupeptin

All embryos in this group grew abnormally, all parameters used to assess embryonic growth and differentiation were very severely affected (Figure 5) and the results are summarised in Table 1.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
		Presence of Heart Beat	Presence of Vitel-line Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Control	34	34 (100)	34 (100)	34 (100)	33 (97)	33 (97)	34 (100)	34 (100)	34 (100)	4.22 ±0.09	25.7 ±0.29	3.416 ±0.06	198.90 n=24 ±7.56
Serum treated with 1 ug/ml leupeptin	24	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	4.0 ±0.10	24.84 ±0.92	3.35 ±0.08	194.5 n=20 ±7.3
Serum treated with 2 ug/ml leupeptin	30	30 (100)	28 (93)	30 (100)	27 (90)	26 (87)	23 (77)	30 (100)	30 (100)	3.872 *** ±0.12	22.84 ** ±0.942	3.12 ** ±0.109	176.18 **** n=20 ±8.76
Serum treated with 4 ug/ml leupeptin	34	32 (94)	10 (29)	31 (91)	8 (23)	10 (29)	11 (32)	12 (35)	32 (94)	2.316 * ±0.102	15.28 * ±0.821	2.26 * ±0.048	89.2 * n=25 ±6.0
Serum treated with 8 ug/ml leupeptin	24	16 (67)	0 -	20 (83)	0 -	0 -	0 -	8 (33)	21 (87)	2.072 * ±0.74	13.611 * ±0.915	1.96 * 2.32 ±0.046	69.55 * n=18 ±6.06

Table 1 Growth and development of 10.5 days rat embryos, cultured for 24 hours in control, Fresh H.I. serum and in serum containing different doses of leupeptin.
Figures in paranthesis denote the percentage number of embryos in the group.

Using Student's 't' test;
* Significant at $p < 0.001$ when compared to the control embryos.
** Significant at $p < 0.01$ when compared to the control embryos.
*** Significant at $p < 0.02$ when compared to the control embryos.
**** Significant at $p < 0.05$ when compared to the control embryos.

Figure 1

Comparison of growth of embryos cultured in fresh serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Embryonic protein content.

Each rectangle in the histogram represents the mean. For details of numbers of embryos and standard errors see Table 1.

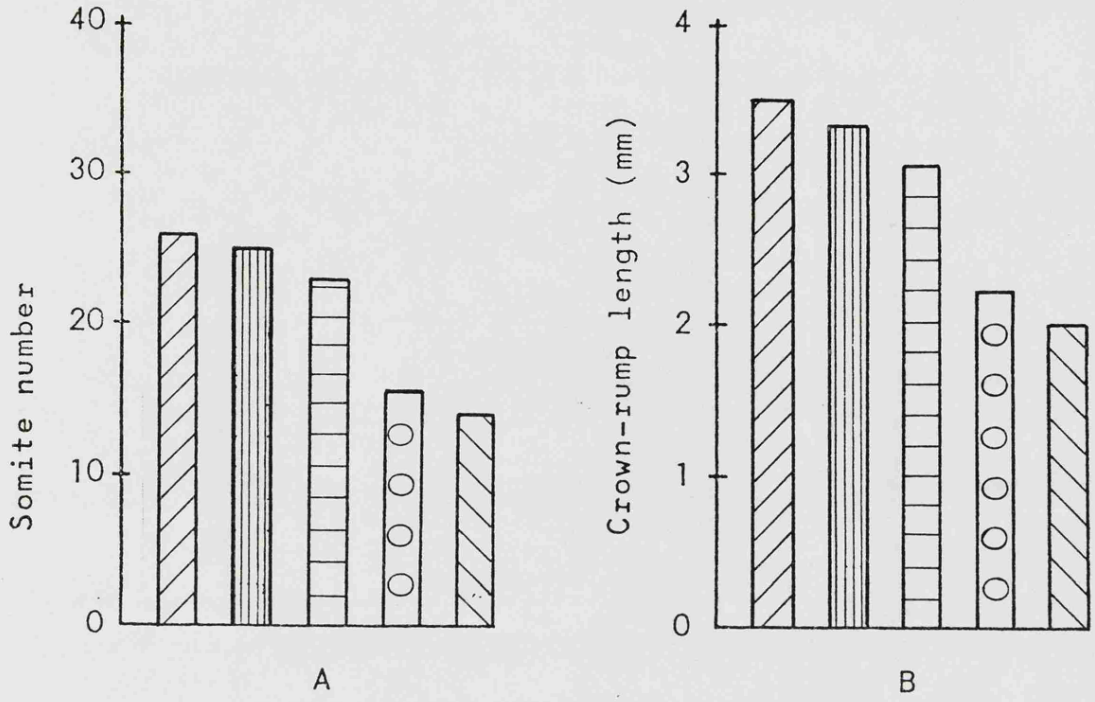
 Fresh H.I. Serum (control).

 Serum containing 1 ug/ml Leupeptin.

 Serum containing 2 ug/ml Leupeptin.

 Serum containing 4 ug/ml Leupeptin.

 Serum containing 8 ug/ml Leupeptin.


Somite number
40
30
20
10
0
A
Crown-rump length (mm)
4
3
2
1
0
B

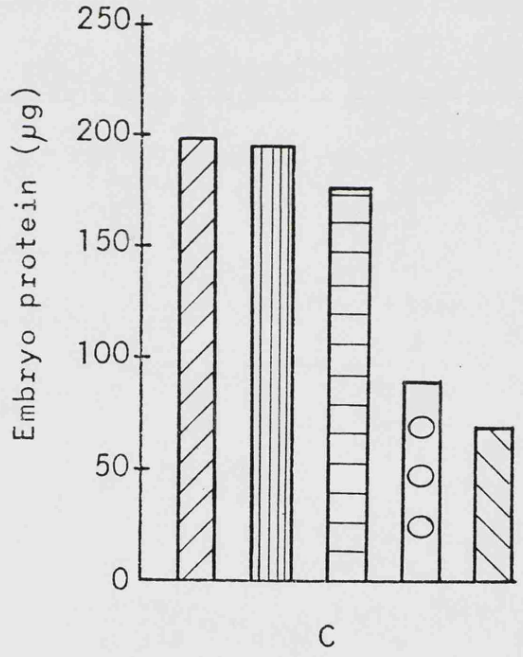

Embryo protein (µg)
250
200
150
100
50
0
C

Figure 2

Normal rat embryo explanted at 10½ days and grown for 24 hours in untreated serum (control). (Mag x 28).

Figure 3

Rat embryo explanted at 10½ days and grown for 24 hours in the presence of 2 ug leupeptin/ml serum culture medium. The embryo is abnormally turned, has an open neural tube in the rhombencephalic region and shows a tail defect (twisted). (Mag x 26).

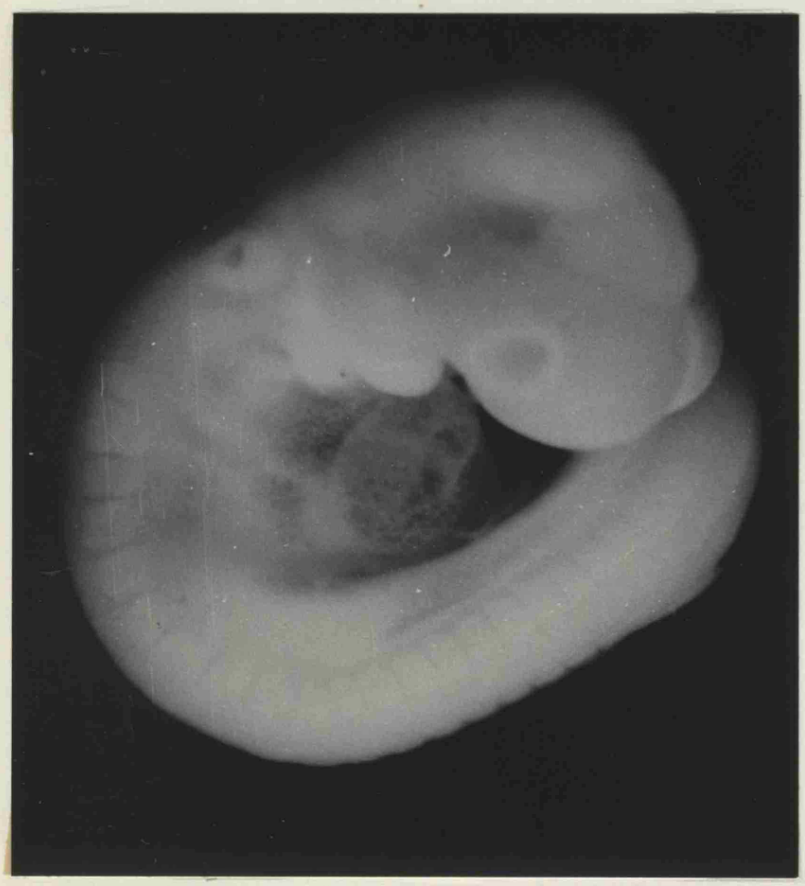


Figure 2.

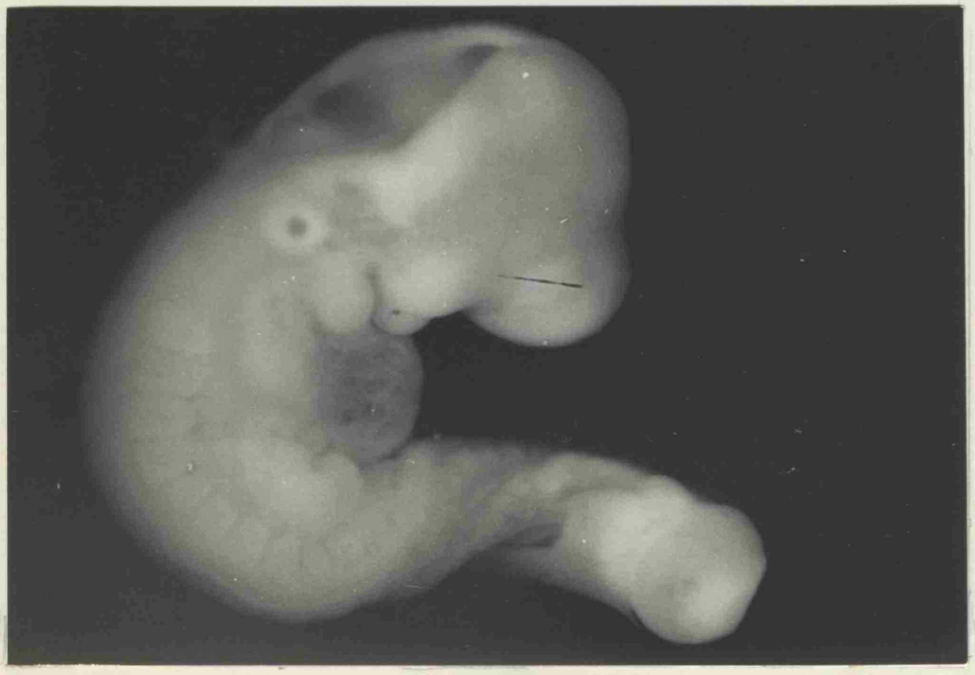


Figure 3.

Figure 4

Rat embryo explanted at 10½ days and grown for 24 hours in the presence of 4 ug leupeptin/ml serum culture medium. Embryo has failed to turn completely and the cranial part of the neural tube is completely open. (Mag x 26).

Figure 5

Rat embryo explanted at 10½ days and grown for 24 hours in the presence of 8 ug leupeptin/ml serum culture medium. Embryo is grossly abnormal. (Mag x 40).

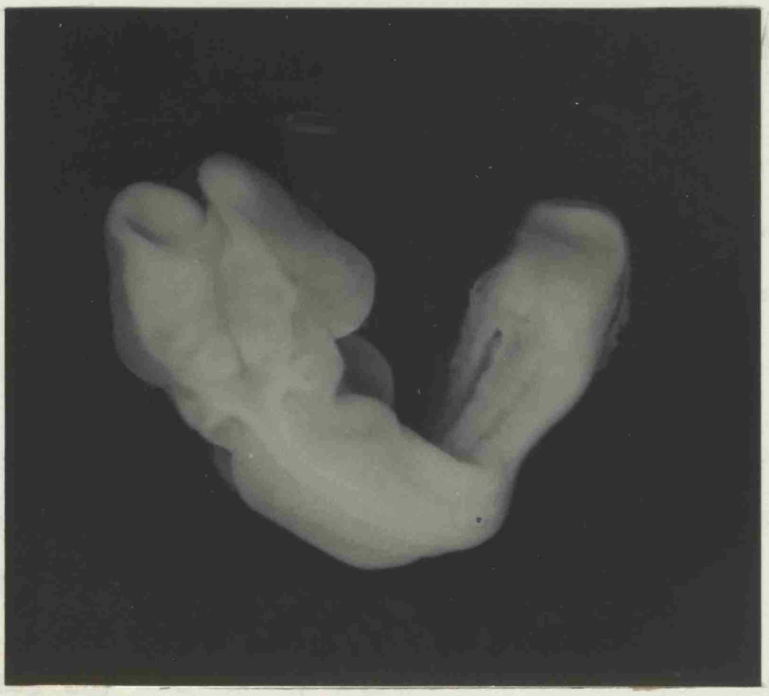


Figure 4.

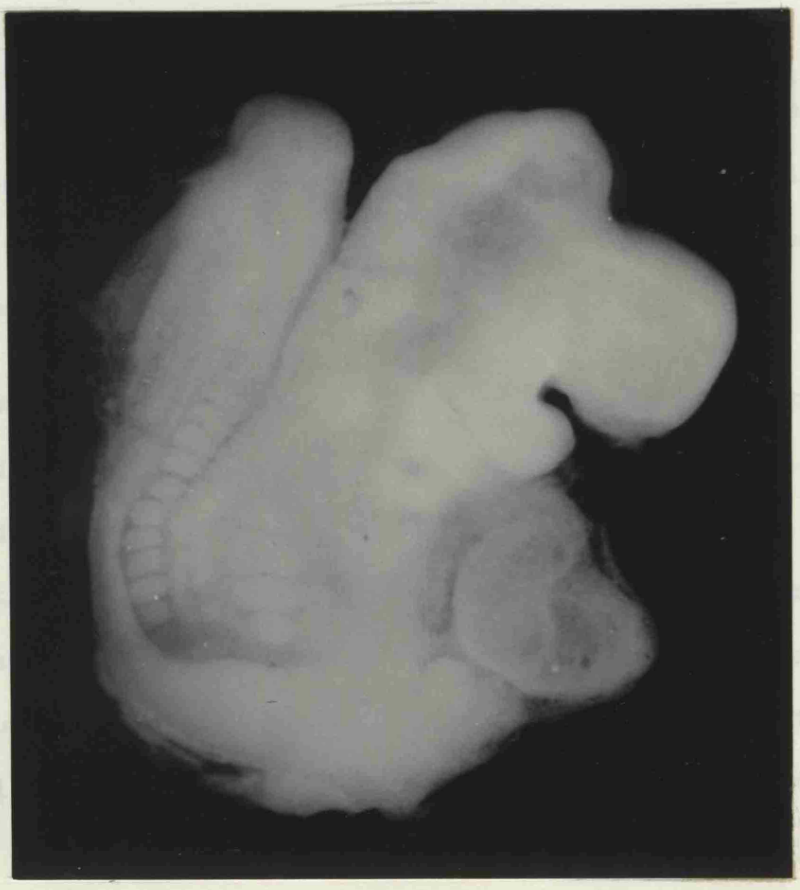


Figure 5.

DISCUSSION

Although the embryos used in this study were of greater age ($10\frac{1}{2}$ day old) and the culture period was 24 hours; the results here have confirmed the observation made by Beck and Lowy (1982) that the presence of leupeptin in the culture medium of growing rat embryos has a dose dependent embryopathic effect on rat embryonic development.

A difference has been found regarding the sensitivity to Leupeptin of $10\frac{1}{2}$ day old embryos when compared to that of $9\frac{1}{2}$ day old embryos used in the above mentioned study.

In my study $10\frac{1}{2}$ day old conceptuses cultured for 24 hours, showed a threshold at 2 ug/ml Leupeptin, below which Leupeptin has no effect on embryonic growth. Leupeptin at a dose of 1 ug/ml did not affect growth and differentiation of $10\frac{1}{2}$ day old embryos. Leupeptin at dose of 2 ug/ml started to effect the embryonic growth, while the presence of 4 ug/ml of leupeptin caused a significant reduction in all parameters used to assess growth and differentiation of the cultured embryos to an extent similar to that described by Beck and Lowy (1982) in the $9\frac{1}{2}$ day old rat embryos cultured for 48 hours in medium containing 2 ug/ml leupeptin.

The higher dose levels necessary to establish an embryopathic effect in the $10\frac{1}{2}$ day old embryo cultured for 24 hours, is probably due to either the shorter period of exposure to the drug, or to the greater age of the embryos. In addition, it is possible that leupeptin has a direct effect upon embryonic tissues. The age at which the rat conceptus is susceptible to the drug is crucial because the drug can pass directly to the embryo through the vittello-intestinal duct only for the 12 hours between $10\frac{1}{2}$ to 11 days before it is sealed (rat embryos invaginate completely into their own yolk sac at 11 days of gestation, Denker, 1977).

The hypothesis put forward by Beck and Lowy (1982) suggests that

Cathepsin B, H and L are essential in the adequate breakdown of maternal proteins in the lysosomal system of the visceral yolk sac endoderm and that the inhibition of these cathepsins by leupeptin can result in the starvation of the cultured embryos of the amino acids essential for embryos to build up their own proteins.

The addition of leupeptin resulted in a dose dependant reduction of embryonic growth so it is likely that not all of the B, H and L enzymes are inhibited by the addition of small amounts of leupeptin. This could allow limited provision of amino acids by the yolk sac and thus limited embryonic growth. However, at 8 ug/ml of leupeptin, marked inhibition of embryonic growth was observed. The severity of embryonic growth impairement produced by 8 ug/ml leupeptin in the culture medium in this study suggests two possible alternative mechanisms. The first possibility may be secondary to the gross physical distortion of the visceral yolk sac endoderm of the conceptuses treated with high doses of leupeptin as shown by Beck and Lowy (1982) using electron microscopic examination. This could produce its effect by interferring with the uptake or digestion of macromolecular nutrients by the visceral yolk sac endoderm or even the release of the end products of the digestion process to the embryo.

The second possibility which has also been suggested by Beck and Lowy (1982) is that leupeptin may have a direct effect upon the embryonic tissue. This has not formally been disproved by the experiments reported here and indeed the gross retardation and the high mortality produced by the presence of 8 ug/ml of leupeptin in the medium suggests that a large amount of leupeptin reaches the embryonic tissue directly before the embryo has completely inverted into its yolk sac, hence affecting embryonic growth at an early stage, rather than indirect embryonic starvation of amino acids produced by

leupeptin action on the visceral yolk sac, which could require a time interval before its embryopathic effect is seen.

It is well-known that there are lysosomal porteinases other than cathepsin B, H and L. A wide variety of lysosomal proteinases have been investigated - A, C, D, E (Barrett, 1969); F, G (Barrett, 1975); FG, GF, Lebez and Kopitar, 1970 and S (Turnesek, et al., 1975), or cathepsin C or D (Huisman, et al., 1974). Therefore it is possible that unless the above cathepsins (A, C, D, E, F, G, FG, GF and S) require the co-operation of cathepsin B, H and L to complete the breakdown of proteins, then the complete inhibition of protein breakdown in the visceral yolk sac endoderm by leupeptin cannot be achieved only by the inhibition of cathepsins B, H and L.

It is therefore necessary either to show that leupeptin does not enter the embryo, perhaps by the use of radio labelled leupeptin or ideally it would be best if a method could be developed to show if leupeptin exerted a direct effect on embryos. In the absence of such methods any apparent variation of susceptibility with time may reflect a variation in the embryo's accessibility to the drug rather than in its sensitivity to disturbance of histiotrophic nutrition.

CHAPTER IV

SECTION 3

EFFECT OF DIFFERENT DOSES OF AMINO ACIDS ON EMBRYO GROWTH

INTRODUCTION

It is well documented that rat serum is the medium which contains all the nutrient requirements for normal growth and differentiation of rat embryos *in vitro* from day $9\frac{1}{2}$ to day $11\frac{1}{2}$ of gestation (see Chapter I). There is no chemically defined medium which can presently replace serum for growing embryos. Some of the standard tissue-culture media have been tested for their capacity to support embryonic growth, but the results have been very poor (New, 1978; Steel, 1975). It seems essential that at least part of the culture medium is serum. Rat serum contains 72.09 mg/ml total protein of which 25.1 ug/ml is free α-amino nitrogen (Tam & Chan, 1977).

In this section the direct effect of excess amino acids on $10\frac{1}{2}$ day old rat embryos growing for 24 hours was investigated. The aim was to produce a medium (serum) containing the maximum amount of amino acids which would not by itself significantly effect the growth of embryos. This medium was used in the subsequent section, where the possibility of reversing the embryopathic effect of leupeptin on rat embryos *in vitro* was investigated.

The source of amino acids used was a 50X concentrated mixture of the

13 essential amino acids used in Eagles Minimum Essential Medium (MEM). The presence of these amino acids was found to be essential for survival and growth of cell or tissue in any artificial medium (Eagle, 1959).

The amino acid concentrate had a pH of 2.3 and preliminary tests revealed that the addition of 40 ul/ml of amino acid concentrate (giving a final level of amino acids 2 times that of MEM), caused a fall in the serum pH to 6.2. Such acidity is clearly not physiological and is not consistent with normal growth.

For this experiment to succeed it was clear that a means of adding amino acids to the serum must be found which would not, by itself, significantly affect the growth of the embryos. Of the factors thought to be most critical in this was the pH. The acidity of the amino acid treated medium was adjusted until the original pH of the serum was reached, i.e. pH 7.2 to 7.4 by titrating to pH 7.2 with NaoH, but this created problems of its own in that the large quantities of Na^+ added disturbed the serum sodium concentration (normal rat serum level, 123.9 mequiv/L, (Tam & Chan, 1977)), and increased serum osmolarity. The Na^+ concentration and osmolarity were corrected to normal physiological levels by adding distilled water containing glucose and vitamins.

MATERIALS AND METHODS

The amino acids concentrate was obtained from Flow Laboratories Ltd, U.K. used in the formulation of Eagles Minimum Essential Medium (MEM), its composition is shown in Table 1. Ten and a half day old conceptuses were obtained from 14 Wistar rats (Chapter II, D). A pool of immediately centrifuged serum, from 30 rats, was prepared as in (Chapter II, C). Serum was always heat-inactivated before use.

Preparation of Amino Acid Supplemented Serum

Serum was supplemented with 40 ul/ml, 60 ul/ml and 80 ul/ml of the 50X amino acid concentrate (MEM), which gave a final level of amino acids 2, 3 and 4 times that of MEM. The resulting media had a pH of 6.8, 6.45, and 6.1 respectively.

Such acidity is not physiological and therefore it is not consistent with normal embryonic growth. To correct this low pH to physiological levels (pH 7.2-7.4) 1M sterile NaoH was used. This corrected the pH, but resulted in increases serum sodium concentration which in turn shifted the osmolarity of the final solution to a range incompatible with normal embryonic growth.

To correct the high sodium concentration and the high osmolarity, the supplemented serum was diluted with sterile distilled water, containing 1.5 mg/ml D-glucose and the eight vitamins used in the formulation for MEM (Appendix C). The dilution required to correct Na^+ concentration and the osmolarity did not exceed 25%. Such dilution is believed to be compatible with normal embryonic growth (Chapter III, Section 3), but this altered the final concentration of the essential amino acids to a very small degree. A series of experiments were performed using serum samples supplemented with 40, 60 and 80 ul/ml of the amino acids concentrate. This gave a final level about 2, 3 and 4 times that of the Eagles MEM. Each medium was neutralised.

TABLE 1

Show The Composition of the Amino Acid Concentrate

	Amino Acid	mg/ml	ug/40ul	ug/60ul	ug/80ul
1	L-Arginine Hcl	6.32	252.8	379.2	505.6
2	L-Cysteine disodium salt	1.42	56.8	85.2	113.6
3	L-Glutamine	14.615	584.6	876.9	1169.2
4	L-Histidine	2.095	83.8	125.7	167.6
5	L-Isoleucine	2.625	105	157.5	210
6	L-Leucine	2.625	105	157.5	210
7	L-Lysine	3.653	146.12	219.18	292.24
8	L-Methionine	0.745	29.8	44.7	59.6
9	L-Phenylalanine	1.651	66.04	99.06	132.08
10	L-Theronine	2.382	95.28	142.92	190.56
11	L-tryptophan	0.51	20.4	30.6	40.8
12	L-tyrosine	1.811	72.44	108.66	144.88
13	L-valine	2.345	93.8	140.7	187.6
	TOTAL:	42.797 mg	1711.88 ug	2567.87 ug	3423.76 ug

The sodium concentration and osmolarity were corrected as above. Control embryos were cultured in Fresh H.I. serum. Conceptuses $10\frac{1}{2}$ day old were cultured in the above media (1ml/conceptus) for 24 hours as in (Chapter II, D). At the end of the culture period conceptuses were examined and assessed as in (Chapter II, E).

RESULTS

Embryos in the control groups grew normally, exhibiting features identical to those of 11½ day old embryos *in vivo*. The results are summarised in Table 2. Only one embryo of this group had failed to grow normally; this could have been damaged during the explantation procedure or it could be a manifestation of retarded development.

Conceptuses cultured in serum supplemented with 40 ul/ml amino acids were also normal and identical to the control group, (Table 2 and Figure 1). The supplementation of serum with 60 ul/ml amino acid caused a significant impairment in embryo growth.

Table 2 summarises the results, the main yolk sac diameter was reduced by 21% when compared with control. The somite number was reduced by 3, crown-rump length was reduced by 18% and the protein content by 9%.

The neural tube was affected in 25% of the embryos and the defect was manifested as non-closure of the rhombencephalic region and the caudal part (Figure 3).

Turning was incomplete in 29% of the embryos, in most of these the tail was lying to the left of the head. Fore-limb buds failed to develop in 18% of the embryos.

Serum supplemented with 80 ul/ml amino acid was the most toxic to embryonic growth. A summary of the results is shown in Table 2 and Figure 1. Almost all the embryos were abnormal or squirrel-shaped (convex anteriorly) (Figure 4).

The neural tube was widely open at the cranial part in all embryos. The yolk sac diameter was reduced by 20.5%, when compared with control; the somite number was reduced by 10; the crown-rump length was reduced by 43% and the protein content by 34%. Each parameter was significantly inferior to that of the control group at $p < 0.001$, using Student's 't' test.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter in mm ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Control (untreated)	28	27 (96)	27 (96)	28 (100)	27 (96)	27 (96)	27 (96)	28 (100)	28 (100)	4.08 ±0.12	24.72 ±0.45	3.4 ±0.043	205 n=20 ± 4.2
Serum supplemented, 40 ul/ml amino acid	26	26 (100)	23 (88)	26 (100)	26 (100)	25 (96)	26 (100)	26 (100)	26 (100)	3.93 ±0.079	25 ±0.34	3.3 ±0.087	207.98 n=20 ± 7.3
Serum supplemented, 60 ul/ml amino acid	28	27 (96)	24 (86)	28 (100)	21 (75)	20 (71)	23 (82)	28 (100)	28 (100)	3.21 * ±0.076	22 * ±0.66	2.79 * ±0.078	186.4 ** n=20 ± 6.86
Serum supplemented, 80 ul/ml amino acid	16	16 (100)	2 (11)	16 (100)	0 -	0 -	0 -	16 (100)	16 (100)	3.24 * ±0.088	14 * ±0.74	1.94 * ±0.066	135.2 * n=14 ±10.6

Table 2 Growth and development of 10.5 days rat embryos, cultured for 24 hours in Fresh untreated serum and serum supplemented with different doses of amino acids.

Figures in parantheses denote the percentage number of embryos in the group. ± standard error of mean.

Using Student's 't' test;

* Significant at $p < 0.001$ when compared with control.

** Significant at $p < 0.02$ when compared with control.

Figure 1

Comparison of growth of embryos cultured in fresh serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Embryonic protein content.

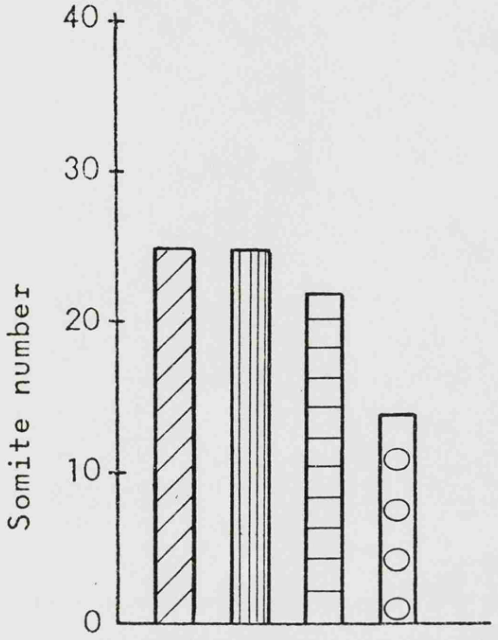
Each rectangle in the histogram represents the mean. The details of numbers of embryos and standard errors are seen in Table 2.

 Fresh H.I. Serum (control).

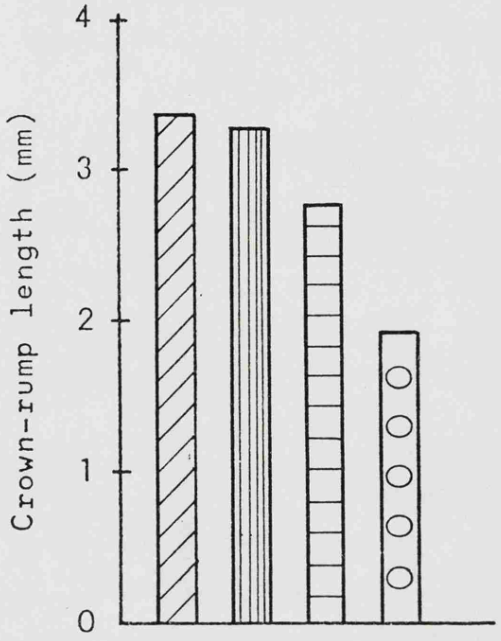
 Serum supplemented with 40 ul/ml amino acids.

 Serum supplemented with 60 ul/ml amino acids.

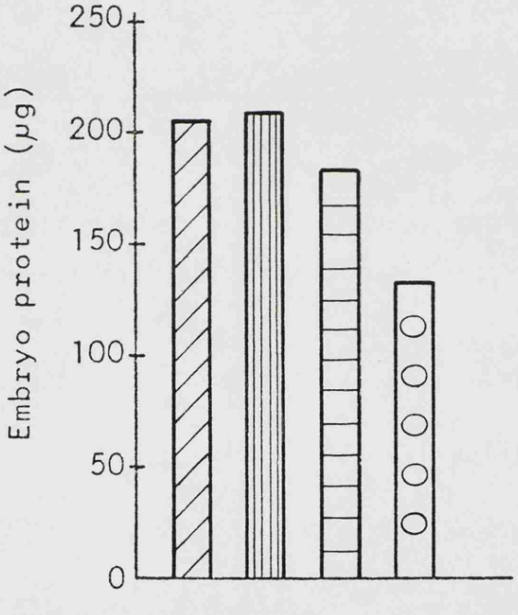
 Serum supplemented with 80 ul/ml amino acids.


Somite number
40
30
20
10
0

A


Crown-rump length (mm)
4
3
2
1
0

B


Embryo protein (μg)
250
200
150
100
50
0

C

Figure 2

Normal rat embryo explanted at 10½ days and grown for 24 hours in untreated serum (control). (Mag x 26).

Figure 3

Rat embryo explanted at 10½ days and grown for 24 hours in the presence of 60 ul of the MEM amino acids concentrate/ml serum culture medium. The neural tube is open in the rhombencephalic region and at the posterior neuropore. Note the abnormal turning. (Mag x 26).

Figure 4

Rat embryo explanted at 10½ days and grown for 24 hours in the presence of 80 ul of the MEM amino acids concentrate/ml serum culture medium. The embryo was grossly abnormal, it failed to turn and was squirrel-shaped. The cranial part of the neural tube is completely open. (Mag x 26).

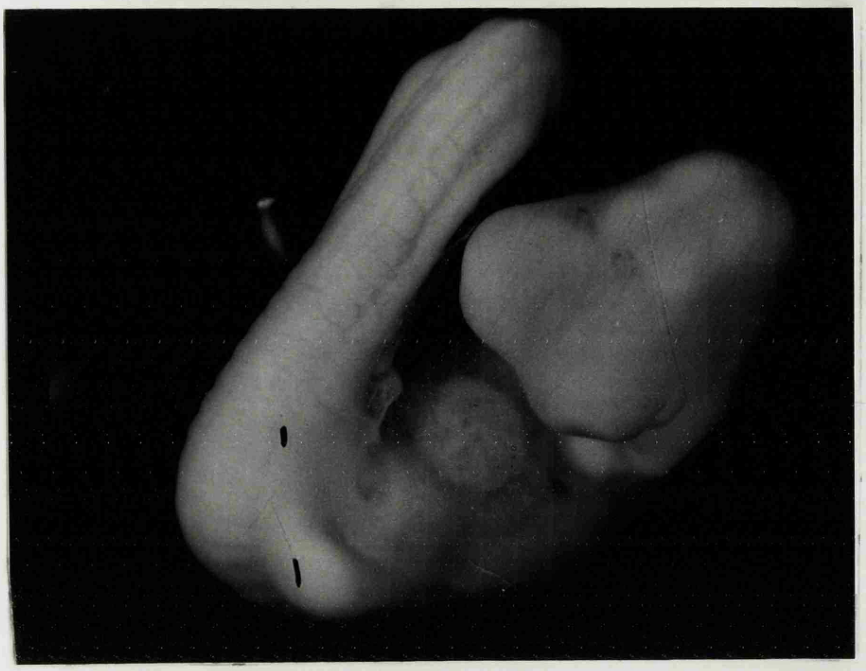


Figure 2.

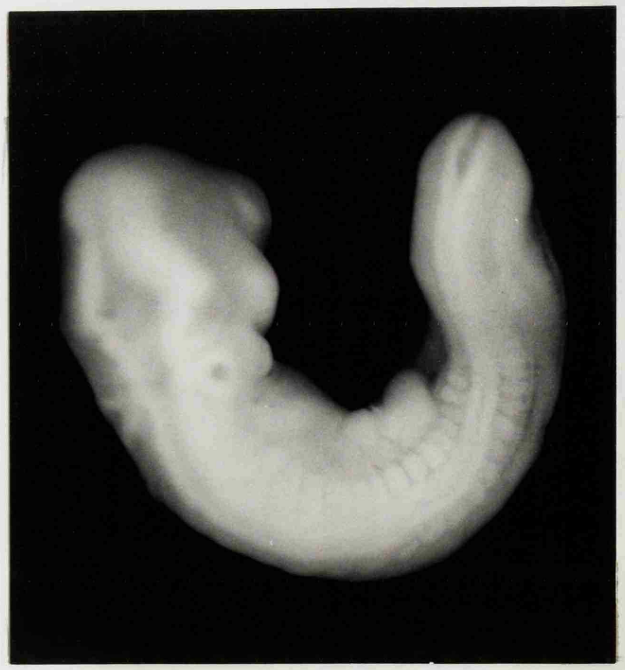


Figure 3.

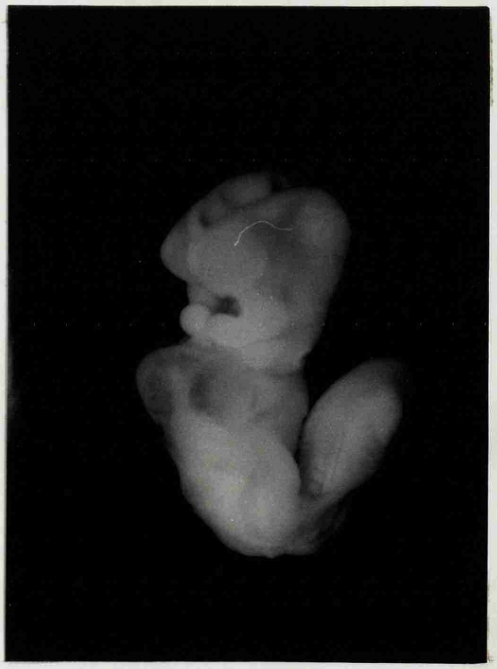


Figure 4.

DISCUSSION

The objective of this study was to establish whether an increase in concentration of the MEM essential amino acids in the culture serum has an effect on the cultured embryo and to establish the concentration of this amino acid mixture which can be added to the serum without affecting embryonic growth.

The results showed that serum supplemented with 40 ul/ml (1.71 mg/ml) MEM amino acids has no effect on the growth. Embryonic development was very similar to that obtained in untreated serum (control). Supplementation of serum with 60 ul/ml (2.56 mg/ml) MEM amino acid had an adverse effect on embryonic growth and differentiation, while serum supplemented with 80 ul/ml (3.42 mg/ml) MEM amino acids supported very little embryonic growth and differentiation.

The toxic effect seen by the presence of high doses of amino acids in serum was unexpected because it is known that during organogenesis, before the establishment of the chorioallantoic placenta, rat embryos derive their nutrients from the maternal histiotroph. Freeman et al., (1981) identified the source and supply route. Exogenous serum proteins and other macromolecules are captured by pinocytosis into the visceral yolk sac epithelial cells, whereupon they are digested within the lysosomes to amino acids, which are used for protein synthesis in both the visceral yolk sac and the embryo. I therefore expected that by raising the amino acid level in the culture medium, I would provide the cultured embryos with extra amino acids which might accelerate embryonic growth. The results have shown otherwise.

The retardation in growth and differentiation in embryos cultured in serum supplemented with high doses of amino acids, may have been due to either the inevitable addition of NaoH and/or the dilution of serum which was necessary to keep the osmolarity and pH within the normal limits.

This could result in disturbances of other electrolytes. Such K^+ and Ca^+ disturbances were found by (Grabowski, 1977) to cause developmental defects. The other possibility is that the high amino acid doses were clearly not physiological and one or more of these amino acids at this high concentration could be toxic by its action on the visceral yolk sac epithelial cells or by a direct action on the embryo. The latter could not be forgotten since amino acids have been shown to be transported to the yolk sac fluid and to the amniotic fluid. The embryos in turn use these amino acids to build up their own proteins. The transport of amino acids across the visceral yolk sac is studied further in Chapter V, section 8 below.

CHAPTER IV

SECTION 4

AN ATTEMPT TO REVERSE THE EFFECT OF LEUPEPTIN BY USING SERUM TREATED WITH AMINO ACIDS

INTRODUCTION

Leupeptin is known to be a specific inhibitor for the hydrolytic lysosomal enzyme cathepsin B, H and L. (Section 1 of this Chapter).

In vitro, the addition of leupeptin to the culture medium of rat embryos $9\frac{1}{2}$ and $10\frac{1}{2}$ days old produces growth retardation and abnormalities of embryonic development (Beck and Lowy, 1982 and the results in Section 2 of this Chapter).

In both studies the hypotheses put forward for the mechanism by which leupeptin produces its embryopathic effect was the possibility that leupeptin inhibits maternal protein breakdown at the level of the visceral yolk sac by the inhibition of cathepsin B, H and L, a process which results in starvation of the cultured embryos of amino acids.

The study described in this section is an investigation of the hypothesis on the mechanism of action of leupeptin on embryonic development. It is possible that if this mechanism is the only way by which leupeptin produces its embryopathic effect, then it might be possible to reverse

these effects simply by providing excess amino acid in the culture medium.

To investigate the above hypothesis, two experiments were performed.

In the first, embryos were cultured in serum treated with 4 ug/ml leupeptin and supplemented with either 40 ul/ml or 60 ul/ml of the 13 essential amino acids concentrate (MEM). While in the second experiment the embryos were cultured in serum diluted 4:6 with M199, a medium which contains 21 amino acids.

The 4 ug/ml leupeptin dose was chosen because it was found (Section 2 of this Chapter) to reduce the protein content per embryo by an average of 50%, when compared to that of the control group. Also it affected all the parameters used to assess embryonic growth. On the other hand, the decision to use the 40 ul/ml and the 60 ul/ml amino acid concentrates was based on the findings in Section 3 of this Chapter.

1. The 40 ul/ml amino acid dose was the maximum amount which can be added to the serum without affecting the embryonic growth.
2. The 60 ul/ml amino acid dose provided an ample amount of amino acids, but it had produced limited embryopathic effects which it was hoped might be neutralised by the amino acid deficiency in the yolk sac fluid created by leupeptin activity at the level of the yolk sac.

MATERIALS AND METHODS

Ten and a half day conceptuses were obtained from 17 Wistar rats (Chapter II, D). Pools of immediately centrifuged serum, each from 20-25 rats, were prepared as described in Chapter II, C, serum was always heat-inactivated before use. Leupeptin was obtained from Peptide Institute Inc. Osaka, Japan as in Section 2 of this Chapter. Leupeptin was again disolved, stored, measured and added to the serum in a similar way to that described in Section 2. As a general rule the leupeptin was added to the serum before the amino acid. The serum, pH, Na^+ concentration and osmolarity were corrected to the normal levels as described in Section 3 of this Chapter.

Experiment 1

In this study 145 conceptuses were used. Conceptuses obtained from each mother were divided into 6 groups, and were cultured for 24 hours, as described in Chapter II, D in the following media:

1. Control serum (Fresh H.I. serum).
   This was to control the growth and differentiation of embryos cultured in experimental media.
2. Serum treated with 4 ug/ml leupeptin.
   This was the reference for the embryopathic effect of this dose of leupeptin.
3. Serum supplemented with 40 ul/ml acid concentrate.
   This was the reference for the growth and differentiation of embryos cultured in serum supplemented with this dose of amino acid.
4. Serum treated with 4 ug/ml leupeptin and supplemented with 40 ul/ml amino acid concentrate.
5. Serum supplemented with 60 ul/ml amino acid concentrate.
   This was the reference for the effect of this dose of amino acid in serum on embryonic growth and differentiation.
6. Serum treated with 4 ug/ml leupeptin and supplemented with

60 ul/ml amino acid concentrate.

Experiment 2

Medium M199 was obtained from GIBCO Europe Limited, Glasgow, U.K. This physiological medium is made in Hanks' Solution and contains 21 amino acids, the amino acids and their concentration are given in Table 1 below. Conceptuses 10½ day old were obtained from 15 Wistar rats. Conceptuses from each mother were divided into 6 groups and cultured (1ml/conceptus) for 24 hours as described in Chapter II, D in the following media:

1. Control (Fresh H.I. serum).
   This was to control the growth and differentiation of embryos cultured in the experimental media.
2. Serum treated with 4 ug/ml leupeptin.
   This was the reference for the embryopathic effect of this dose of leupeptin.
3. Serum diluted 4:6 with Medium M199 treated with 4 ug/ml leupeptin.
4. Serum diluted 4:6 with Medium M199.
   This was the reference for embryonic growth in medium 3 above.
5. Serum diluted 4:6 with Hanks' solution supplemented with 0.5 mg/ml D-glucose and the 8 vitamins from MEM (Appendix C), treated with 4 ug/ml leupeptin.
6. Serum diluted 4:6 with Hanks' solution supplemented with 0.5 mg/ml D-glucose and the 8 vitamins from MEM.
   This was the reference for embryonic growth in medium 5 above.

At the end of the culture period conceptuses were examined and assessed as in (Chapter II, E).

TABLE 1

Showing the Amino Acid Composition of Medium M199 (ug/ml) and their Final Concentrations in the Culture Medium (ug/ml)

		Ug/ml M199	Ug/ml 4:6 Dilution of Serum: M199
1	DL-Alpha Alanine	50.0	30.0
2	L-Arginine Hcl	70.0	42.0
3	DL-Aspartic Acid	60.0	36.0
4	L-Cystine, Hcl H_2O	0.110	0.066
5	L-Cystine	20.0	12.0
6	DL-Glutamic Acid H_2O	150.0	90.0
7	L-Glutamine	100.0	60.0
8	Glycine	50.0	30.0
9	L-Histidine Hcl H_2O	21.88	13.128
10	L-Hydroxyproline	10.0	6.0
11	DL-Isoleucine	40.0	24.0
12	DL-Leucine	120.0	72.0
13	L-Lysine Hcl	70.0	42.0
14	DL-Methionine	30.0	18.0
15	DL-Phyenylalanine	50.0	30.0
16	L-Proline	40.0	24.0
17	DL-Serine	50.0	30.0
18	DL-Threonine	60.0	36.0
19	DL-Tryptophan	20.0	12.0
20	L-Tyrosine	40.0	24.0
21	L-Valine	50.0	30.0
		1101.99 µg/ml	661.194 µg/0.6 ml

RESULTS

1. Experiment 1

A. Controls

All embryos cultured in control serum grew normally exhibiting features identical to that of 11½ day old embryos *in vivo*. The results are summarised in Table 2, Figure 3.

Again, in agreement with the results in Chapter IV, Section 2, the presence of 4 ug/ml leupeptin resulted in a significant impairment of embryo growth (Table 2, Figure 1).

As has been found previously in Section 2 of this Chapter, there was no significant difference (using Student's 't' test) between the growth and differentiation of embryos cultured in this medium and in the control serum.

All embryos cultured in serum supplemented with 40 ul/ml amino acid grew normally (Table 2, Figure 1).

Supplementation of serum with 60 ul/ml amino acids brought about some impairment of embryonic growth and differentiation. The results are seen in Table 2, Figure 1 and are in agreement with the results in Section 3 of this chapter.

B. Experimental

Conceptuses cultured in serum treated with 4 ug/ml leupeptin and supplemented with 40 ul/ml amino acids

All embryos of this group grew abnormally. The results are summarised in Table 2. The differentiation of embryos in this group, in some of the parameters measured, was found to be worse than those of embryos cultured in serum treated with 4 ug/ml leupeptin. The results show that only 65%

of the embryos survived the culture period, with none of them showing the presence of an active vitelline circulation, closure of neural tube (it was open at the cranial part only), Figure 4, full development of the fore-limb buds. At the same time all the embryos had failed to turn completely but most of them were posteriorly convex. Interestingly, a higher percentage of embryos had developed normal optic vesicles when compared to embryos cultured in serum treated with 4 ug/ml leupeptin only, (61%, 37% respectively). Also the mean values for yolk sac diameter, somite number, crown-rump length and protein content per embryo were significantly improved (using Student's 't' test) compared to embryos cultured in serum treated with 4 ug/ml leupeptin.

Conceptuses cultured in serum treated with 4 ug/ml leupeptin and supplemented with 60 ul/ml amino acids.

The state of development of these embryos was significantly inferior to that of the previous set of experimental embryos (Table 2, Figure 1). Only 46% of the embryos survived the culture period. None of them showed the presence of an active vitelline circulation, closure of neural tube (it was open on both cranial and caudal parts), or full development of fore-limb buds. Also all embryos were squirrel-shaped (Figure 5). The mean values for yolk sac diameter, somite number, crown-rump length and protein content per embryo were not significantly different (using Student's 't' test) from values obtained from embryos cultured in serum treated with 4 ug/ml leupeptin.

Experiment 2

All embryos cultured in fresh H.I. serum grew normally, exhibiting features identical to those of 11½ day old embryos grown *in vivo*. The results are summarised in Table 3.

Embryonic growth was markedly affected by the presence of 4 ug/ml leupeptin and all parameters used to assess growth and differentiation

were significantly inferior ($p < 0.001$, using Student's 't' test) to those of embryos cultured in fresh H.I. serum.

Embryos cultured in media consisting of 40% serum + 60% M199, or 40% serum + 60% Hanks' solution supplemented with glucose and vitamins, were all normal, and all parameters used to assess their growth and differentiation were not significantly different from those of embryos cultured in fresh H.I. serum 'Student's 't' test), the results are seen in Table 3 Figure 2.

Growth of embryos cultured in medium consisting of 40% serum and 60% M199 and 4 ug/ml leupeptin is summarised in Table 3. Although all parameters used to assess growth and differentiation of this group of embryos were significantly inferior at $p < 0.001$ (Student's 't' test) to those of embryos cultured in fresh H.I. serum or in serum diluted 4:6 with M199, they still showed an improvement in growth and differentiation over that of embryos grown in serum containing 4 ug/ml leupeptin (Figure 2). Improvements were shown in the mean values of yolk sac diameter which was increased by 34.5% somite number which was increased by average 3 somites, crown-rump length which was increased by 22% and protein content per embryo which was increased by 34.5%. All these parameters were significant at $p < 0.001$ (Student's 't' test). Vigorous vitelline circulation was present in 89% of embryos, 78% of embryos had closed neural tubes, 82% had completed turning, 57% of the embryos had developed paddle-shaped limb buds and 96% of embryos developed normal optic vesicles.

Both growth and differentiation of embryos cultured in serum diluted 4:6 with Hanks' solution containing 4 ug/ml leupeptin were retarded; all parameters used to assess the growth and differentiation of this group of embryos were significantly inferior at $p < 0.001$ (Student's 't' test), to those of embryos cultured in fresh H.I. serum or in serum diluted 4:6

with Hanks' solution (Figure 6). On the other hand, when the growth parameters were compared to those of embryos cultured in serum containing 4 ug/ml leupeptin some parameters from the former group showed significant improvement. The results are shown in Table 3, Figure 2.

	Number of Embryos	Number of Embryos:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter in (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
Control	28	28 (100)	28 (100)	28 (100)	28 (100)	28 (100)	28 (100)	28 (100)	28 (100)	4.02 ±0.146	25.61 ±0.34	3.5 ±0.056	211 n=26 ± 6.2
Serum treated with 4 ug/ml leupeptin	19	18 (95)	10 (53)	19 (100)	6 (32)	6 (32)	5 (26)	7 (37)	19 (100)	2.67 * ±0.081	16.31 * ±0.92	2.24 * ±0.071	93.8 * n=16 ± 7.8
Serum treated with 40 ul/ml amino acid	22	22 (100)	22 (100)	22 (100)	22 (100)	21 (95)	22 (100)	22 (100)	22 (100)	3.8 ±0.064	24.9 ±0.59	3.28 ±0.042	196.8 n=20 ± 6.8
Serum treated with 40 ul/ml amino acid + 4 ug/ml leupeptin	26	17 (65)	0 -	26 (100)	0 -	0 -	0 -	16 (61)	26 (100)	3.32 * ±0.055	18.5 * ±0.73	2.58 * ±0.08	117.4 * n=24 ± 7.7
Serum treated with 60 ul/ml amino acid	22	21 (95)	21 (95)	22 (100)	15 (68)	16 (73)	20 (91)	22 (100)	22 (100)	3.3 * ±0.092	20 * ±0.72	2.6 * ±0.048	165.6 * n=20 ± 8.2
Serum treated with 60 ul/ml amino acid + 4 ug/ml leupeptin	28	13 (46)	0 -	26 (93)	0 -	0 -	0 -	10 (36)	28 (100)	3.11 * ±0.32	15.32 * ±0.76	2.27 * ±0.065	97.4 * n=22 ± 8.5

Table 2 Growth and development of 10.5 days rat embryos cultured for 24 hours in Fresh serum and serum treated with leupeptin alone or with amino acids plus leupeptin.

Figures in parantheses denote the percentage number of embryos in each group ±standard error of mean.

* Significant at $p < 0.001$ when compared with the control group. (Using Student's 't' test).

Figure 1

Comparison of growth of embryos cultured in fresh H.I. serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Embryonic protein content.

Each rectangle in the histogram represents the mean. The details of number of embryos and standard errors are seen in Table 1.

 Fresh H.I. Serum.

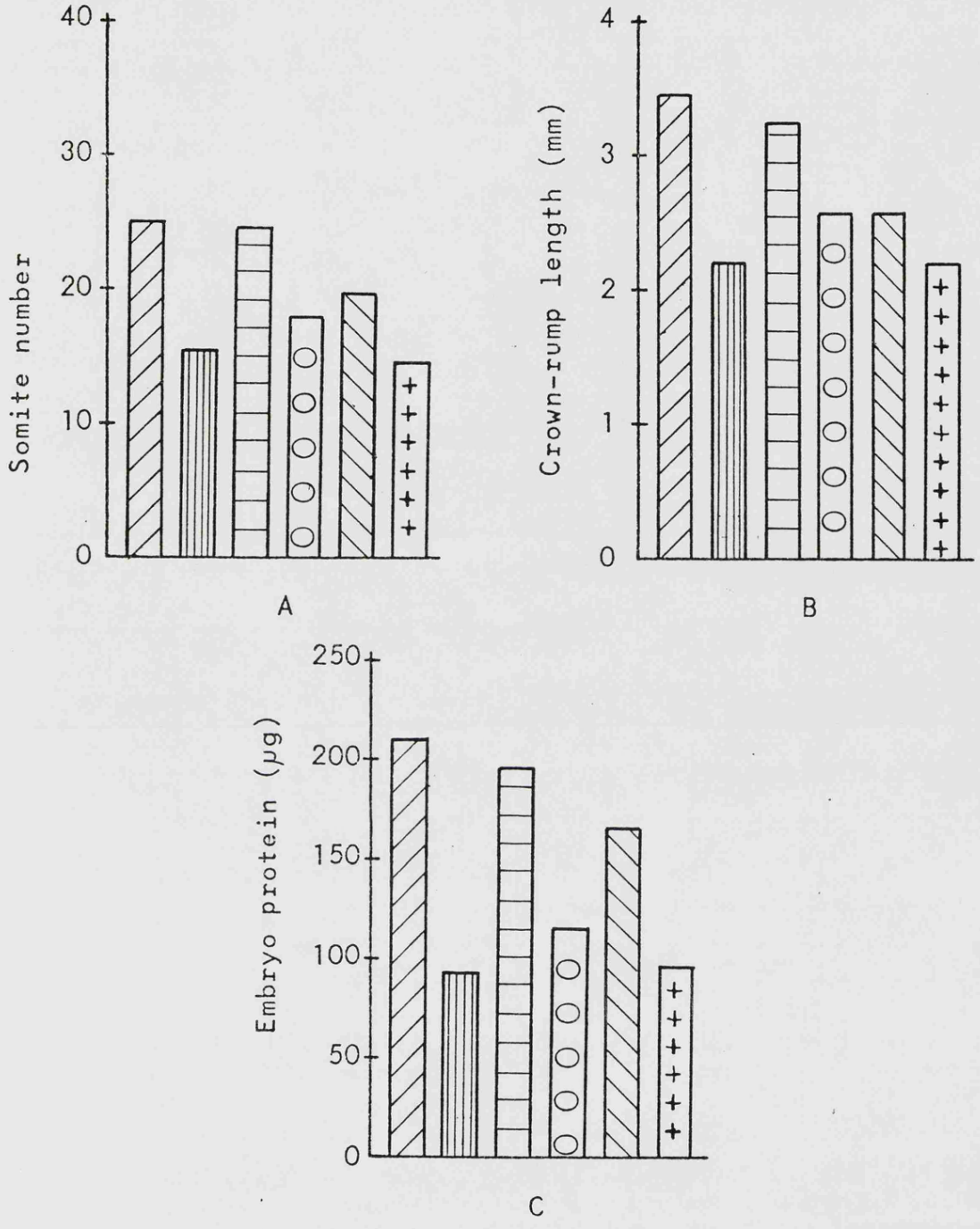
 Serum containing 4 ug/ml Leupeptin.

 Serum containing 40 ul/ml Amino Acids.

 Serum containing 40 ul/ml Amino Acids and 4 ug/ml Leupeptin.

 Serum containing 60 ul/ml Amino Acids.

 Serum containing 60 ul/ml Amino Acids and 4 ug/ml Leupeptin.


Somite number
40
30
20
10
0
A
Crown-rump length (mm)
4
3
2
1
0
B
Embryo protein (μg)
250
200
150
100
50
0
C

	Number of Embryos	Number of Embryos Showing:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter in (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
Control Untreated Serum	18	18 (100)	18 (100)	18 (100)	17 (94)	18 (100)	18 (100)	18 (100)	18 (100)	4.16 ±0.17	25.5 ±0.39	3.43 ±0.13	198.6 n=18 ± 8.4
Serum containing 4 ug/ml leupeptin	18	18 (100)	7 (39)	17 (94)	3 (17)	4 (22)	2 (11)	7 (39)	17 (94)	2.35 * ±0.18	15.6 * ±0.78	2.22 * ±0.07	82.2 * n=18 ± 6
40% Serum + 60% M199	12	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	4.03 ±0.092	24.7 ±0.45	3.5 ±0.7	201.3 n= 12 ±12.6
40% Serum + 60% M199 containing 4 ug/ml leupeptin	28	28 (100)	25 (89)	28 (100)	22 (78)	23 (82)	16 (57)	27 (96)	28 (100)	3.59 * ±0.047	21.28 * ±0.34	2.835 * ±0.07	125.417 * n=24 ± 4.685
40% Serum + 60% Hanks' Solution	12	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)	11 (92)	12 (100)	12 (100)	4.12 ±0.13	24.8 ±0.52	3.4 ±0.66	198.1 n=12 ± 9.8
40% Serum + 60% Hanks' containing 4 ug/ml leupeptin	27	25 (93)	18 (67)	25 (93)	18 (67)	20 (74)	12 (44)	26 (96)	26 (96)	3.511 * ±0.049	18.22 * ±0.378	2.348 * ±0.073	105.088 * n=24 ± 5.184

Table 3 Growth and development of 10.5 days rat embryos cultured for 24 hours in Fresh H.I. serum, serum treated with leupeptin, serum diluted with M199, serum diluted with M199 containing leupeptin, serum diluted with Hanks' solution and serum diluted with Hanks' solution containing leupeptin.

Figures in parantheses denote percentage of the number of embryos in the group ±standard error of mean.

* Significant at $p < 0.001$ when compared with the control (using Student's 't' test).

Figure 2

Comparison of growth of embryos cultured in fresh serum and in different culture media.

The parameters compared are:

A- Somite number.

B- Crown-rump length.

C- Embryonic protein content.

Each rectangle in each histogram represents the mean. The details of numbers of embryos and standard errors are seen in Table 2.

Fresh H.I. Serum (control).

Serum containing 4 ug/ml Leupeptin.

Medium containing 40% Serum plus 60% M199.

Medium 40% Serum and 60% M199 containing 4 ug/ml Leupeptin.

Medium containing 40% Serum and 60% Hanks' Solution.

Medium 40% Serum and 60% Hanks' Solution containing 4 ug/ml Leupeptin.

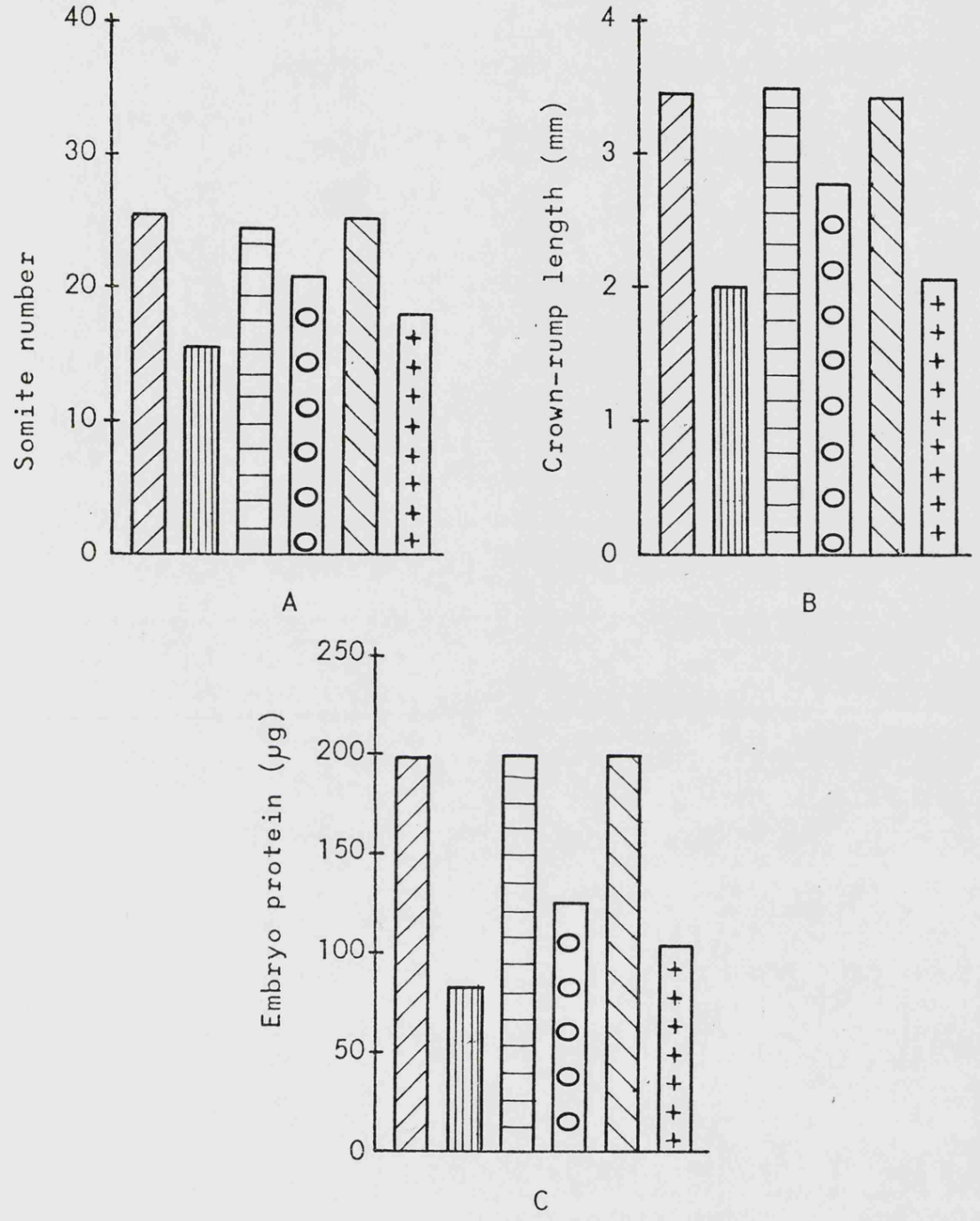

Somite number
40
30
20
10
0
A
Crown-rump length (mm)
4
3
2
1
0
B
Embryo protein (µg)
250
200
150
100
50
0
C

Figure 3

Normal rat embryo explanted at 10½ days and grown for 24 hours in serum (control). (Mag x 22).

Figure 4

Rat embryo explanted at 10½ days and grown for 24 hours in serum treated with 4 ug/ml leupeptin and supplemented with 40 ul/ml MEM amino acids concentrate. The embryo is abnormal, note that the tail is stunted and the cranial part of the neural tube is open. (Mag x 25)

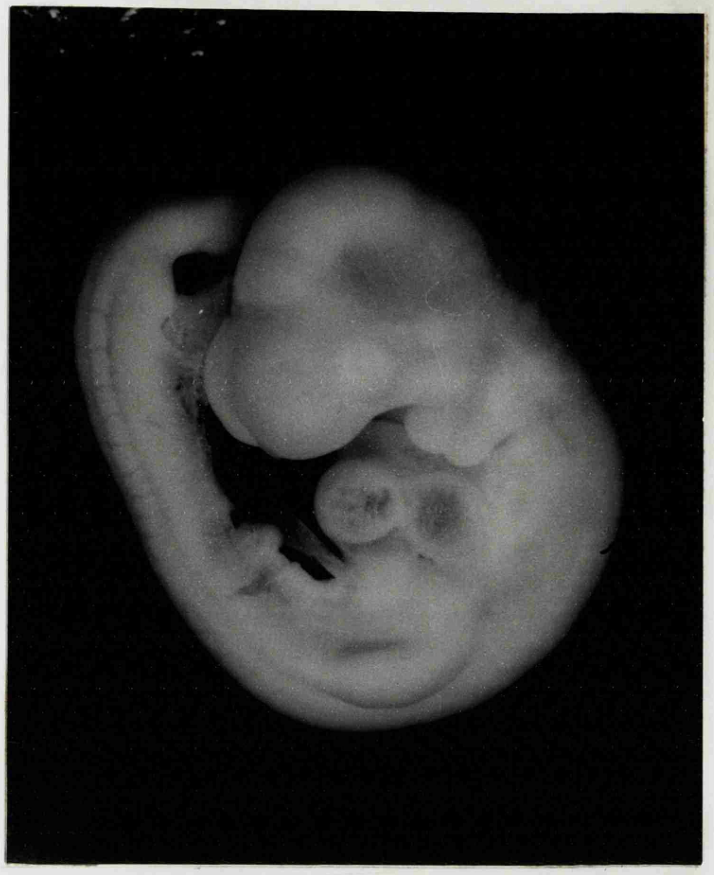


Figure 3.

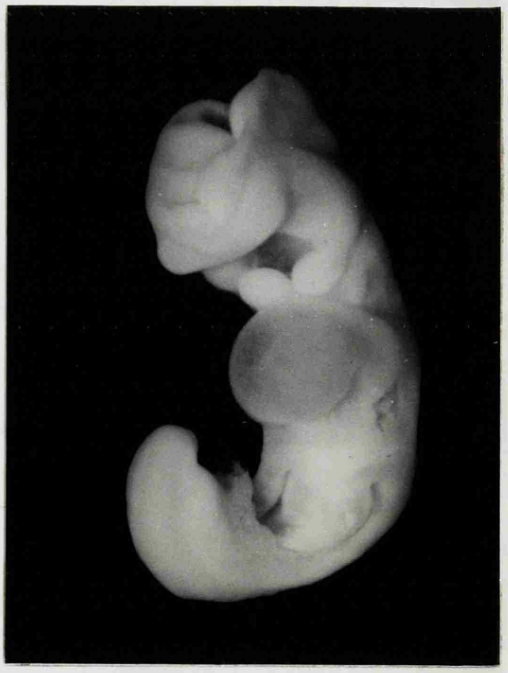


Figure 4.

Figure 5

Grossly abnormal rat embryo. It was explanted at 10½ days and grown for 24 hours in serum treated with 4 ug/ml leupeptin and supplemented with 60 ul/ml MEM amino acids concentrate. The embryo has failed to turn and is squirrel shaped. The neural tube is open completely at the cranial part and the caudal part. (Mag x 30).

Figure 6

Embryos explanted at 10½ days gestation and grown in (A) untreated serum, (B) serum diluted 4:6 with M199 treated with 4 ug/ml leupeptin, (C) serum diluted 4:6 with Hanks' solution treated with 4 ug/ml leupeptin. (Mag x 12).

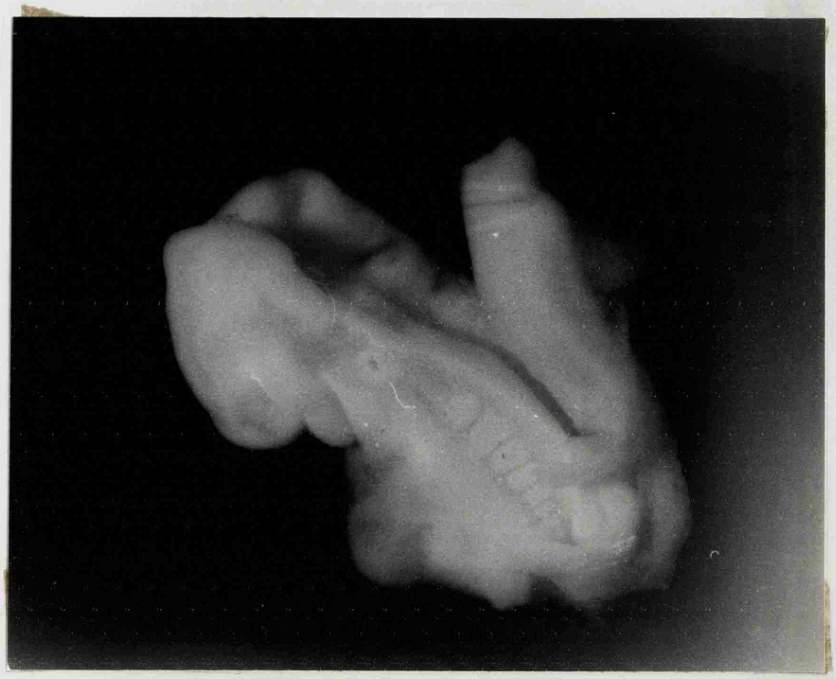


Figure 5.

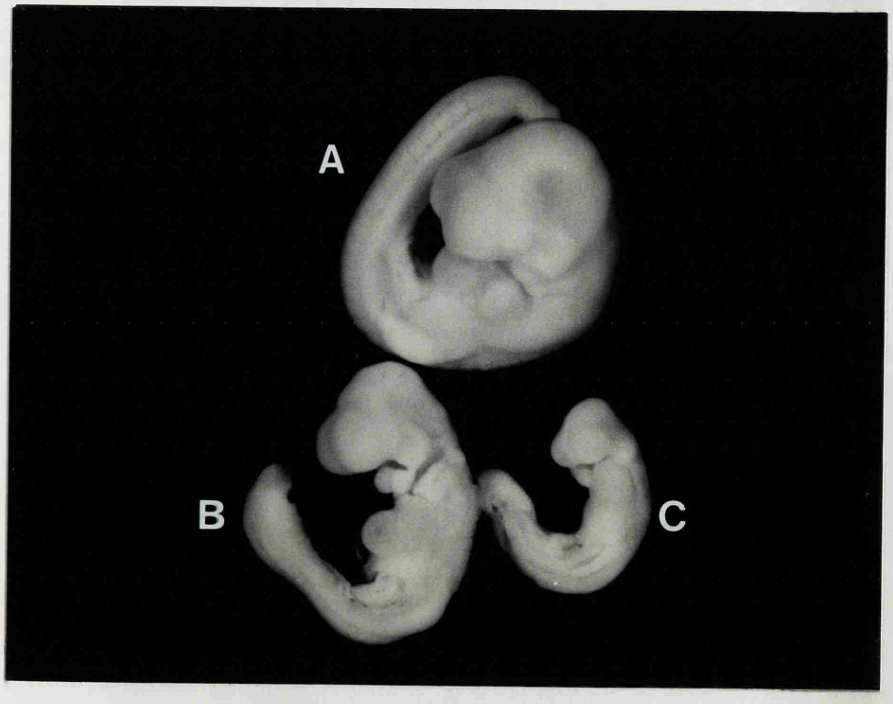


Figure 6.

DISCUSSION

In this study two attempts were made to reverse the embryopathic effect of leupeptin. In the first attempt, leupeptin treated serum was supplemented with 40 ul/ml (1.72 mg/ml) of the essential amino acids. The results showed very little improvement in embryonic growth over that of embryos cultured in leupeptin treated serum. Increasing the concentration of essential animo acid in the culture medium to 60 ul/ml (2.3 mg/ml) was found actually to increase the embryopathic effect of the leupeptin.

From the above we can suggest that the essential amino acids as used in these experiments were not sufficient to compensate for the deficiency in amino acids, resulting from inhibition of protein breakdown by leupeptin in rat embryo culture. It might be possible that embryos could need 'non-essential' amino acids in addition to the essential ones in order to build up their own proteins.

In view of the above suggestion it is interesting that serum proteins contain 20 amino acids, and 20-25 free amino acids can be separated by column chromatography from protein filtrates of plasma, (Yong, 1979). Therefore, the possibility that a wider variety of amino acids are needed cannot be overlooked.

In the second attempt, dilution of serum 4:6 with M199 provides a medium containing 661 ug/ml of 21 free amino acids. Embryos cultured in the above medium showed a significant improvement in all parameters used to assess growth and differentiation over embryos cultured in leupeptin treated serum.

Comparison between results from both attempts, shows a clear superiority of the 21 amino acids from M199 in reversing the embryopathic effect of leupeptin to that of the 13 'essential' amino acids (Tables 2 and 3).

The improvement in growth and differentiation of embryos, achieved in

the second attempt, leads us to believe that the likely deficiency of amino acids supplied to the embryos as a result of leupeptin treatment may to some extent involve amino acids which can be synthesised by the adult organism but remain necessary for embryos to build up their own proteins. Rat embryos during organogenesis can use free amino acids provided in the culture medium for building up their own proteins, at least to a certain extent, but still the amino acids produced from protein breakdown are essential for rat embryos to grow and develop normally. Previous studies by Cockroft (1979) and Freeman et al (1981) are in keeping with the suggestion that the amino acids needed for synthesis of embryonic proteins must be derived from proteins and not from free amino acids.

Two further interpretations of the results can be made:-

a) the relative amount of each amino acid present in the amino acid mixture used is likely to be different from the amounts of the same amino acids produced by the breakdown of protein within the lysosomes.

b) the conditions under which amino acids are transported or diffuse from the lysosome could be changed or shifted away from optimal values when the lysosome is presented with a mixture of free amino acids in the form found in M199 and MEM amino acid composites.

It remains necessary to bear in mind the possibility that in addition to leupeptin's inhibitory effect of protein breakdown, there are other pathways through which leupeptin might affect embryonic growth (Discussion, Section 2 of this Chapter).

CHAPTER FIVE

CHAPTER V

GIANT YOLK SAC

SECTION 1

INTRODUCTION

The importance of the extra-embryonic membranes in normal development in mammals has long been recognised. These membranes constitute inter alia structures essential for the nutrition, excretion, respiration as well as the protection of the embryo and the fetus during development.

The rat visceral yolk sac is the principal organ of embryonic nutrition until the chorioallantoic placenta becomes functional (between days 11 and 12 of gestation). Even after the establishment of the 'true' placenta, the digestive processes in the visceral yolk sac epithelium continue (Beck et al., 1967; Davies, et al., 1969) (see Chapter I for a more detailed review on the role of the rat visceral yolk sac in embryonic nutrition).

Unlike the chorioallantoic placenta the yolk sac placenta can be grown in vitro (Sorokin and Padykula, 1960). Sorokin and Padykula (1964) cultured 13 day old rat yolk sac fragments (1-2 mm) on "solid media" (containing either chicken or human cord serum) for two weeks. Yolk sac fragments were found to be developing and differentiating along the same general lines as in vivo yolk sac. Also, Williams et al., (1975a) described a

method for the *in vitro* culture of 17.5 days rat visceral yolk sac in liquid media consisting of 10% calf serum and 90% Medium 199. They found that tissue survival was good, as judged by light and electron microscopy, even after 24 hours in culture.

The two culture systems outlined above were each designed to study one functional aspect of the yolk sac with consequent experimental exclusion of other possibly related aspects. Thus, these techniques cannot provide adequate experimental models for yolk sac function as a whole.

Rat embryos can now be grown routinely with their visceral yolk sacs intact during most of the period of organogenesis (i.e. from day 9.5 to 11.5) in specially prepared rat serum. The rate and quality of embryonic growth and differentiation *in vitro* is virtually identical to that *in vivo* (Cockroft, 1976). The digestive function of the yolk sac at 9.5 and 11.5 days of gestation has also been found to be identical to that *in vivo* (Gupta, *et al.*, 1982). The limitation of this extremely useful technique is simply the small amount of extra-embryonic material and the products of histiotrophic nutrition it provides.

When $9\frac{1}{2}$ day old conceptuses are cultured in serum by New's technique for a period of more than 48 hours, the embryonic circulation ceases and though the embryos quickly die, the yolk sac continues to grow to reach an extremely large diameter. This is subsequently referred to as the 'giant yolk sac'.

It was decided to take advantage of this phenomenon. It essentially constituted growing the yolk sac as a closed sac beyond the usual 48 hours. Basically, the culture methods developed by New (1976), continued to be used during the period of prolonged growth although minor modifications in the culture protocol were required to make it compatible with the increasing nutritional demands of the growing yolk sac.

In vivo, the rat yolk sac survives only until term (21 days), but *in vitro* the giant yolk sac could be cultured for 14 days (i.e. 2 days beyond "term"). Similarly, Sorokin and Padykula (1964) found that 13 and 15 day old rat yolk sac fragments survive *in vitro* beyond "term". The importance of the giant yolk sac lies initially in its large size which provides enough material for extensive physiological and cytological studies of the yolk sac cells and their products.

Secondly, being a closed system, it can provide a model which has advantages over the previously described method of culture because the products of the yolk sac meant for the embryo are separated from the culture medium. Thus the method of Williams *et al.*, (1975a) for culturing yolk sac *in vitro* has been extensively used to study pinocytic activity, but there is still little information about the secretory function of the yolk sac and its possible regulatory role in the preferential transport of biological materials. It is possible that investigations using the giant yolk sac will provide a working model for such studies.

CHAPTER V

SECTION 2

THE CULTURE OF THE GIANT YOLK SAC

MATERIALS AND METHODS

Nine and a half day old conceptuses were obtained from Wistar rats (Chapter II, D). Pooled immediately centrifuged serum, from batches of 25 - 30 rats, were prepared (see Chapter II, C); serum was always heat-inactivated before use.

Conceptuses were cultured (see Chapter II, D) but gassing at 36 hours was omitted. At the end of the 48 hours' incubation the following procedure was adopted to grow the giant yolk sac:

1. The culture serum was changed at 48-hour intervals, the first and second changes being at the level of 1.5 ml/ conceptus and the third at 2 mls/conceptus.
2. Culture bottles were gassed at 24-hour intervals with 20% O_2, 5% CO_2, 75% N_2.
3. The culture period was extended to 216 hours (9 days) at the conclusion of which the giant yolk sacs were washed 3 times in Hanks' solution, and then transferred to a petri-dish for examination where the yolk sac fluid was collected. Anti-biotics were added to the fluid (Appendix F) which was then

stored in a sterile container at -20°C.

The size of the giant yolk sac and the amount of fluid it contains makes manipulation difficult. The methods used for handling the giant yolk sac are, therefore, described in detail:

1. After day 4 of culture, the culture bottles were permanently held and transported in a horizontal position.
2. For changing the culture serum and washing with Hanks' solution, a syringe with a bent needle was used (Figure 1), the medium being removed very slowly and away from the giant yolk sac. Addition of new medium to the culture bottles was also done very slowly, against the culture bottle wall.
3. At the end of the culture period the yolk sacs were washed and transferred from culture bottles to petri-dishes using the following method:
   a) The Hanks' solution in the culture bottle was made up to 10 mls.
      The culture bottle was inverted slowly, with the index finger sealing its opening (Figure 2).
   b) A staining trough (size: 10 x 10 x 5 cm) was filled with Hanks' solution and the neck of the inverted culture bottle was immersed in the solution (Figure 3).
      Gravity then caused the yolk sacs to pass to the staining trough (Figure 4).
   c) They were then transferred from the staining trough using small petri-dishes (Figure 5).

For comparison, yolk sac fluid from 18½ day old conceptuses <u>in vivo</u> was collected. Wistar rats were killed on the morning of day 19 of pregnancy and conceptuses carefully dissected out with the visceral yolk

sac still intact. The yolk sac fluid was collected with a sterile syringe and antibiotics were added (Appendix F) followed by storage in sterile containers at -20°C.

Estimation of total nitrogen in yolk sac fluid

This was carried out using the Ciocaltean phenol reagent (see Appendix H).

Measurement of glucose levels in yolk sac fluid

This was carried out using the Boehringer glucose oxidase colorimetric standard assay kit (Boehringer MannHeim GmbH, Diagnotica).

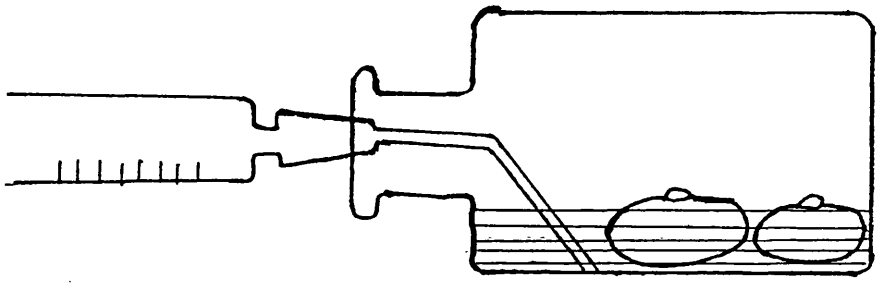


Figure 1

Illustrates the method followed for changing the culture medium, using a specially bent needle.

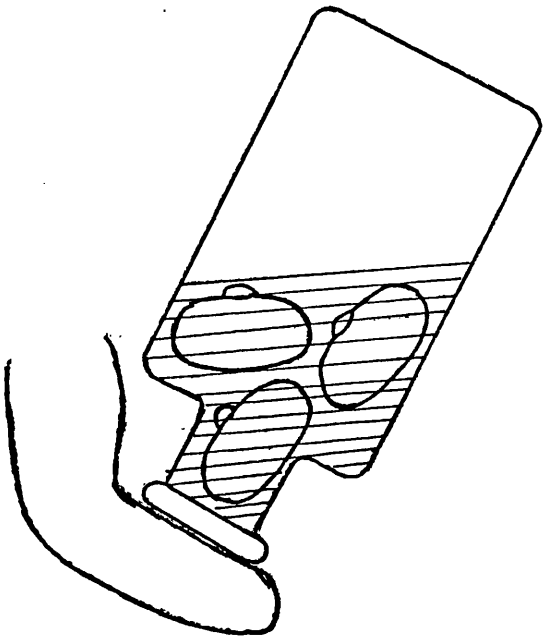


Figure 2

To transfer giant yolk sacs from culture bottles the bottle is inverted slowly with the index finger sealing the opening.

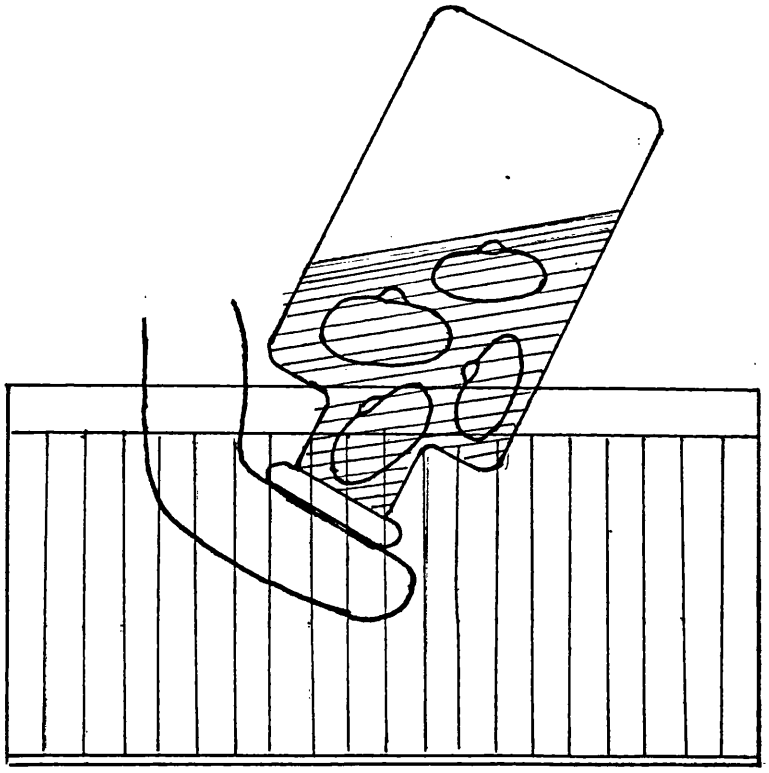


Figure 3

The inverted culture bottle is immersed in Hanks' solution and the index finger is released.

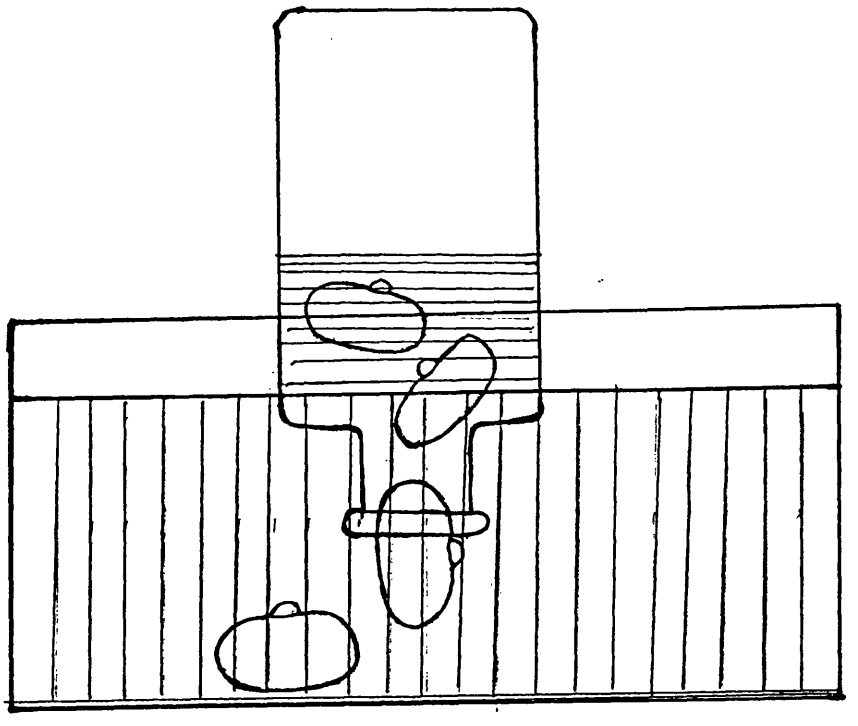


Figure 4

The giant yolk sacs travel freely from the culture bottles into the staining trough.

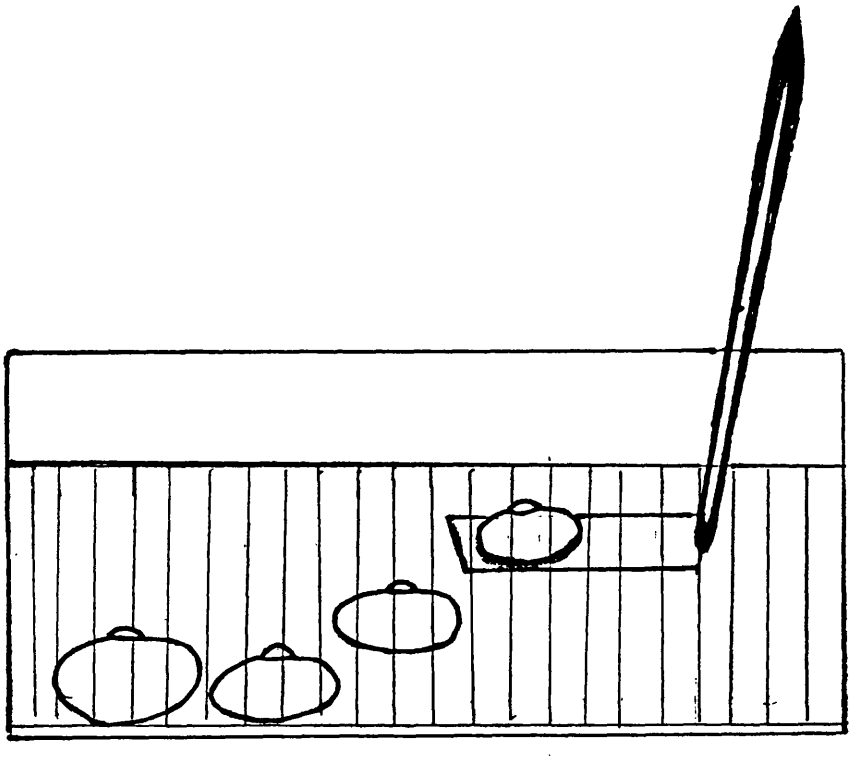


Figure 5

Transfer of giant yolk sacs by petri-dishes for examination.

Results

The thirty-six 9½ day old conceptuses cultured for 9 days (216 hours) had a mean visceral yolk sac diameter of 19.0 mm (Figure 6), forming a membrane which enclosed the amniotic sac and the embryonic remains.

The ectoplacental cone was still present.

Under the dissecting microscope:

i) vitteline capillaries were seen to be collapsed;

ii) the allantois was always well developed and connected to the ectoplacental cone;

iii) the amniotic sac, the diameter of which varied in size from 40 mm to 100 mm, appeared spherical in shape and contained embryonic remnants.
The quantity of fluid collected from giant yolk sacs varied from 400 ul to 750 ul, depending upon the degree of amniotic sac development. The volume of the amniotic fluid varied between 150 ul and 350 ul.

iv) the embryos were always dead, most probably due to inadequate nutrition and an insufficient oxygen supply.
The dead embryos in the giant yolk sacs were always surrounded by the amniotic sac and, in most cases, fragmented.

Comparison of the giant yolk sac diameter with that of the yolk sac of 18½ day *in vivo* conceptuses showed the former to be smaller than the latter by an average of 4.3 mm and the difference was significant at $p < 0.001$, using student's 't' test (Table 1). The total nitrogen content in the giant yolk sac fluid (Table 1) was significantly higher than that in the yolk sac fluid collected from 18½ day old conceptuses grown *in vivo* ($p < 0.001$, using student's 't' test) and this could be the result of continuous synthetic and transport activity of the growing giant yolk

sac in the absence of a live embryo.

On the other hand the glucose concentration in the giant yolk sac fluid was significantly less than in the *in vivo* comparison ($p < 0.001$, using student's 't' test) and this could be due to the exhaustion of the glucose from the limited culture medium *in vitro*, or perhaps consumption by giant yolk sac cells rather than passage into the cavity.

In the culture method adopted for growing giant yolk sacs the frequent change of the culture medium and the gradual increase in the volume per conceptus was designed to meet the increasing nutritional demand of the growing yolk sac and to avoid the accumulation of toxic material and osmolarity changes which could affect both the yolk sac and early embryonic growth (see Chapter III, Section 1).

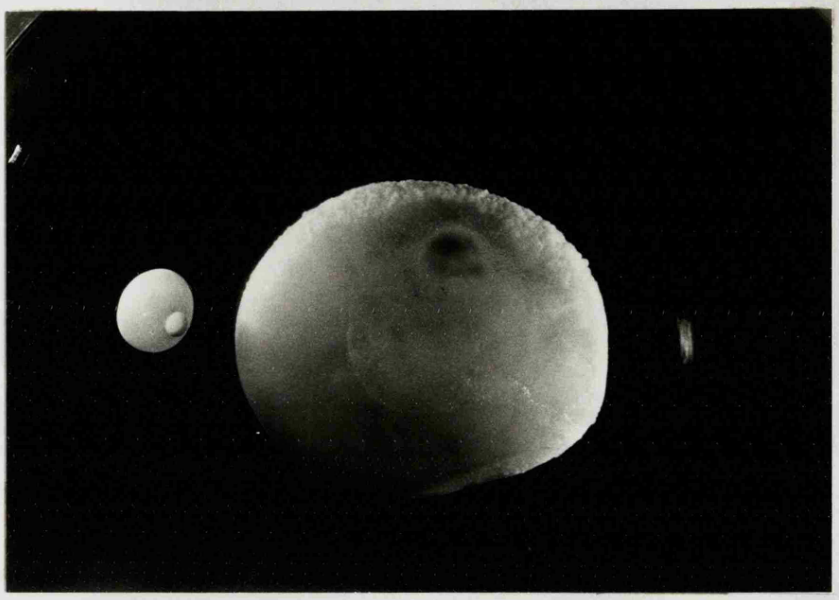


Figure 6.

Photograph showing the difference in size between the giant yolk sac(right) and 11½ day yolk sac(left) Mag X 2.5.

TABLE 1

Comparison Between Giant Yolk Sac and 18½ Day Conceptuses Grown in vivo. Results represent Mean ± S. E. M. Using Student's 't' Test, the Giant Yolk Sac Diameter was significantly different from the 18½ day yolk sac diameter. There were significant differences in the nitrogen content and glucose concentration of yolk sac fluids

* = $p < 0.001$

	Giant Yolk Sac	18½ Day Conceptus
Yolk Sac Diameter in (mm)	19.0 ± 0.36 n=36	23.3 ± 0.81 n=20 *
Total Nitrogen Concentration of Yolk Sac Fluid in mg/ml	5.27 ± 0.151 n=12	2.92 ± 0.116 n=12 *
Glucose Conentration of Yolk Sac Fluid in mg/ml	0.18 ± 0.016 n=10	0.79 ± 0.033 n=10 *

CHAPTER V

SECTION 3

MORPHOLOGICAL STUDIES ON THE GIANT AND 18½ DAY VISCERAL YOLK SACS

Aim

In this section the ultrastructure of the giant yolk sac will be studied and compared to the 18½ day visceral yolk sac from *in vivo* material.

Materials and Methods

The morphological studies on the giant yolk sac and the 18½ day visceral yolk sac grown in vivo were performed on specimens obtained as shown in the chart overleaf (p.151).

Tissue pieces were fixed in 3% freshly prepared glutaraldehyde fixative in 0.1 M phosphate buffer at pH 7.3 (Sabatini, Bensch and Barnett, 1963) for a period of 2 hours at 4°C. They were then washed 3 x 30 minutes in a phosphate buffer containing 6.84% sucrose.

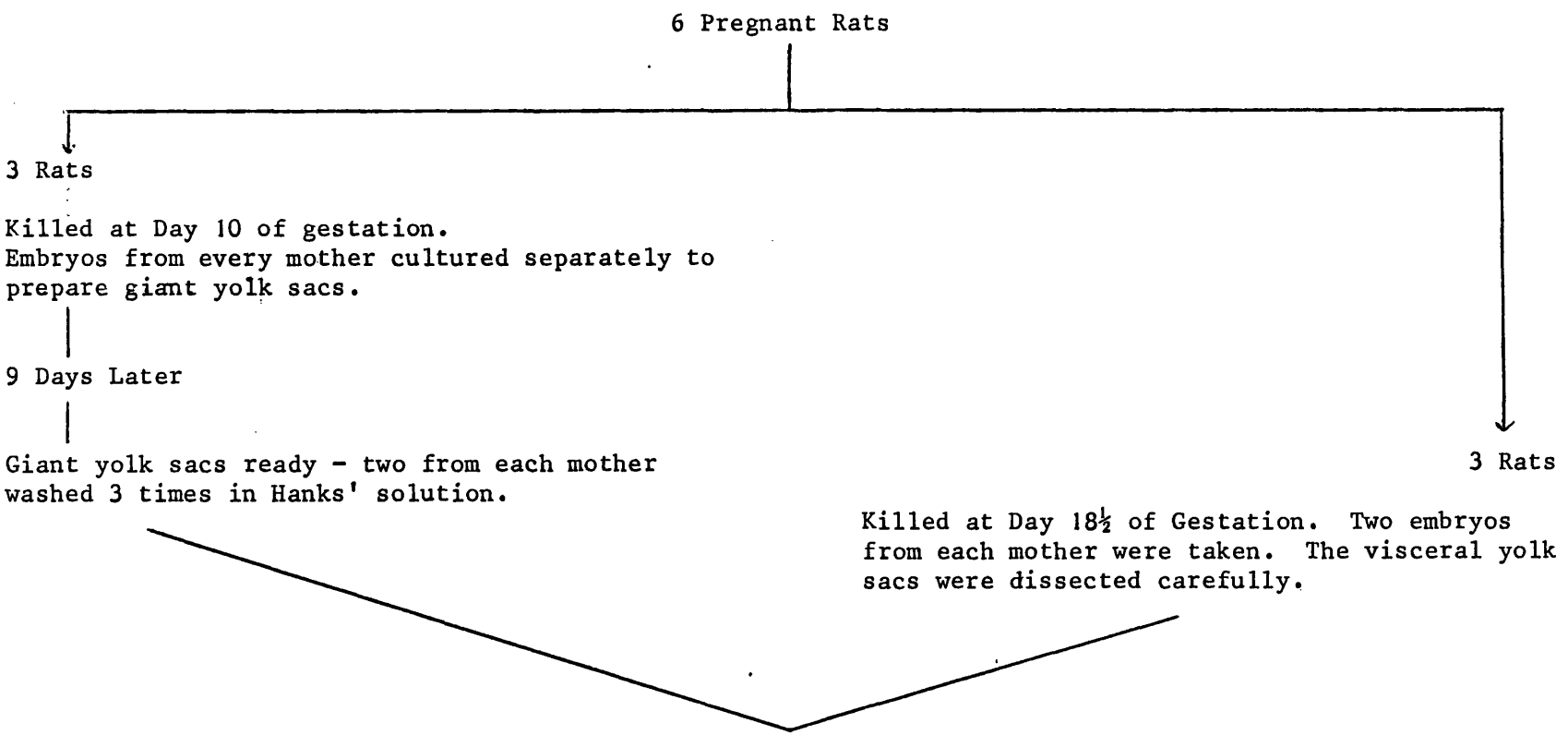
The tissue was left overnight in sucrose phosphate buffer at 4°C.

Secondary fixation was effected in 2% osmium tetroxide in water for 1 hour at 4°C and the tissue then washed several times in distilled water followed by dehydration in increasing concentrations of ethanol. The dehydrated tissue was cleared in propylene oxide for 20 minutes, left overnight in a 1 : 1 mixture of propionyl acetate and araldite containing an accelerator and then placed (with fresh araldite plus accelerator) in silicone rubber embedding moulds. These were then left in an oven at 60°C for polymerisation over a period of 48 hours.

Using glass knives made with an LKB knife-maker thin pale gold/silver sections were cut on a Reichert or OMU-4 ultramicrotome and copper 200 mesh grids were used to lift them. They were stained first with 10% uranyl acetate in absolute methanol then with lead citrate for 4 - 5 minutes according to the method of Reynolds (1963), after which they were washed in filtered distilled water, dried and stored to await examination.

The sections were examined in a JEOL 100-S electron microscope operating at 80 kv.

SPECIMEN COLLECTION CHART



Tissue pieces measuring 3 x 4 mm were taken from specific areas (see diagram 1) of the villous part of the visceral yolk sac and fixed for electron microscopy.

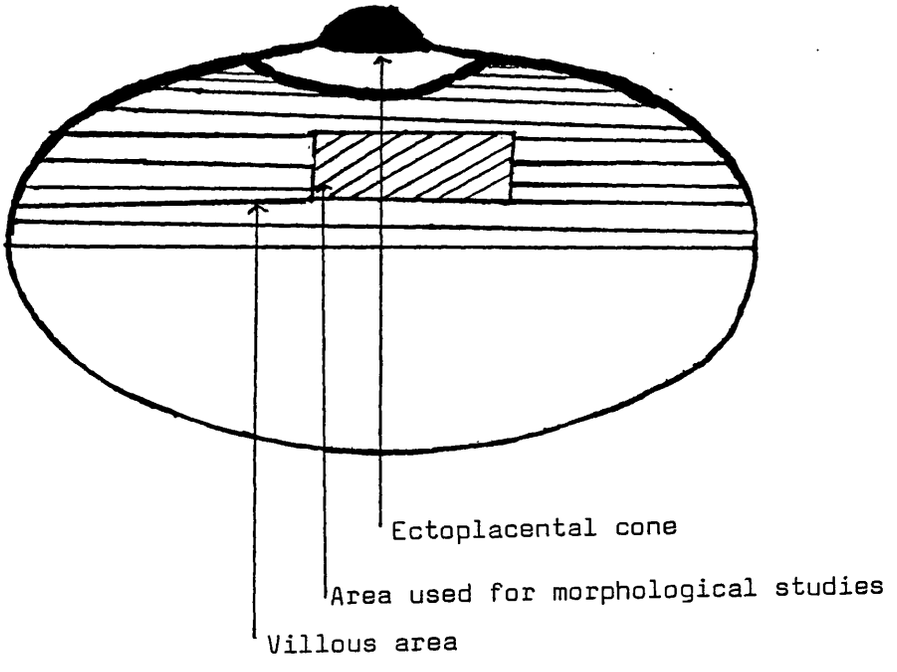


Diagram 1

Showing the giant yolk sac and the area of visceral yolk sac endoderm used for morphological studies.

Results

Examination of the sections showed that the giant yolk sac is composed of three cellular layers separated by two basement membranes (Figure 7).

i) the outermost endodermal layer consists of simple columnar epithelial cells (Figure 1) resting on the visceral basement membrane (Figure 5), which separates it from the second cellular layer;

ii) the second cellular layer comprises scattered mesenchymal cells and fetal capillaries (Figures 7 and 5);

iii) the third cellular layer, which lines the exocoelom, consists of flattened mesothelial cells and is separated from the second cellular layer by means of the serosal basement membrane (Figure 7).

Examination of the visceral endodermal cells showed that the plasma membrane on the free surface forms a microvillous border between which caveolae are present.

At the bases of the microvilli the plasma membrane frequently forms deep tubular invaginations, the fuzzy surface coat of which is quite conspicuous and the membrane appears more dense, forming coated vesicles (Figure 9).

The cytoplasm beneath this level is occupied by numerous, relatively large vacuoles - some stained homogenously whilst others are filled with material of varying electron density (Figures 1 and 3). Presumably the empty-looking vacuoles arise from the canalicular system (Figures 3 and 9) and the homogenously staining vacuoles represent absorptive profiles that have moved deeper into the cytoplasm.

The supranuclear cytoplasm below the superficial canalicular system also contains mitochondria, granular endoplasmic reticulum and free

ribosomes (Figures 3 and 5). The Golgi elements are mainly in a paranuclear position and the nuclei of the epithelial cells (Figure 1) are basally located.

The sections revealed typical junctional complexes between all epithelial cells in the giant yolk sac. The junction is composed of three elements:

(i) the tight junction (zonula occludens);

(ii) intermediate junction (zonula adherens), and

(iii) desmosome (macula adherens).

At the tight junction the adjacent outer leaflets of adjacent cell membranes are apparently fused. At the base of the junctions the membranes diverge and the second element of the complex, the intermediate junction, (Figure 11) begins; this is characterised by the presence of a true intercellular space measuring approximately 200 A across. The cytoplasm along this junctional element is dense and has a fine fibrillar texture. The desmosome (Figure 11) is usually located at a distance from the basal end of the intermediate junction. Here the intercellular space is about 240 Å across and is occupied by a disc of moderately dense material.

The sections show that the desmosome consists of 2 straight parallel plaques of dense material with bundles of cytoplasmic fibrils that converge on the inner aspect of the plaques.

The intercellular spaces in the apical one-third of the lateral cell margins are moderately convoluted (Figure 3). The basal two-thirds of these spaces are frequently dilated (Figure 1) which results in a loose and irregular association of the lateral cell surfaces. Shelf-like and finger-like projections extend across the intercellular spaces to interdigitate loosely and irregularly (Figure 3).

The basal plasma surface is somewhat irregular in contour; finger-like projections of the surfaces extend into the visceral basement membrane

and coated vesicles are frequently seen just within the cytoplasm in this area.

Sections from 18½ day *in vivo* material showed the visceral yolk sac to be composed of three cellular layers separated by two basement membranes, as in the giant yolk sac (Figures 2 and 8).

Although the general morphology in the 2 yolk sacs was similar (Figures 2, 4, 6, 10 and 12), the following distinguishing points of the 18½ day yolk sac derived from *in vivo* conceptuses could be demonstrated:

1. in the visceral endoderm cells glycogen areas were seen in the paranuclear region (Figures 2, 6 and 12), a feature not present in the giant yolk sac;
2. the serosal basement membrane was thicker in 18½ day visceral yolk sacs and contained more collagen fibres (Figure 8).

Figure 1

Giant Yolk Sac (visceral)

This micrograph shows details of the first cellular layer (composed of columnar epithelial cells) of visceral yolk sac. The free surface of these cells possesses microvilli (mv).

The apical cytoplasm contains fine tubules and vacuoles (v) of varying density.

Granular endoplasmic reticulum (ER) is mainly present in the perinuclear cytoplasm. The lateral cell junctions (Jc) are intact. (Mag x 7,560).

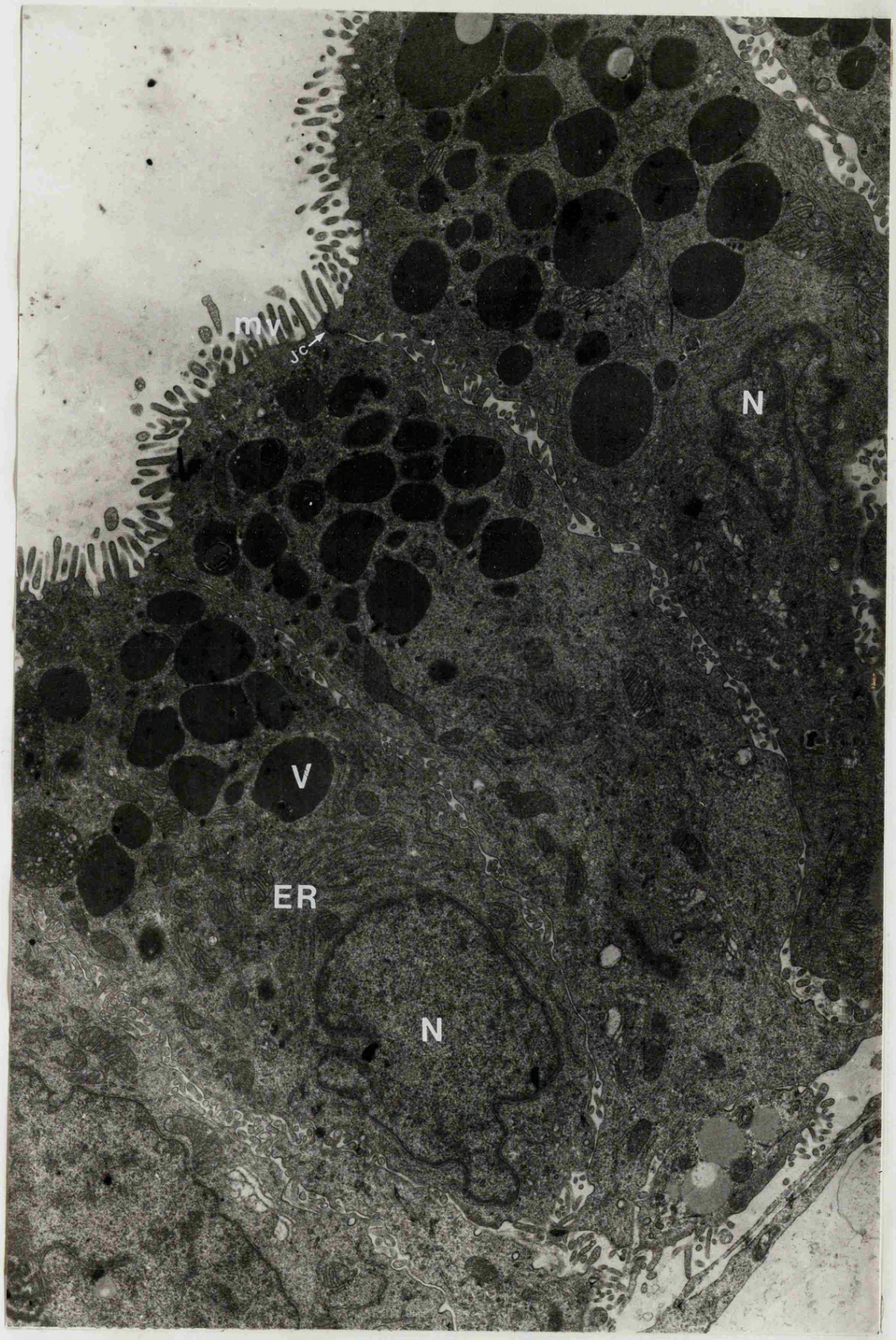


Figure 1.

Figure 2

Eighteen and a half days visceral yolk sac in vivo:

The composition of the visceral yolk sac is shown in this micrograph.

The columnar epithelial cells with well-developed microvilli are spearated from the mesenchymal cells (M) by the visceral basement membrane (VBM). The cytoplasm contains vacuoles, granular endoplasmic reticulum (ER), glycogen deposition (Gly) and nuclei (N). The lateral cell junctions (Jc) are intact. (Mag x 5,335).

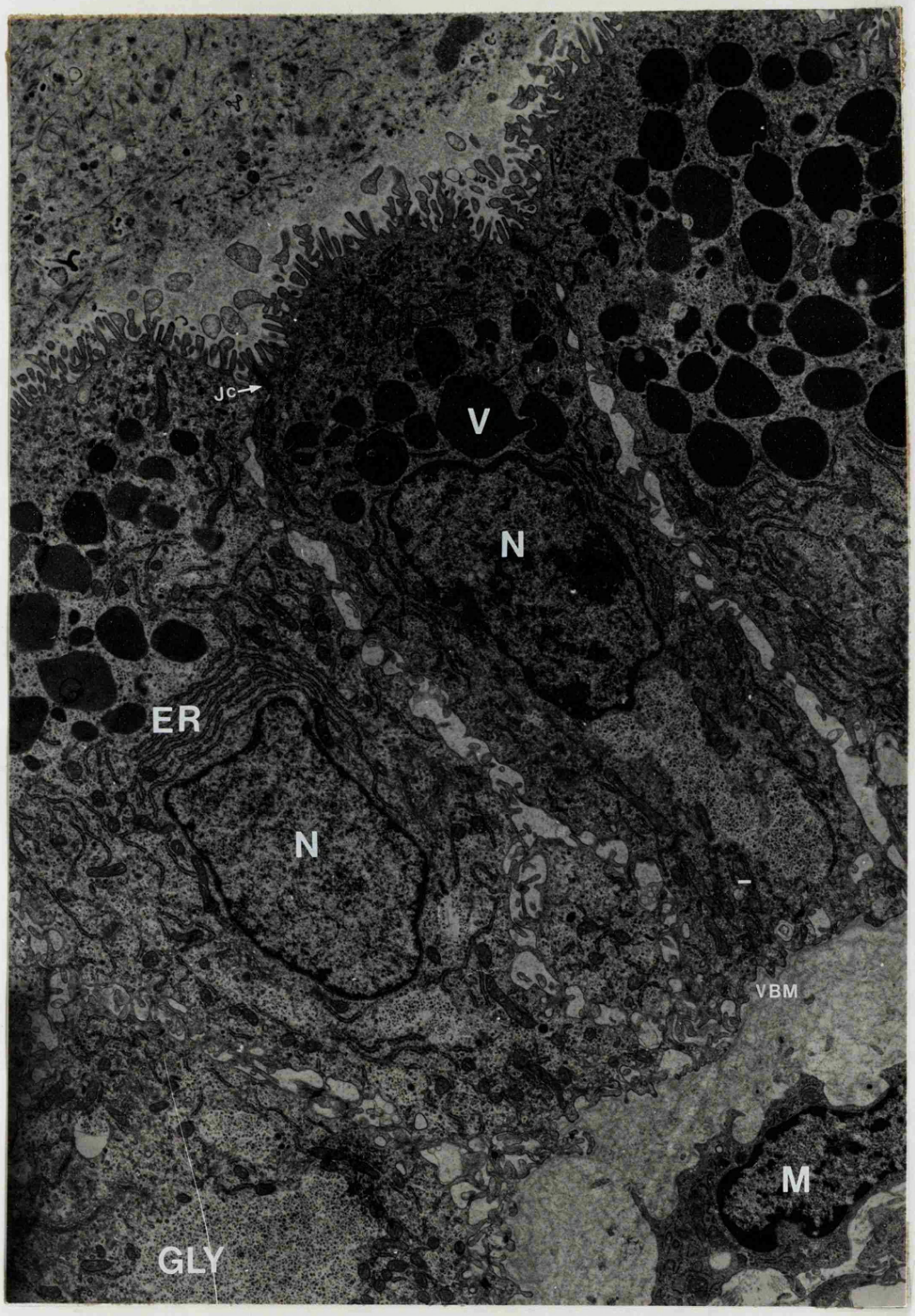


Figure 2.

Figure 3

Giant Yolk Sac (Visceral).

On the free (maternally exposed) surface of the epithelial cells, there are numerous microvilli (mv). The surface plasma membrane invaginates to form cavulae (arrows).

Tubules or canaliculae (c) are located beneath the surface plasma membrane, large vacuoles (V) of varied density occupy most of the supra-nuclear cytoplasm, which contains mitochondria (M) and cisterne of granular endoplasmic reticulum (ER).

Junctional complexes (Jc) seal the junctions near the free surface. (Mag x 12,880).

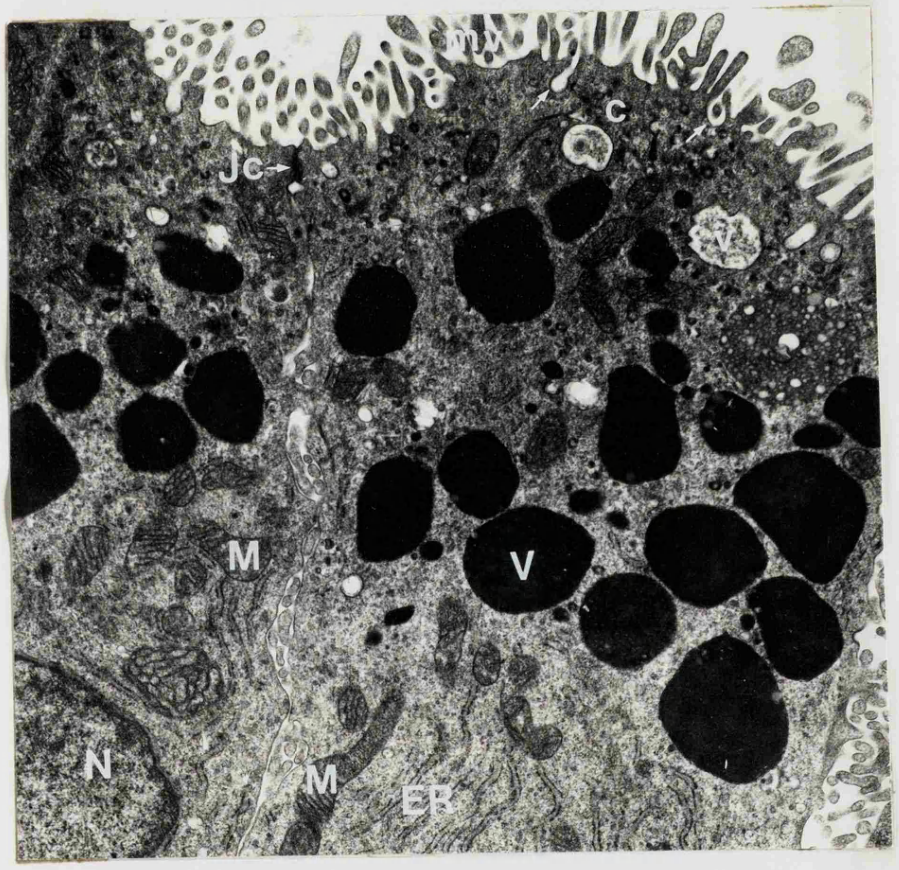


Figure 3.

Figure 4

Eighteen and a half days Visceral Yolk Sac in vivo.

On the free surface of the epithelial cells, there are numberous microvilli (mv). The surface plasma membrane invaginates to form cavulae (arrows).

Dense-walled tubules or canaliculae (c) are located beneath the surface plasma membrane, large vacuoles (v) of varied density occupy most of the supra-nuclear cytoplasm. There are also mitochondria (M) and cisterne of granular endoplasmic reticulum (ER).

Junctional complexes (Jc) seal the junctions near the free surface. (Mag x 15,400).

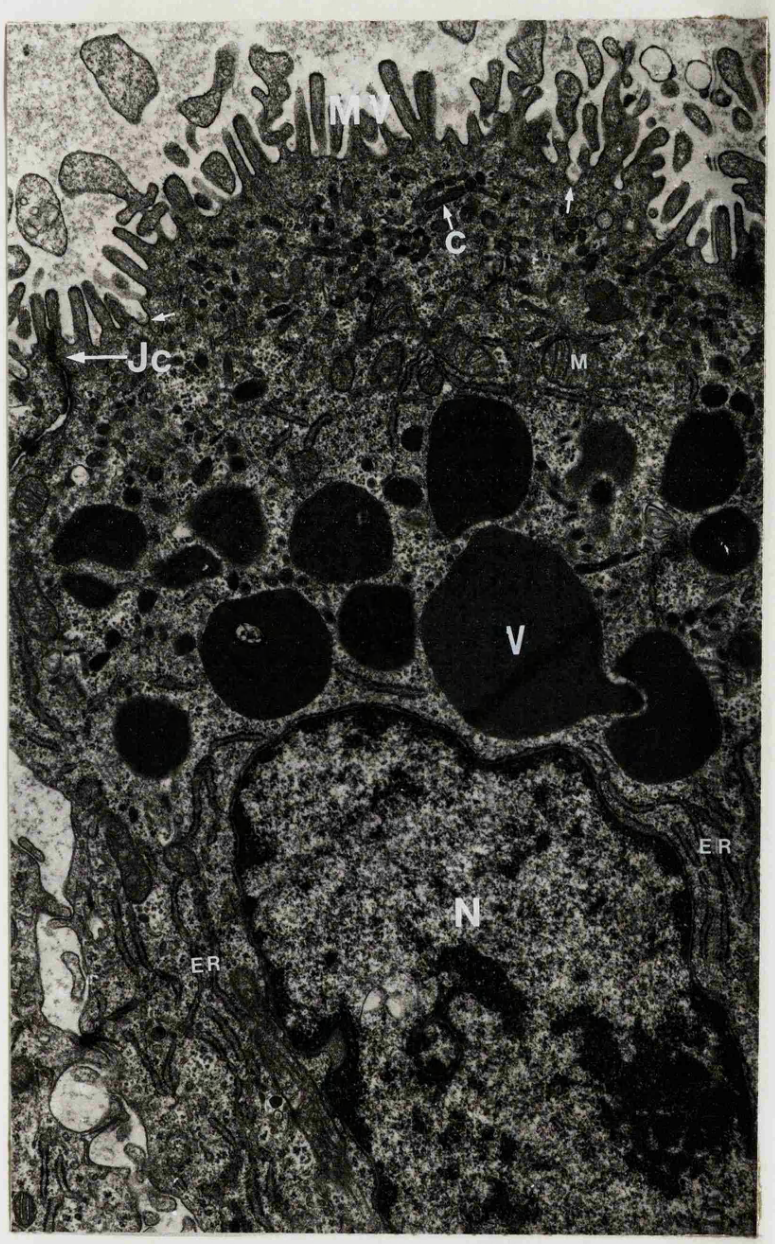


Figure 4.

Figure 5

Giant Yolk Sac (Visceral).

The basal area of the columnar epithelial cells is shown. The cytoplasmic region is filled with mitochondria (M) and cisterne of the granular endoplasmic reticulum (ER). The basal plasma membrane rests on the visceral basement membrane (VBM), which is irregular in form sending finger-like and foot-like processes (arrows) into the substance of the basement membrane. Beneath the visceral basement membrane is the second cellular layer (Mesenchymal cells), it rests on the serosal basement membrane (SBM). (Mag x 16,100).

Figure 6

Eighteen and a half days Visceral Yolk Sac in vivo.

The basal area of the columnar epithelial cells is shown. The surface rests on the visceral basement membrane (VBM) and is irregular in form, sending processes (arrows) into the substance of the visceral basement membrane.

The columnar epithelium cell cytoplasm contains glycogen deposition (Gly). There are also cisterne of the granular endoplasmic reticulum (ER), mitochondria (M), golgi apparatus (G) and coated vesicles (cv). In both visceral and serosal basement membranes (SBM) there are collagen fibres (c). (Mag x 19,600).

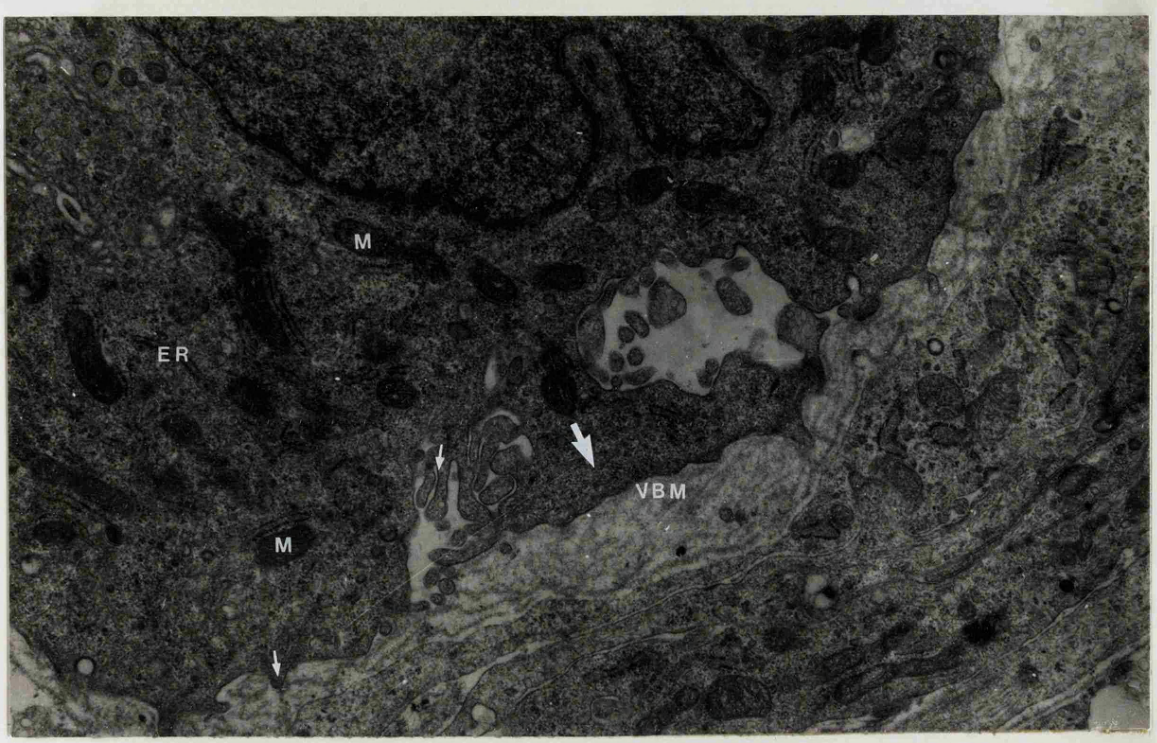


Figure 5.

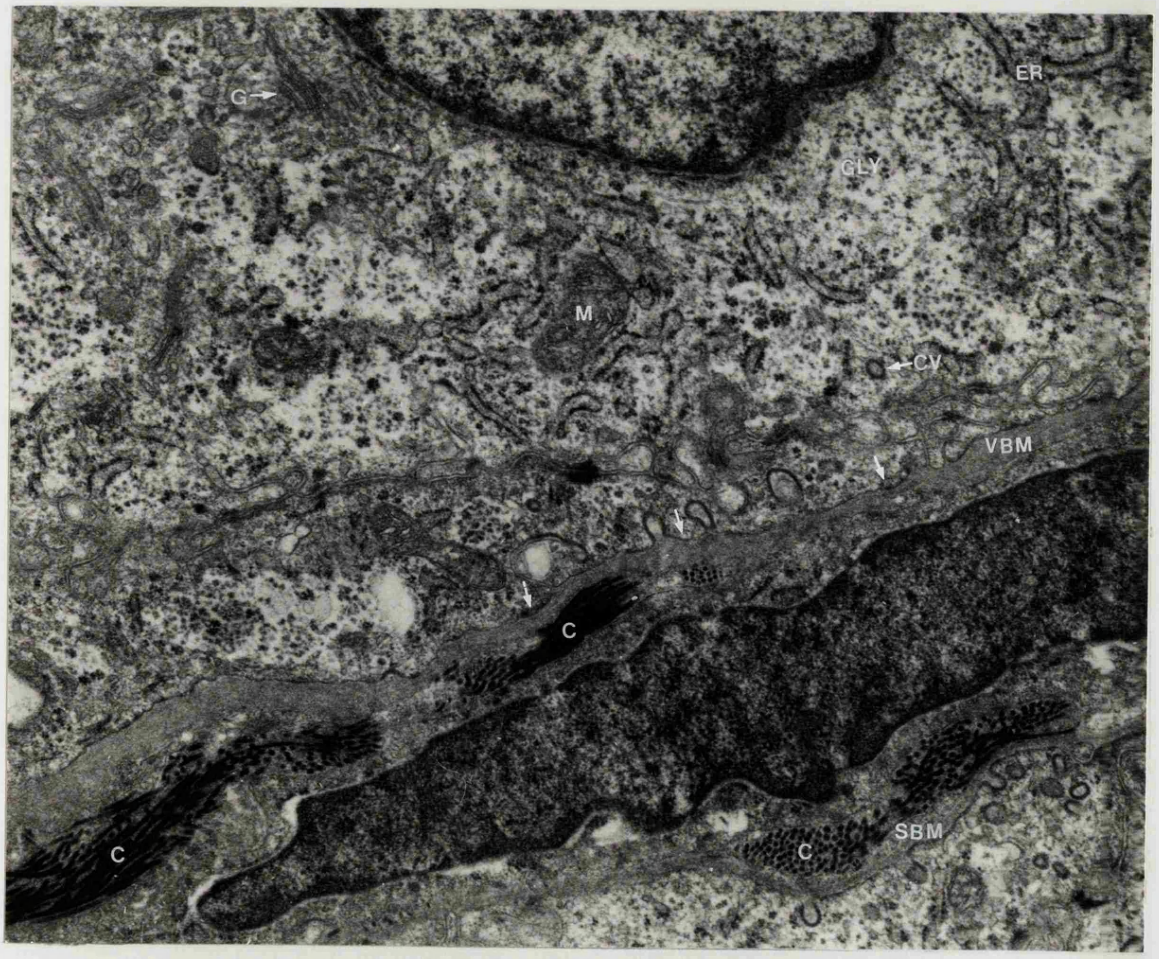


Figure 6.

Figure 7

Giant Yolk Sac (Visceral).

This micrograph shows the basal area of the columnar epithelium. The main features are the visceral basement membrane (VBM), the mesenchymal cells (M) of the second cellular layer, the serosal basement membrane (SBM) containing collagen fibres (c) and the mesothelial cells (Mes). (Mag x 17,500).

Figure 8

Eighteen and a half days Visceral Yolk sac in vivo

This micrograph shows the basal area of the columnar epithelium. The main features include the visceral basement membrane (VBM), the mesenchymal cells (M) of the second cellular layer, a thick serosal basement membrane (SBM) containing collagen fibres (c) and the mesothelial cells (Mes). (Mag x 36,750).

Figure 7.

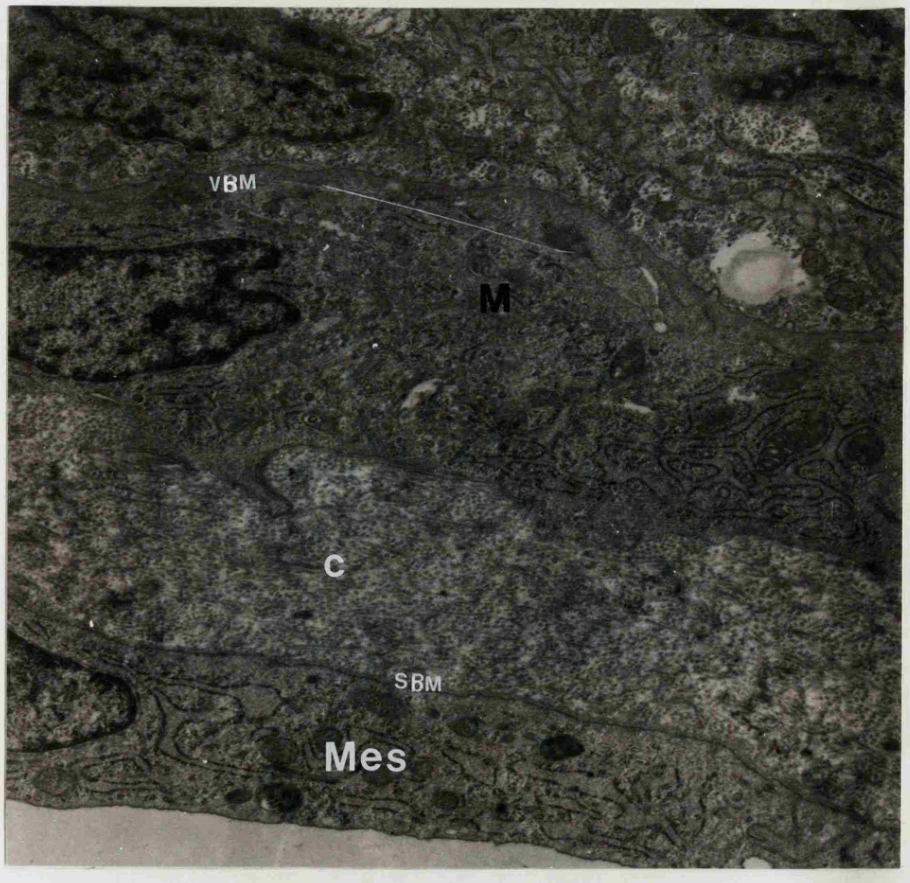


Figure 8.

Figure 9

Giant Yolk Sac (Visceral).

This micrograph shows the ultrastructural details of the microvilli and the cavulae.

The microvilli are somewhat irregular in form and may be branched. At the base of the microvilli the plasma membrane invaginates (arrow), such pocketings are limited by a dense membrane and have a fuzzy coat in their inner surfaces (coated vesicles (cv)). (Mag x 49,000).

Figure 10

Eighteen and a half days Visceral Yolk Sac in vivo.

In this micrograph the supra-nuclear cytoplasm of the epithelial cells shows extensive areas of glycogen deposition. (Mag x 4,830).

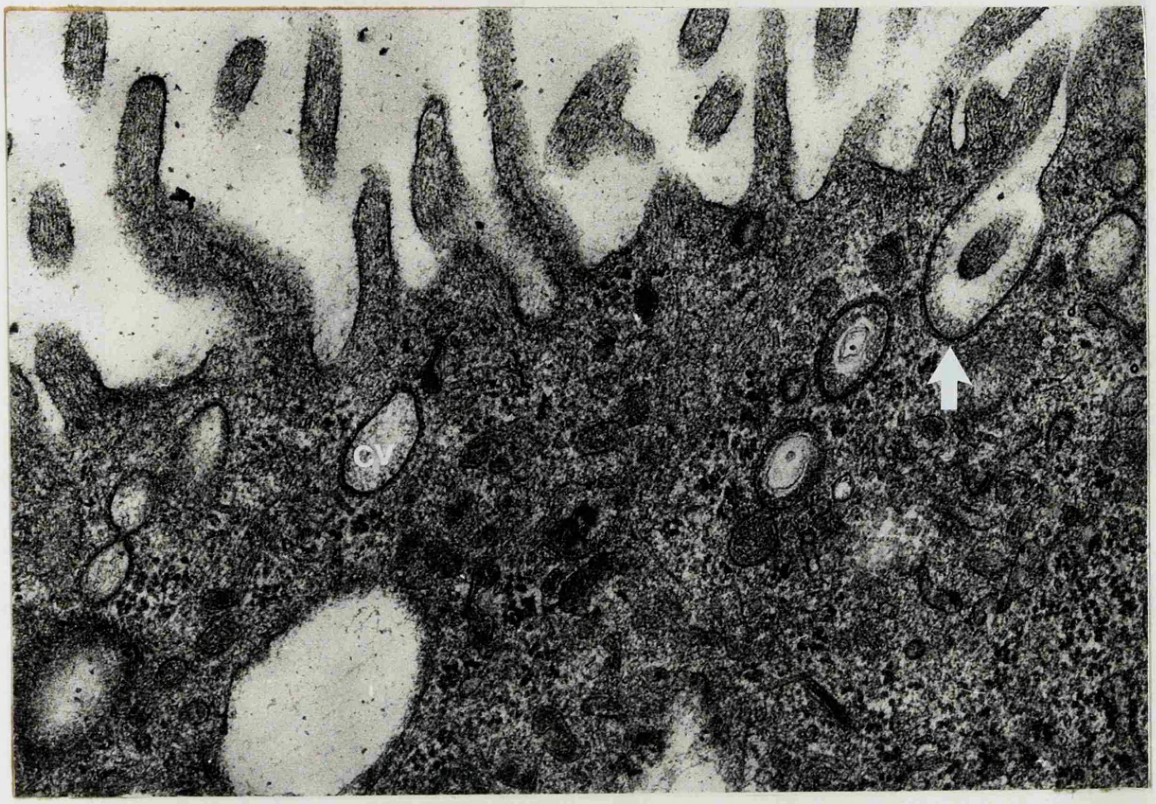


Figure 9.

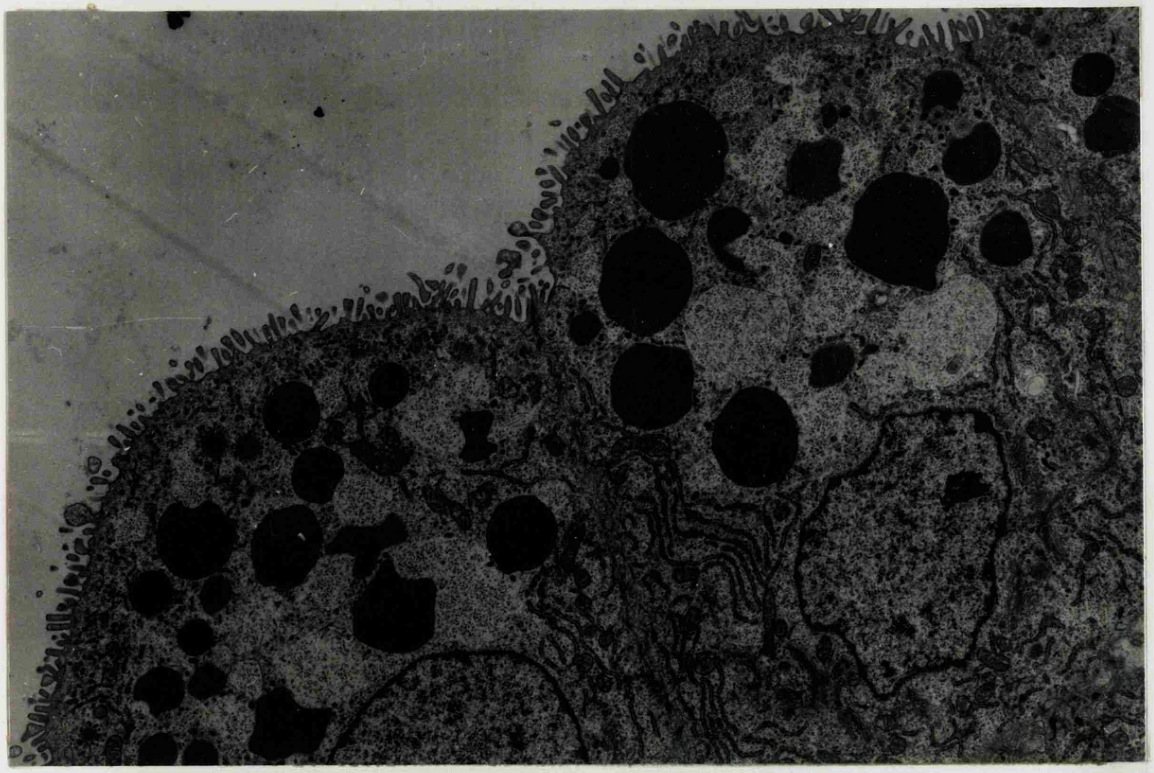


Figure 10.

Figure 11

Junctional Complex Between Two Cells in The Epithelium of the Giant Yolk Sac (Visceral).

The tight junction, located nearest to the brush border is shown between arrows 1 and 2, there is little accumulation of dense cytoplasmic material along this part of the complex.

The intermediate junction extends from arrow 2 to arrow 3 and shows a relatively wide intercellular space measuring (~ 200 A^{o}) maintained throughout the junction with extensive condensation of cytoplasmic fibrils as a fine felt work along either side of the junction. The desmosomes between arrows 4 and 5 show a wide intercellular space and bundles of coarse cytoplasmic fibrils converge into dense plates on each side of the desmosome. (Mag x 64,000).

Figure 12

Junctional Complexes Between Two Cells in the endoithelium of the Visceral Yolk Sac of 18½ day old Embryos.

The tight junction, located nearest to the brush border, extends from arrow 1 to arrow 2 and the intermediate junction from arrow 2 to arrow 3. There is extensive condensation of cytoplasmic fibrils as a fine felt work along either side of the intermediate junction. The desmosomes are marked between arrows 4 and 5. These elements show wide intercellular spaces filled with laminar densities, and ahigh local concentration of dense amorphous and fibrillar material in the subjacent ctyo-plasm matrix (Mag. x 48,300).

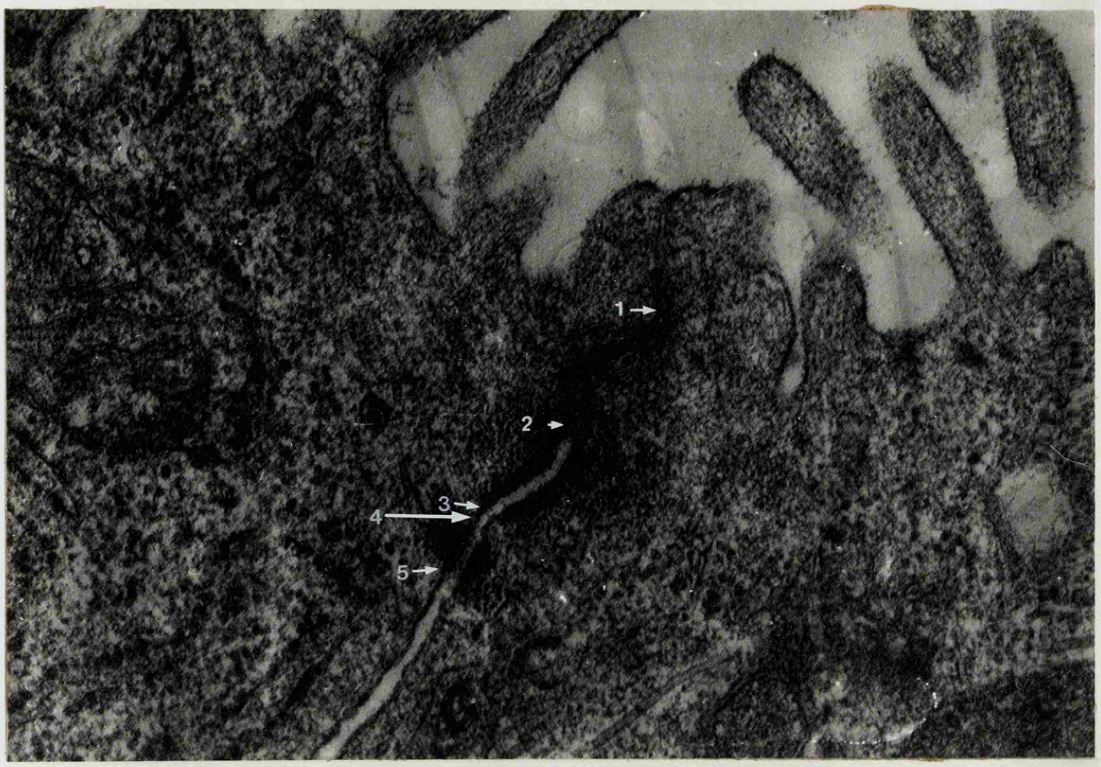


Figure 11.

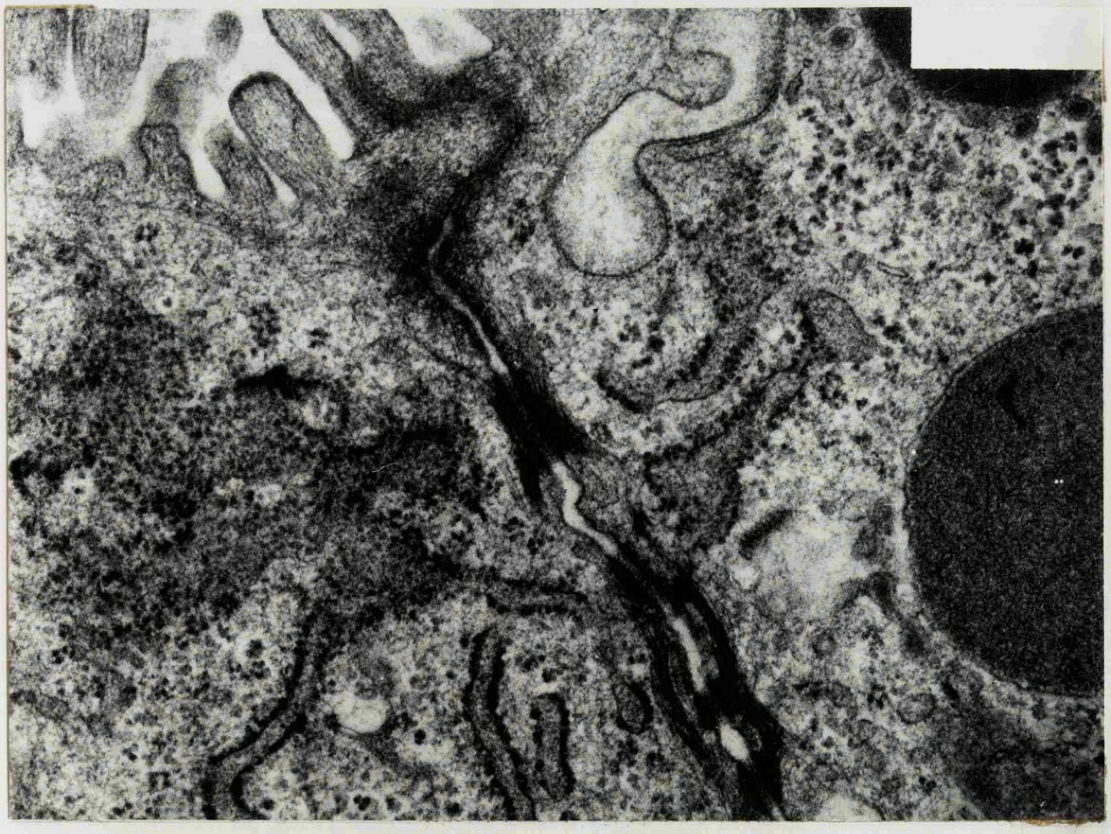


Figure 12.

DISCUSSION

The results in this section have shown that at the end of day 9 in culture, the giant yolk sac wall is composed of three cellular layers, separated by two basement membranes. The fine structure of the cellular layers and the basement membranes is in general similar to that of the 18½ day old visceral yolk sac from *in vivo*. These observations also agree with previous descriptions of the rat visceral yolk sac during the third part of gestation (Wislocki and Dempsey, 1955; Padykula, et al., 1966; Carpenter & Dishaw, 1979). Two main differences were observed between the giant yolk sac and 18½ day visceral yolk sac. There is an abundance of glycogen and a thickening of the serosal membrane in 18½ day *in vivo* yolk sac.

Previous *in vivo* studies have underlined several changes in the fine structure of rat visceral yolk sac from day 10-21 of gestation (of these changes are glycogen and thickening of the serosal basement membrane) (Lambson, 1966) and from day 13 of gestation to term (Padykula & Wilson, 1960; Wislocki & Padykula, 1961; Padykula & Richardson, 1963 and Padykula et al., 1966). Guinea pig visceral yolk sac has also been found to undergo changes within the serosal basement membrane during gestation (26 days until term) (King & Enders, 1970a).

It has been generally agreed that glycogen storage in rat visceral yolk sac *in vivo* commences around day 14 and reaches a peak at day 18 to 19 of gestation. Also thickening of the serosal basement membrane starts at day 13 of gestation and continues until term. These changes *in vivo* are believed to be imposed by modification of the milieu and not a result of inherent senility (Sorokin & Padykula, 1964). On the other hand, Sorokin and Padykula (1960 and 1964) found that glycogen accumulation in visceral yolk sac fragments cultured at 13 days was slower than *in vivo*, reaching a peak between 20-25 days. This was associated with the thickening of the serosal basement membrane, which begins after 14 days *in vitro*.

The two differences between giant yolk sac and the 18½ day visceral yolk sac depicted in our studies are in line with the above *in vitro* findings. The changes appear to commence at a later stage than *in vivo* which could be due to the fact that the giant yolk sac during the culture period is free from changes of the milieu normally associated with pregnancy *in vivo* (e.g. hormonal changes).

CHAPTER V

SECTION 4

A STUDY OF PERMEABILITY OF THE GIANT YOLK SAC USING LANTHANUM

Introduction

In a careful analysis of thin sections from various epithelia, Farquhar and Palade (1963) first defined the junctional complex. They demonstrated that this epithelial structure is composed of a zonula occludens (tight junction), zonula adherens (intermediate junction) and a macula adherence (desmosome). Ultrastructural studies of the rat visceral yolk sac have demonstrated the presence of typical junctional complexes between the visceral epithelial cells at the luminal surfaces (Padykula et al., 1966; Jollie and Triche, 1971). Similar junctional complexes have been observed between visceral epithelial cells in the mouse (Schluter, 1978) and in the guinea pig (King and Enders, 1970 a; King, 1982).

The visceral yolk sac epithelium plays a role in maintaining the barrier between the maternal (uterine) compartment and the underlying fetal compartment. Simple epithelia vary greatly in their ability to maintain ionic and osmotic gradients between solutions on either face of the epithelium and this ability is related to the transepithelial resis-

tance of the epithelium. For example, the amphibian urinary bladder maintains a steep gradient across it whilst others - such as the fluid transporting gallbladder and the proximal convoluted tubule of the kidney - do not (Fromter and Diamond, 1972).

The yolk sac epithelium is characterised by the presence of the zonula occludens which encircles the cells at the boundary separating the apical surface from the basolateral surface of the endoderm cells. This forms a transepithelial barrier preventing the diffusion of macromolecules into the underlying fetal tissue. Evidence exists that these junctions inhibit passive diffusion of certain macromolecular tracer substances such as thorotrast (Carpenter and Ferm, 1969), ruthenium red (Jollie and Triche, 1971) and horseradish peroxidase (Seibel, 1974). However, there is little information available regarding the permeability of these junctions to water, ions and small non-electrolytes.

Tracers like lanthanum nitrate have been used to demonstrate the site of barriers to diffusion and communication between compartments (Revel and Karnovsky, 1967; Martinez-Palomo et al., 1971; Whittembury and Rawlins, 1971; Tisher and Yarger, 1973).

Lanthanum is a polyvalent cation with a tendency to bind at the cell surface to form an electron dense deposit. Revel and Karnovsky (1967) demonstrated that lanthanum can penetrate gap junctions (20 Å wide or less) but not the tight junctions in mouse heart and liver cells. In Section 3 of this chapter the ultrastructural study of the giant yolk sac revealed the presence of typical junctional complexes between epithelial cells of the visceral yolk sac. In this section the integrity of the giant yolk sac as a closed system is tested using lanthanum nitrate. The persistence of a functional barrier (zonula occludens), which seals the lateral junctions between the epithelial cells and prevents leakage, would be confirmed by this technique.

Materials and Methods

This study was carried out on 6 giant yolk sacs obtained from 3 mothers using Lesseps' (1967) technique, which was a modification of the method developed by Doggenweiler and Frenk (1965).

For 1 hour at $0^{o}C$ the specimens were placed in fixative made up of -1 gm $La(NO_3)_3$ $6H_2O$ (BDH), -1 gm $KMNO_4$ (BDH), -20 mls veronal acetate buffer (Appendix I), -6 mls zetterquist salt solution (Appendix J); the pH was corrected to 7.8 by 0.1N HCl and the final volume made up to 100 mls. Fixation with potassium permanganate buffered with veronal acetate gives good fixation and staining of membraneous structures and cytoplasmic dense bodies (lysosomes), but produces very faint staining of other nuclear and cytoplasmic structures (Zebrun and Mollenhauer, 1960; Porter and Machado, 1960). The fixed giant yolk sacs were then washed several times in Tyrode's solution (Appendix K), dehydrated by graded series of ethanol and finally embedded in araldite after which thin sections were cut with LKB-made glass knives on a Reichert OMU-4 ultramicrotome, and examined in a JEOL 100-S electron microscope.

Results

Sections from giant yolk sacs (visceral yolk sac) stained with lanthanum show a thick electron-opaque layer of lanthanum staining material, which is external to the cell membrane (Figure 1).

In all sections examined this dense and continuous electron-opaque layer outlines the microvilli of the brush border and the various invaginations of the cell surface, down to the level of the zonula occludens (tight junction) (Figure 2). Below this level the electron opacity in the material is considerably less than that of the surface layer (lanthanum-staining material) and there is no evidence of a concentration gradient down the junctional complex.

The penetration of lanthanum ions is effectively halted along the line of fusion with the adjoining cell membranes in the tight junction (Figure 3). In any event the concentrated tracer does not reach the intermediate junction, the desmosomes or the intercellular space beyond the level of the tight junction. The lanthanum stain does not appear on any membraneous structures within the cells and on no occasion does it enter the cell cytoplasm (Figure 4).

Figure 1

This micrograph shows the dense continuous layer of lanthanum staining material. It completely covers the outlines of the brush border, including the microvilli and also fills the plasma membrane invagination. (Mag x 7,000).

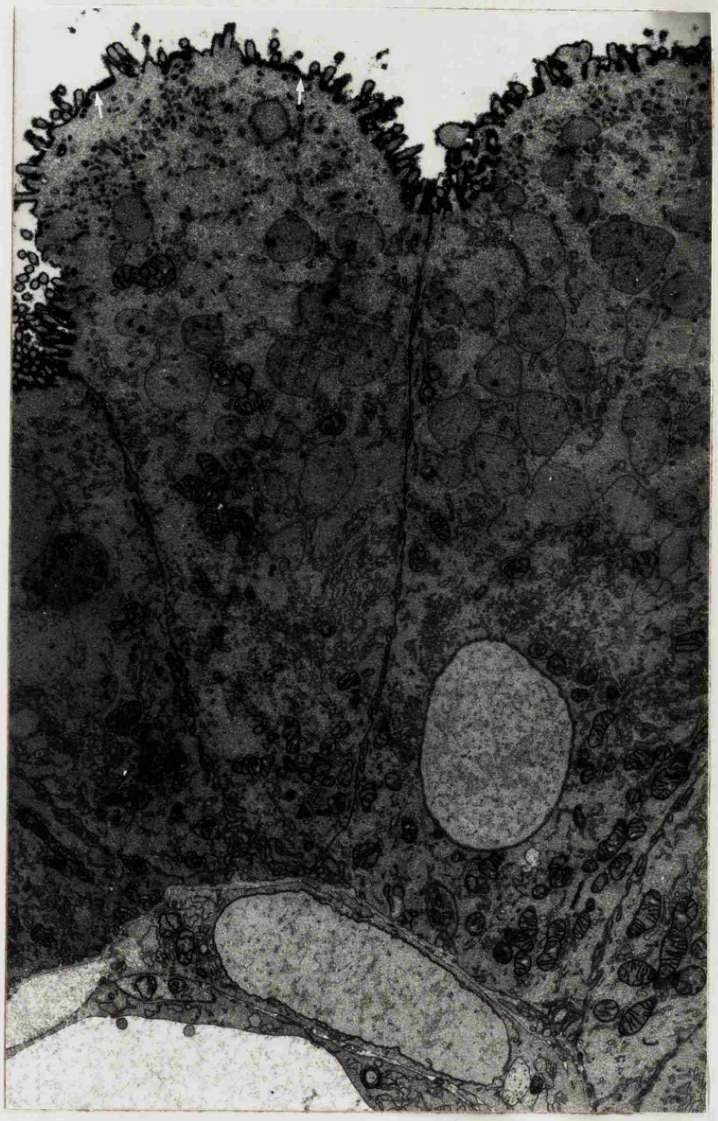


Figure 1.

Figure 2

In this micrograph two cell junctions appear. The lanthanum stain is concentrated on the outlines of the brush border, its microvilli (v), plasma membrane invagination (c), down to the level of the tight junction. (Mag x 35,000).

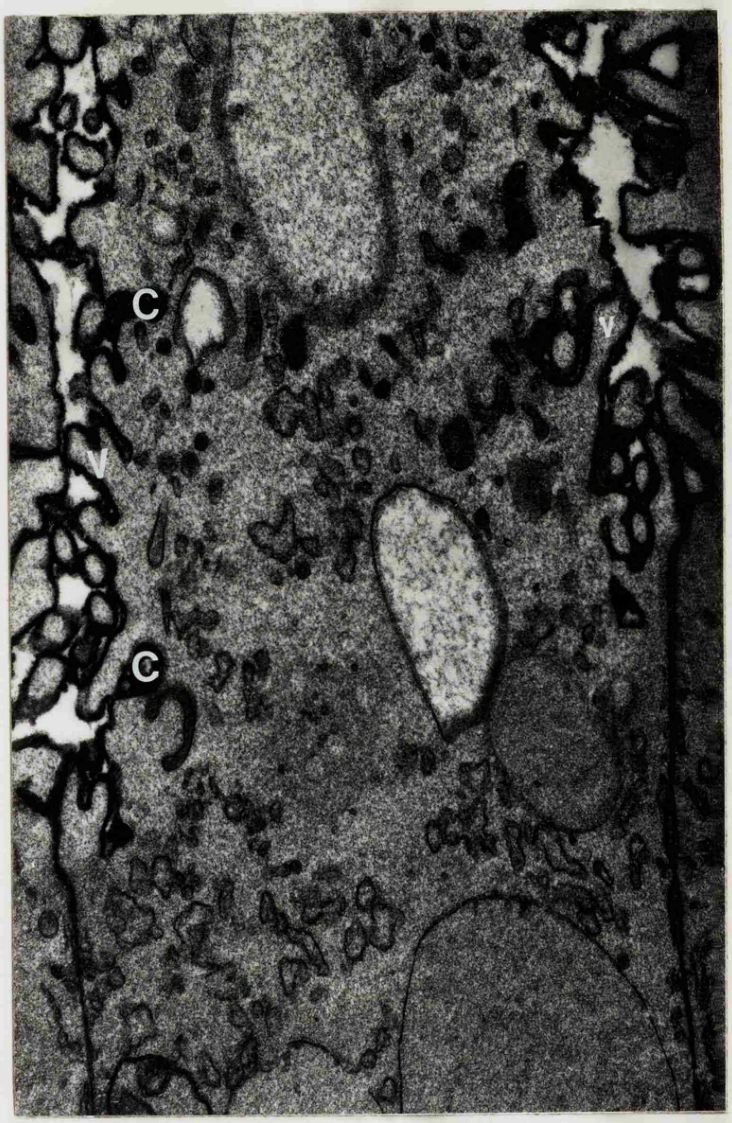


Figure 2.

Figure 3

Intercellular junction between two visceral yolk sac epithelium cells. The dense layer of lanthanum-stain stops at the point where the membranes merge at the two opposed cells in a tight junction (arrow 1). The intercellular space beyond the tight junction (arrow 2 - 3) appears free of lanthanum.

The inner dark leaflet of the cell membrane is visible (arrow 4) but the outer dark leaflet is indistinguishably merged with lanthanum staining. (Mag x 175,000).

Figure 4

This micrograph shows the intercellular spaces (ics). The intracellular structure, mitochondria (M), Vacuoles (V), and rough endoplasmic reticulum are all free of lanthanum staining material. (Mag x 35,000).

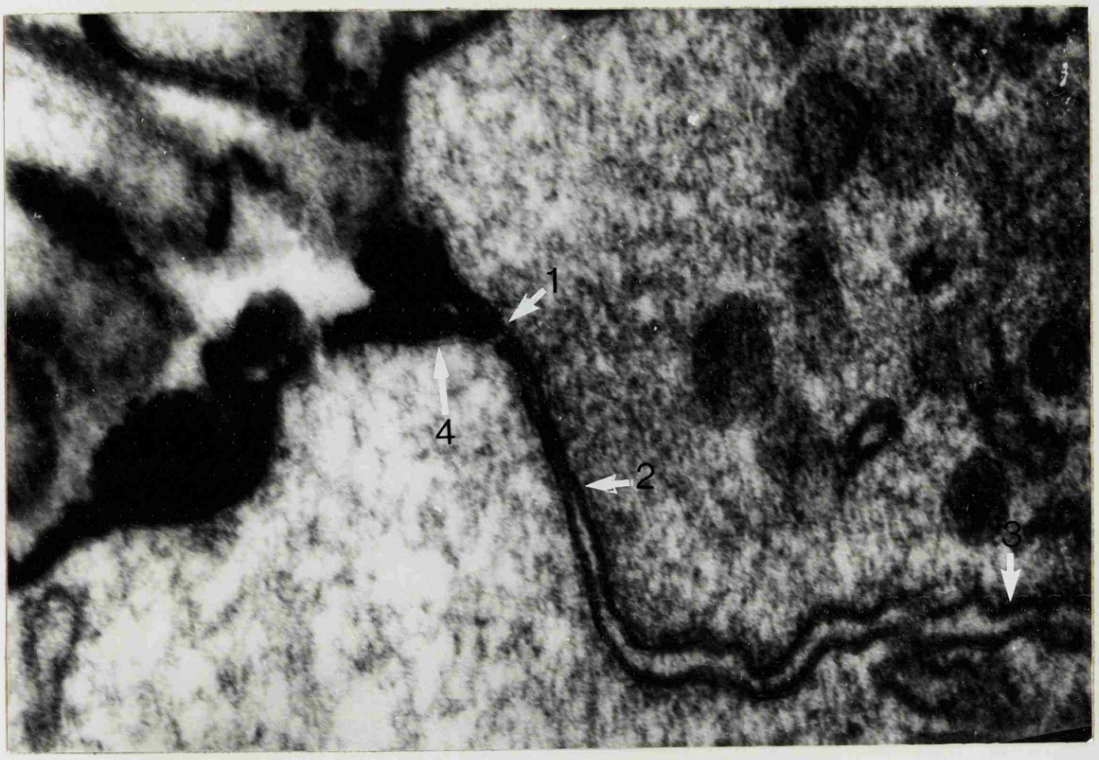


Figure 3.

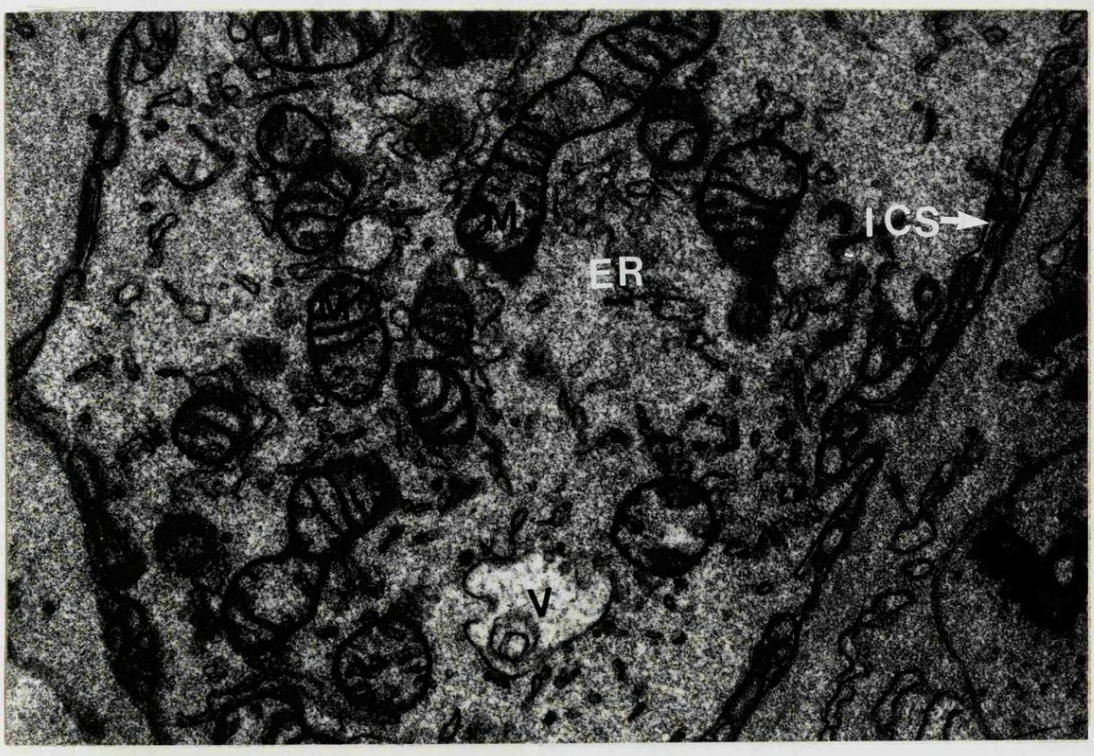


Figure 4.

Discussion

Lanthanum has been widely used to differentiate between tight junctions and leaky (gap) junctions in different epithelia (Rēvel and Karnovsky, 1967; Friend and Gilula, 1972; Martinez-Paloma et al, 1973). It can pass through gap junctions which are 20 - 40 angstrom wide, but not through tight junctions (zonula occludens). The results of this section have shown that lanthanum forms an electron-opaque layer external to the plasma membrane, covering the free (apical) surface of the giant yolk sac epithelium. On the lateral surfaces lanthanum stopped short at the tight junction leaving the intercellular spaces free; also it was not present in other layers of the giant yolk sac.

From these observations we can surmise that, in the giant yolk sac, the tight junctions form 'gasket-like' barriers around each cell to prevent transepithelial movement of lanthanum by passive intercellular diffusion.

Our results are in agreement with previous findings in the visceral yolk sac using the freeze-fracture technique in rats (Carpenter and Dishow, 1979) and in guinea pigs (King, 1982).

CHAPTER V

SECTION 5

HISTOCHEMICAL AND BIOCHEMICAL STUDIES OF ACID PHOSPHATASE IN THE GIANT YOLK SAC AND 18½ DAY YOLK SAC

Introduction

The rat visceral yolk sac has been shown to be the principal site of histiotrophic nutrition where macromolecules are endocytosed and either digested intralysosomally and transferred to the embryo or transported intact to the embryo (see Chapter I for more details on this and the intralysosomal digestion by lysosomal enzymes).

Many of the hydrolytic enzymes capable of breaking down embryotroph within the cells of the visceral yolk sac can be explained histochemically, the most reliably demonstrable of these being acid phosphatase which hydrolyses a wide variety of phosphatase monoesters and phosphoproteins. Histochemically, acid phosphatase activity can be shown at the light microscopic level using either the lead sulphide method (Gomori, 1952) or simultaneous coupling techniques employing derivatives of naphthyl phosphate or phosphate esters of naphtol as substrate (Seligman & Mannheimer, 1949; Burstone, 1958). The most reliable coupling technique is that of Barka and Anderson (1962) which utilises hexazonium pararosanilin (Davis

& Ornstein, 1959) as a coupling reagent.

Histochemical studies in the rat visceral yolk sac using Gomori's staining method (Wislocki & Padykula, 1953; Padykula, 1958; Beck et al. 1967) as well as Barka and Anderson's method have demonstrated the presence of strong acid phosphatase activity in the supranuclear region endodermal cells.

In this section the acid phosphatase activity in the giant yolk sac was studied both histochemically and biochemically and was compared to that of the 18½ days yolk sac *in vivo*.

Materials and methods

Histochemistry

The study of acid phosphatase activity in the giant yolk sac and $18\frac{1}{2}$ day visceral yolk sacs was carried out on specimens obtained as detailed in Chapter V, Section 2. Tissue pieces were fixed for 4 hours at $4^{o}C$ in 10% formaldehyde in 30% sucrose (Straus, 1964) and stored in 30% sucrose for up to 3 days.

A technique devised by Barka and Anderson (1962) was used to demonstrate the presence of acid phosphatase.

Stock solutions

A) Pararosanilin solution: 1gm pararosanilin hydrochloride (BDH), dissolved in 20 mls distilled water and 5 mls concentrated (IIM) HCl.

B) Sodium nitrite solution: 4 gms. sodium nitrite (Analar), dissolved in distilled water to give a 4% solution.

C) Substrate solution: 400 mg sodium α-naphthyl phosphate (Sigma) dissolved in 100 mls michaelis buffer [5.85 gm sodium acetate (anhydrous) and 14.719 gm sodium barbiturate dissolved in CO_2-free distilled water to a final volume of 500 ml.]

Working solution - equal volumes (0.8 ml) of stock solutions A and B were mixed and added to 5 mls of stock solution C, previously diluted with 10 mls H_2O and adjusted to pH 6 with 1N NaoH. The final volume of this solution was made up to 20 mls.

Since the yolk sac is a thin membrane sectioning was not required and tissues were incubated in the working solution for 20 minutes at room temperature.

As a control fixed giant yolk sac and 18½ days yolk sac pieces were incubated in working solution without α-naphthyl phosphate.

After incubation the tissue was washed in normal saline, dehydrated in alcohol and embedded in paraffin after which sections 5 u thick were cut, counter-stained using 1% methyl green and mounted in D.P.X.

Biochemistry: quantitative measurement of acid phophatase

This was carried out using the method described by Barrett (1972) - a modification of the original technique of Fiske and Subbrow (1925) - and it involves the measurement of inorganic phosphate release from the substrate (glycero-2 phosphate).

1. Preparation of giant yolk sacs for acid phosphatase measurement

These were washed three times in 10 mls ice-cold Hanks' solution and transferred to petri-dishes containing quantities of similar solution. The visceral yolk sac was freed from the ectoplacental cone and the individual membrane placed in a test-tube. It was homogenized in 3 mls ice-cold distilled water for 30 seconds using an Ultra-Turrax Homogeniser. The homogenate was cleared by centrifugation at 4°C for five minutes at 600 g and the supernatant used for acid phosphatase assay.

2. Tissue preparation of 18½ day visceral yolk sac for quantitative measurement of acid phosphatase

Pregnant rats at 18½ days' gestation were killed by an overdose of diethyl-ether and the embryos removed. The visceral yolk sac membranes were dissected from embryos in ice-cold Hanks' solution, processed and homogenised as for the giant yolk sac.

Assay procedure for acid phosphatase

Two mls buffered glycerophosphate were added to 1 ml yolk sac supernatant at 37°C and, after 10 minutes' incubation, the reaction was

halted with 3 mls ice-cold 10% (W/V) trichloroacetic acid (BDH); the precipitate was allowed to flocculate for 15 minutes on ice and the mixture was then centrifuged for 10 minutes at 4°C. Two mls of the supernatant were mixed with 0.8 mls ammonium molybdate and 0.3 mls Elon reagent and the resulting colour was measured at E660 after a period of 10 minutes.

Inorganic phosphate standards were used to establish the amount of substrate hydrolysed.

Blanks were prepared by adding the enzyme after the addition of 10% trichloroacetic acid.

Reagents

Buffered glycerophosphate: Glycero-2-phosphate (Sigma) 5 mg/ml, dissolved in 0.1M sodium acetate/acetic acid buffer pH 5.0.

Ammonium molybdate reagent: 2.5gm(NH_4) 6 Mo_7 O_{24} - $4H_2O$ (BDH), dissolved in N H_2SO_4 to a total volume of 100 mls.

Elon reagent: 0.5gm Elon (P-methyl aminophenol sulphate) (Sigma) dissolved in 10% (W/V) sodium metabisulphite solution to make 100 mls.

Phosphate standard - a 30.0 mM stock solution of kH_2PO_4 (BDH) in 5N H_2SO_4 was used as a 1:1 dilution containing 0.30 uMol/ml.

Protein estimation of the yolk sac membrane - half a millilitre of the yolk sac homogenate was digested in 0.5 ml of 1N NaoH for 30 minutes at room temperature and the protein was then estimated by a modification of the method of Lowry et al. (1951) (Appendix A).

Results

A) Histochemistry

The microscopic appearance of the giant yolk sac stained for acid phosphatase is similar to that of the 18½ day visceral yolk sac - both showed a strong reaction to the localisation of acid phosphatase activity in the supranuclear region of the endodermal cells (Figures 1 and 2) Only the endodermal cells showed appreciable staining for acid phosphatase; the mesenchymal cells, mesothelial cells and the two basement membranes did not stain.

The control giant yolk sac and 18½ day yolk sac pieces incubated in the same way without the substrate showed no acid phosphatase activity (Figures 3 and 4).

B) Biochemistry

The quantitative study of the acid phosphatase activity in the homogenates from the giant yolk sac and 18½ day yolk sac (*in vivo*) showed that there was no significant difference between the two visceral yolk sacs (see Table 1).

Figure 1

Light micrograph of a giant yolk sac (visceral) stained for acid phosphatase activity, showing strongly positive reaction in the supranuclear region (Mag x 500).

Figure 2

Light micrograph of an 18½ day yolk sac from *in vivo* stained for acid phosphatase activity, showing strongly positive reaction in the supranuclear region (Mag x 500).

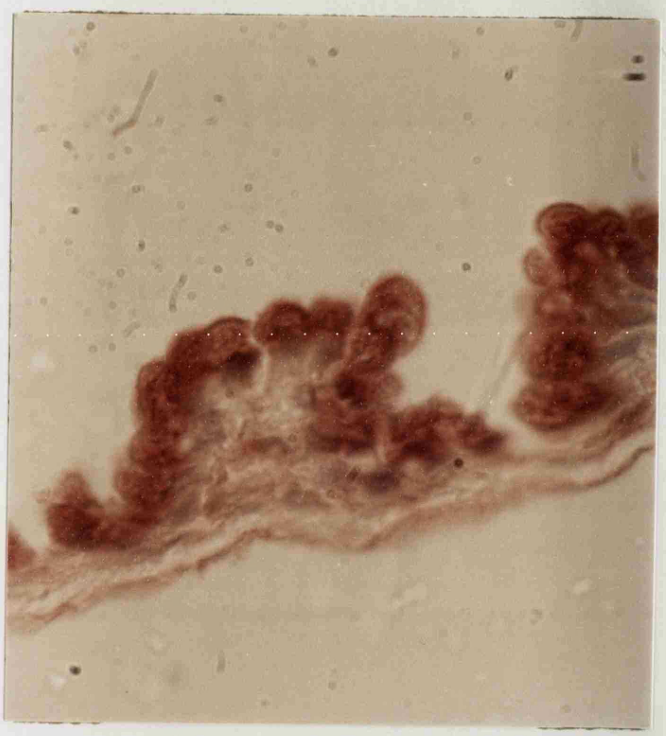


Figure 1.

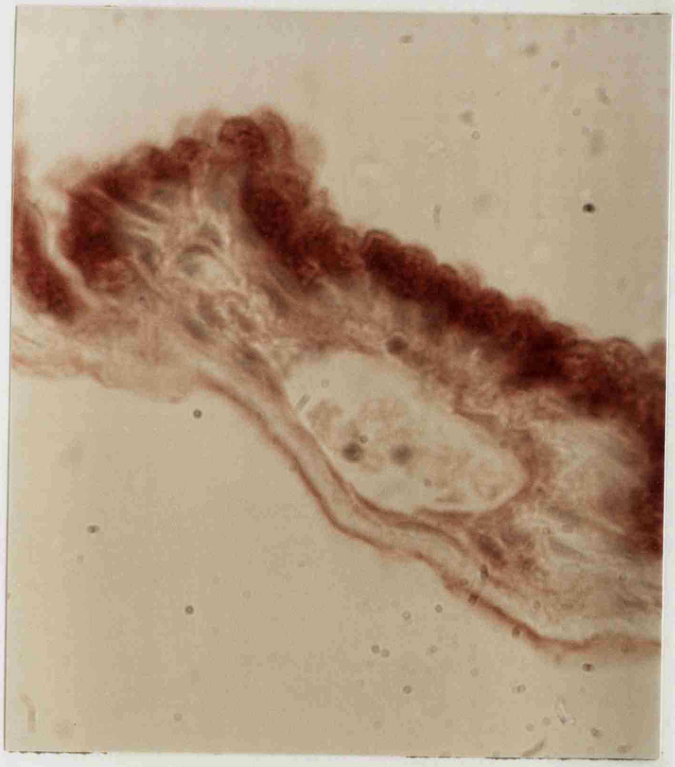


Figure 2.

Figure 3

Light micrograph of a giant yolk sac (visceral) stained for acid phosphatase activity in medium lacking the substrate (sodium α-naphthyl phosphate) shows no acid phosphatase activity. (Mag x 500).

Figure 4

Light micrograph of an 18½ day yolk sac from *in vivo* stained for acid phosphatase activity in medium lacking the substrate (sodium α-naphthyl phosphate) shows no acid phosphatase activity. (Mag x 500).

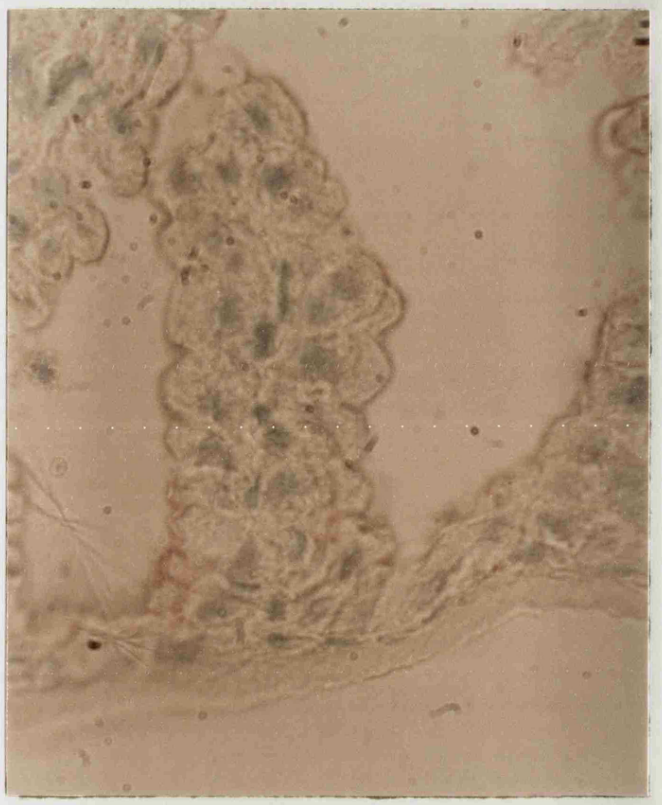


Figure 3.

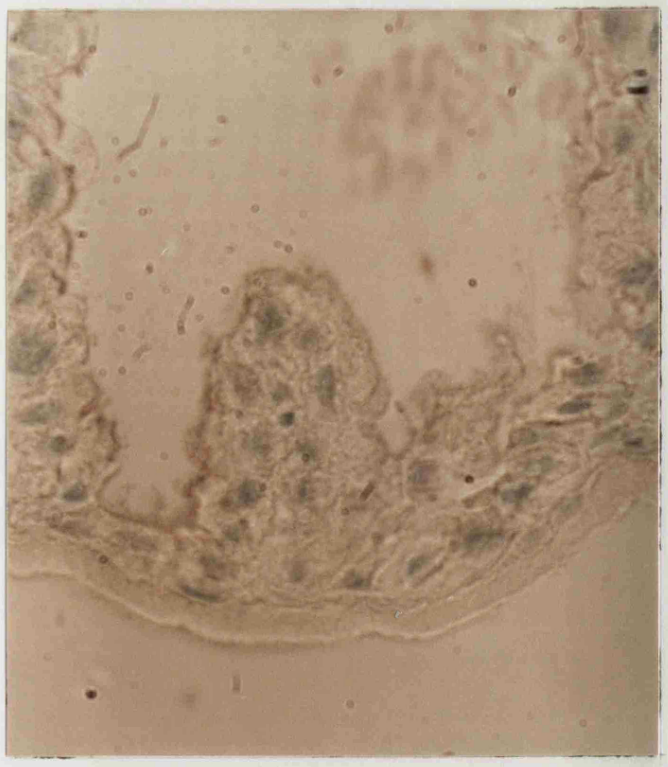


Figure 4.

TABLE 1

Specific activities of acid phosphatase in the giant yolk sac and 18½ day visceral yolk sac *in vivo*. (Sodium glycerophosphate substrate).

Using the Student's 't' test there is no significant difference between the two values.

Source of Visceral Yolk Sac	Activity expressed as milli-micromoles of glycerophosphate substrate liberated per minutes per mg protein (pH 5)
Giant Yolk Sac	61.88 se ± 1.89
18½ Day *in vivo*	60.91 se ± 1.31

Discussion

Histochemical studies of acid phosphatase in the rat visceral yolk sac have shown a strong enzyme activity localised in the supranuclear region endodermal cells (Wislocki & Padykula, 1953; Padykula, 1958; Bulmer, 1965; Beck et al. 1970). These last named workers (1970) studied acid phosphatase activity in 17½ days old rat visceral yolk sacs cultured *in vitro* and compared it to that in same stage *in vivo* visceral yolk sacs and showed that, in both membranes, strong acid phosphatase activity was localised in the supranuclear region of the endodermal cells. However, their biochemical results showed that the visceral yolk sac cultured in T199 for 24 hours contained 74% of the mean specific activity of the visceral yolk sac *in vivo*.

However, the results shown in the present study indicate that acid phosphatase activity in the visceral layer of the giant yolk sac was similar to that in the 18½ days old rat visceral yolk sac *in vivo*, both histochemically and biochemically. The differences between the present results and those of Beck et al. (1970) might be attributed to the absence of rat serum in the culture medium used in the earlier work. If it is assumed that acid phosphatase activity within the yolk sac cell reflects the digestive activity within the visceral yolk sac as a whole then it is likely that the visceral layer of the giant yolk sac has a comparable digestive activity to that of the 18½ days old rat visceral yolk sac grown *in vivo*.

CHAPTER V

SECTION 6

UPTAKE OF COLLOIDAL GOLD BY THE ENDODERMAL CELLS OF THE GIANT YOLK SAC

Introduction

Endocytic activity of the rat visceral yolk sac is well documented at different gestational stages (see Chapter 1).

In this section the endocytic activity of the giant yolk sac was studied by incubating the giant yolk sac in a medium containing 31.5 nm gold particles which have proven to be a very useful electronmicroscopical marker (Goodman et al., 1981). The accumulation of gold colloid by the giant yolk sac was assessed by examining tissue sections for the electron dense marker at different time intervals.

It was not possible to carry out a meaningful study *in vivo* because, at an equivalent yolk sac gestational age ($18\frac{1}{2}$ days):

a) the concentration of colloid reaching the conceptuses would be variable;

b) the stability of the colloidal gold solution is unknown *in vivo*;

c) an accurate experimental exposure to colloid would be

difficult to achieve since:

(i) it is not known how long it would take for the marker to reach the conceptuses;

(ii) the time taken to remove the yolk sac from the pregnant uterus is likely to add yet another variable to the procedure.

Unlike the situation *in vivo*, the giant yolk sac *in vitro* can be quickly removed simply by transferring the yolk sac from marker containing medium to fresh medium or fixative.

In the present study the endocytic vacuoles of the yolk sac endoderm will be defined according to Gupta et al. (1979) who classified the endocytic vacuoles of yolk sac epithelium into four types:

Type I - small vacuoles apically located in association with the microvillous surface of the cell;

Type II - larger vacuoles containing lightly flocculent material situated somewhat deeper in the cytoplasm;

Type III- vacuoles containing flocculent material together with homogeneous electron-dense areas;

Type IV - vacuoles consisting entirely of homogeneous electron-dense material.

Materials and Methods

Preparation of colloidal gold particles

A colloidal gold solution, containing particles of 31.5 nm., was prepared according to the original technique used by Horrisberger and Rosset (1977) modified by Huxham and Beck (1981).

All solutions used were centrifuged or filtered through a 0.22 μm millipore filter before use.

To 200 mls distilled water 1.5 ml 1% sodium citrate was added and boiled in a flask. The size of the gold particles is determined by the extent of reduction of aurochloric acid by sodium citrate, smaller particles are produced when the concentration of the reducing agent is increased. A millilitre of 4% aurochloric acid (Raymond A. Lamb, London) was then added and the solution boiled for approximately 5 minutes under reflux until no further colour change was observed.

The solution was cooled and adjusted to pH 7.0 with 0.2 Nk_2CO_3 and then stabilised by the addition of 3 mls 1% polyethyleneglycol (20 M) sigma. The solution was centrifuged at 28,000 x g for 30 minutes at $4^{o}C$ on a Sorvall ultracentrifuge. It was then resuspended twice in Hanks' solution containing 0.3 mg/ml polyethyleneglycol. Finally, the colloidal gold was collected and made up in 10 mls Hanks' solution containing 0.3 mg/ml polyethyleneglycol. The 10 mls contained approximately 40 x 10^{12} of 31.5 nm colloidal gold particles.

Before use the colloidal gold solution was diluted 1 : 1 with Hanks' solution containing 0.3 mg/ml polyethyleneglycol.

When the intact giant yolk sacs were ready they were washed 3 times in warm Hanks' solution at $37^{o}C$. They were then incubated in 4 mls diluted colloidal gold solution at $37^{o}C$ for 5 minutes, 10 minutes, 15

minutes, 20 minutes or 60 minutes. Three giant yolk sacs were used in each incubation condition.

The culture bottles were gassed with 20% O_2, 5% CO_2 and 75% N_2 at the beginning of the incubation period. After incubation the giant yolk sacs were washed in Hanks' solution 3 times and prepared for electron microscopy as in Section 3 of this chapter.

Results

The ultrastructure of the endodermal cells has already been described. During the incubation of the tissue in culture medium containing gold particles no morphological changes were apparent in the endodermal cells (i.e. the cells appeared to be undamaged).

5 minutes - within this time the gold particles were seen in the plasma membrane invaginations in between the microvilli (Figure 1) and inside small vesicles (Figure 2). The vesicles, which have been referred to as 'Type I' by Gupta et al. (1979), were located in the apical region of the cells and associated with the microvillous surface.

10 minutes - by this time gold particles were seen in vacuoles situated more deeply in the cytoplasm (Type II) which were either completely empty or contained small amounts of flocculent material of moderate electron density (Figure 3).

15 minutes - the gold particles were seen in large vacuoles (Type III) containing flocculent material together with homogeneous electron-dense areas (Figure 4).

20 minutes - the gold particles were seen in Type IV vacuoles, consisting of homogeneous electron-dense material.
In addition, gold particles were present in Types I, II and III vacuoles (Figure 5).

60 minutes - further incubation of the tissue in culture medium containing colloidal gold resulted in the accumulation of gold particles in vacuoles containing small or large amounts of flocculent material (Figures 6, 7) and in the electron-dense vacuoles (Figure 8).

It is important to note that at all periods of incubation no free gold particles were evident in the cytoplasm or in the intercellular spaces, the particles remaining in the vacuolar system.

Figure 1

The apical region of giant yolk sac endodermal cells, after 5 minutes of incubation in medium containing colloidal gold. Note the gold particle (arrow) entering the plasma membrane invagination. (Mag x 23,100).

Figure 2

The apical region of giant yolk sac endodermal cells, after 5 minutes incubation in medium containing colloidal gold. This figure shows a gold particle inside a small endocytic vesicle (arrow). (Mag x 46,200).

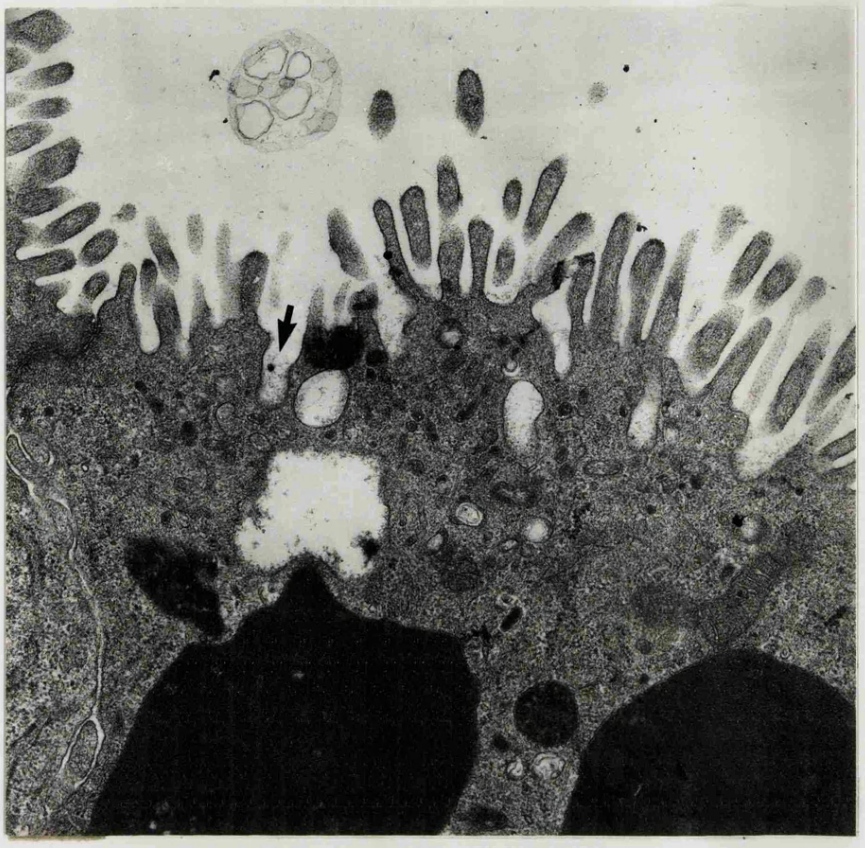


Figure 1.

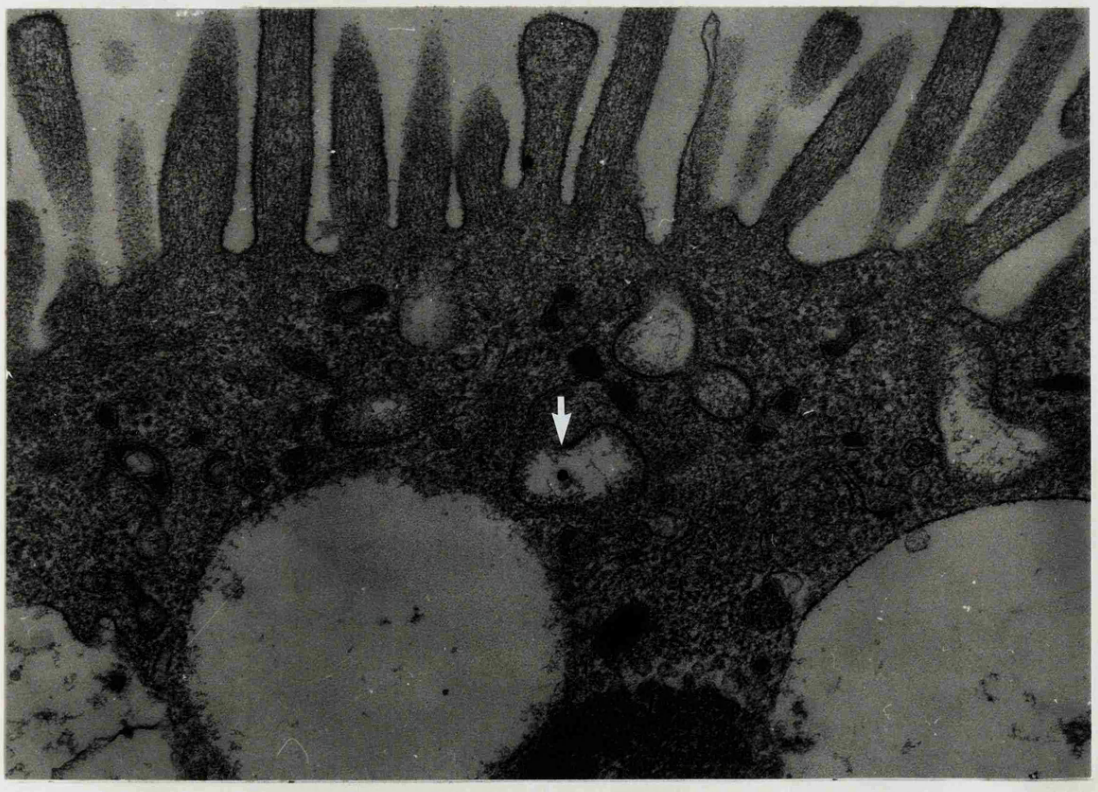


Figure 2.

Figure 3

After 10 minutes of incubation, in medium containing colloidal gold particles, vacuoles with small amounts of flocculant material (arrow) are seen to contain colloid. (Mag x 30,000).

Figure 4

After 15 minutes of incubation in medium containing colloidal gold, gold particles are present in flocculant filled vacuoles. Note the contrast between type 2 and type 3 vacuoles. (Mag x 23,100).

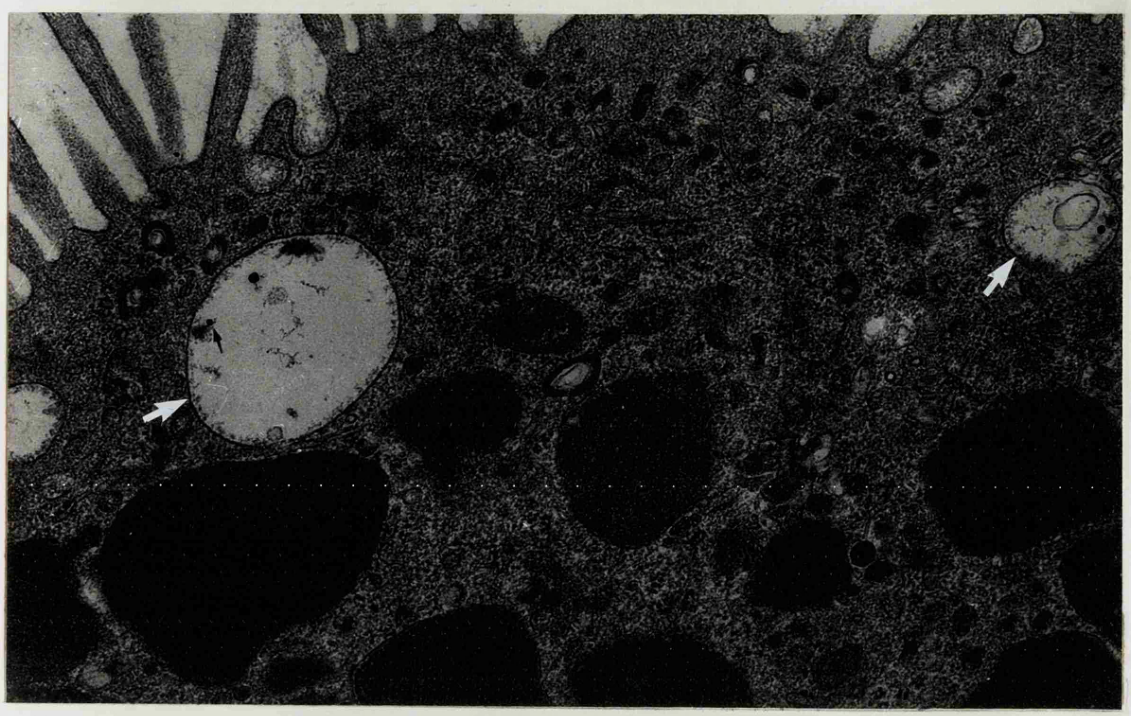


Figure 3.

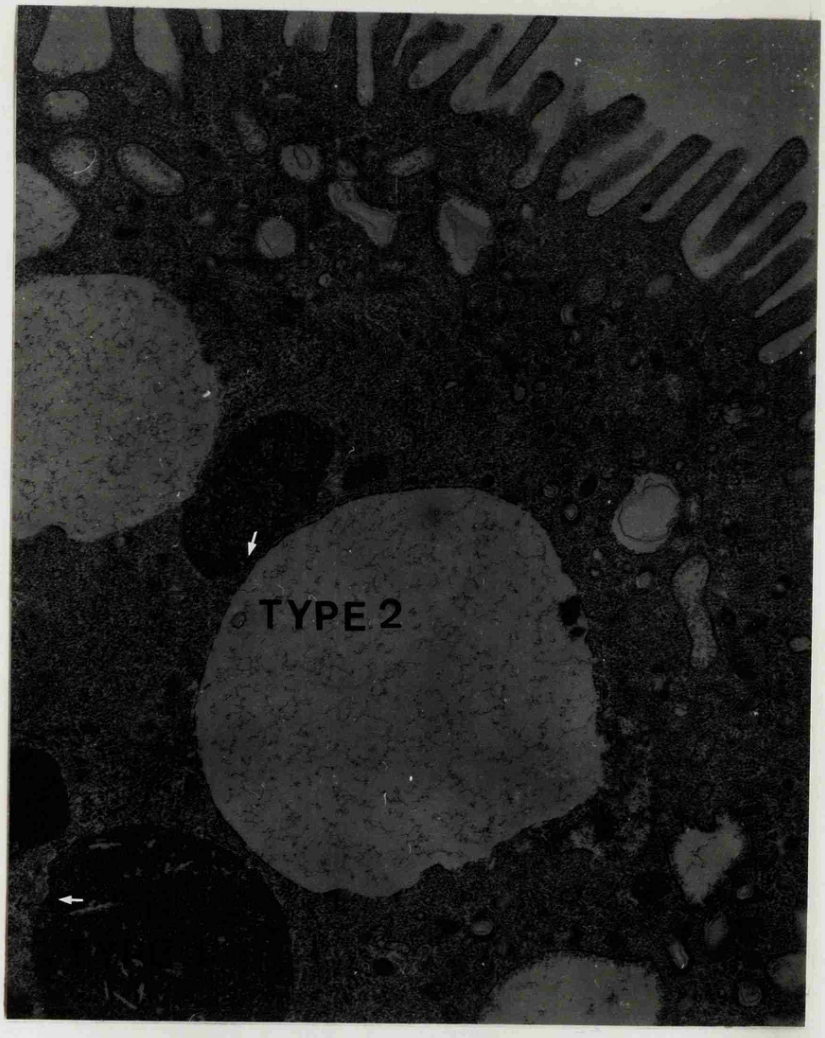


Figure 4.

Figure 5

After 30 minutes incubation in medium containing colloidal gold, gold particles (arrow) were present in electron dense vacuoles and also present in vacuoles of type 1, 2 and 3. (Mag x 26,250).

Figure 6

High magnification micrograph of the apical region of giant yolk sac endodermal cells, showing the accumulation of gold particles in type 2 vacuoles. The arrow indicates a gold particle lying in the plasma membrane invaginating, thereby showing the continuous uptake of gold particles during 60 minutes of incubation. (Mag x 46,200).

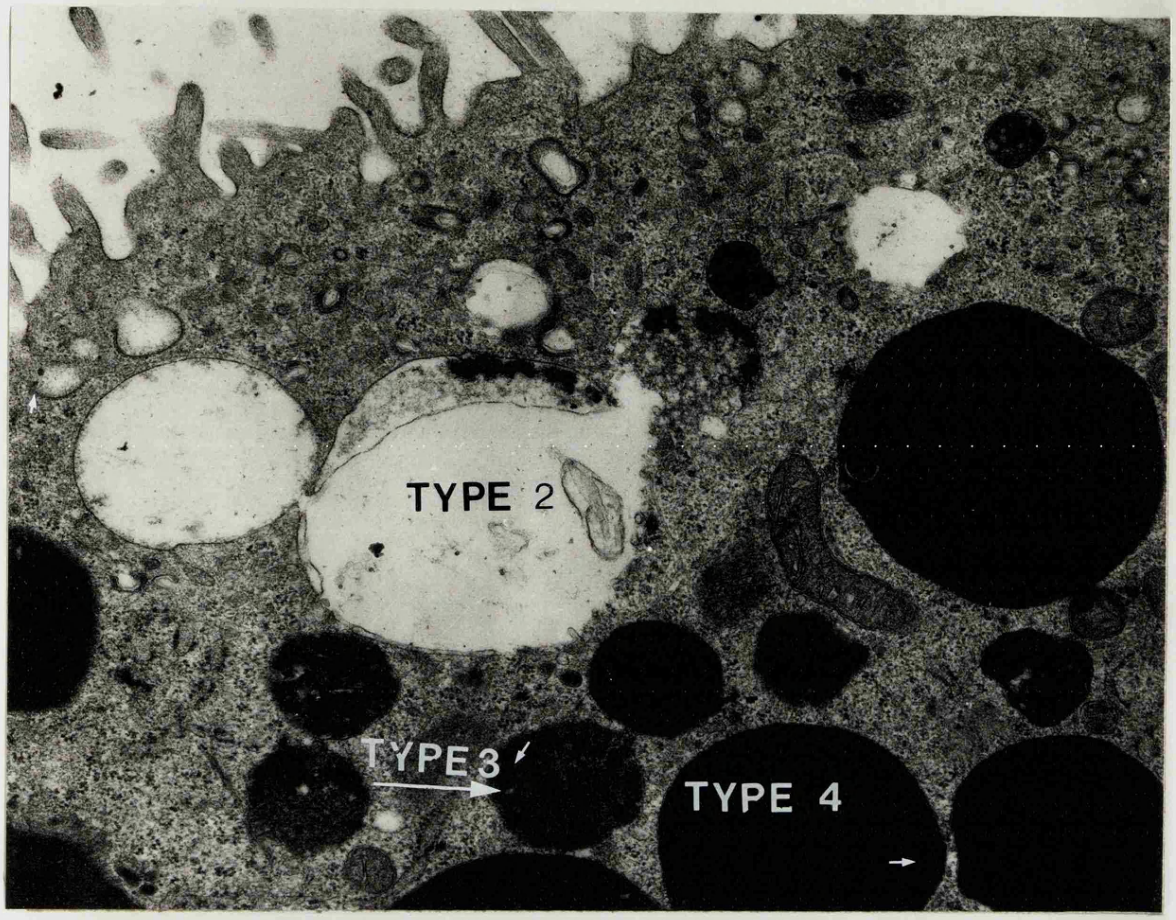


Figure 5.

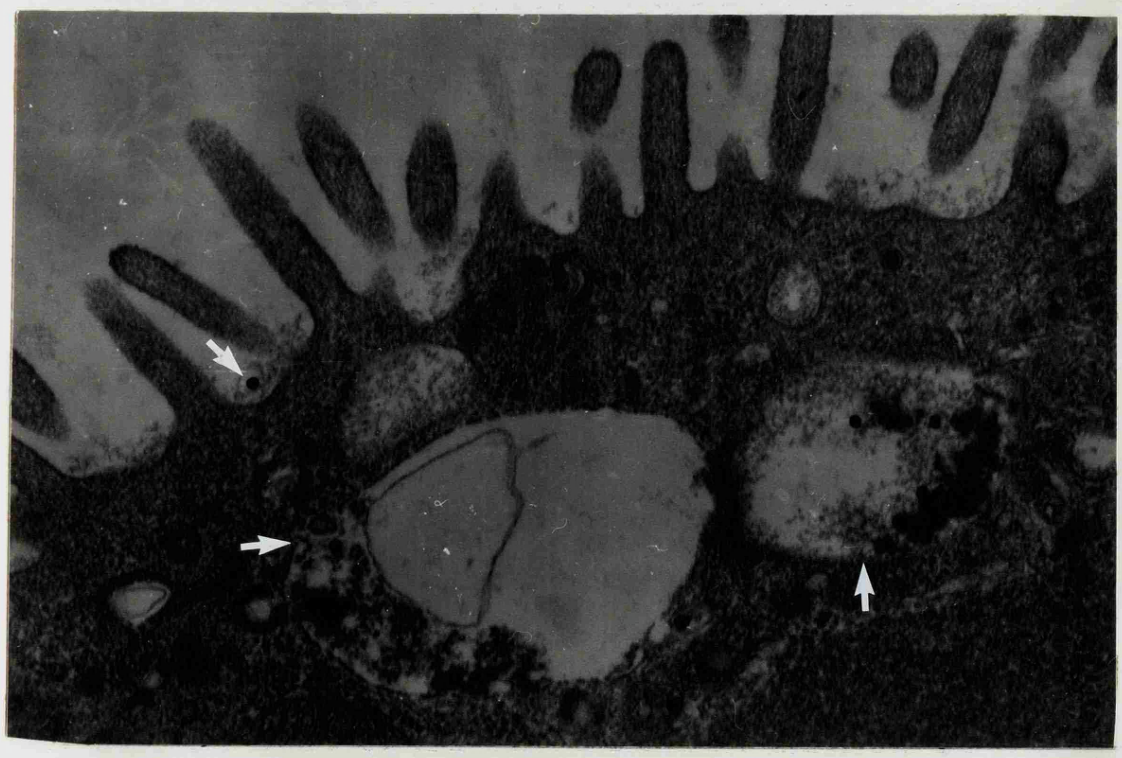


Figure 6.

Figure 7

After 60 minutes of incubation in medium containing colloidal gold particles, vacuoles of type 3 and 4 contain colloid (arrows). (Mag x 38,500).

Figure 8

High magnification macrograph. This shows the accumulation of gold particles in electron dense vacuoles after 60 minutes incubation in medium containing colloidal gold. (Mag x 77,000).

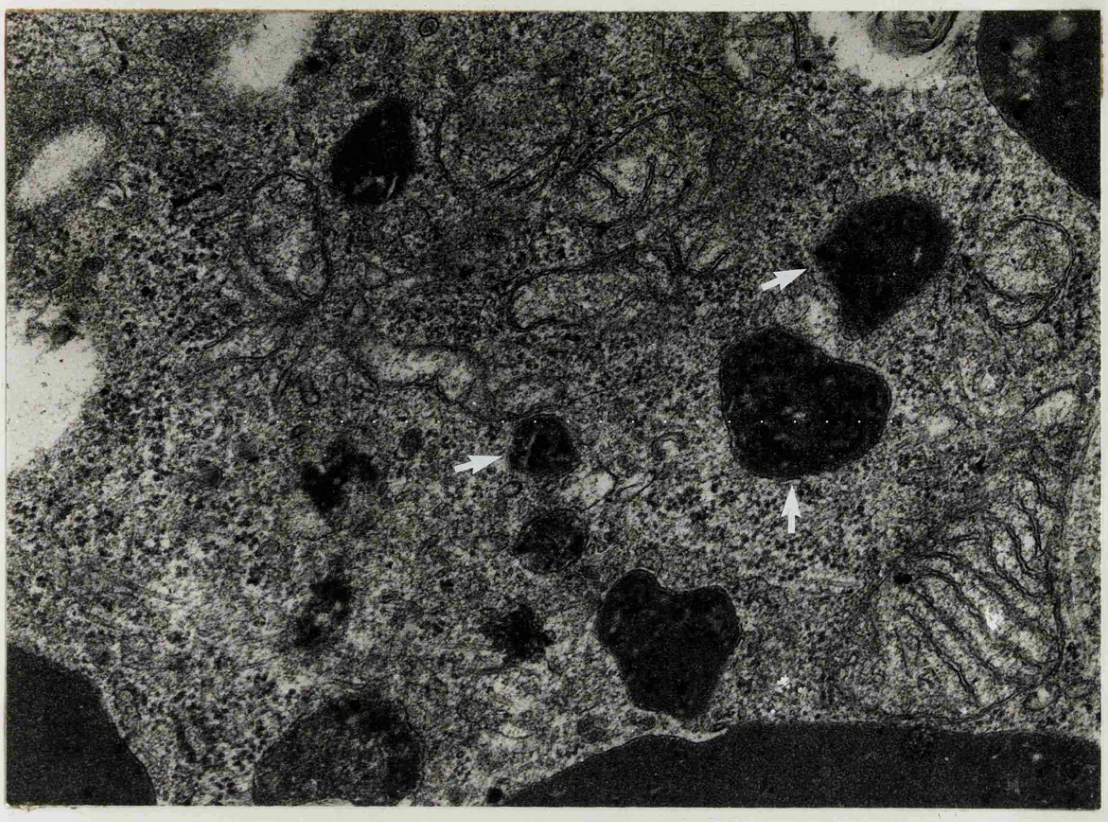


Figure 7.

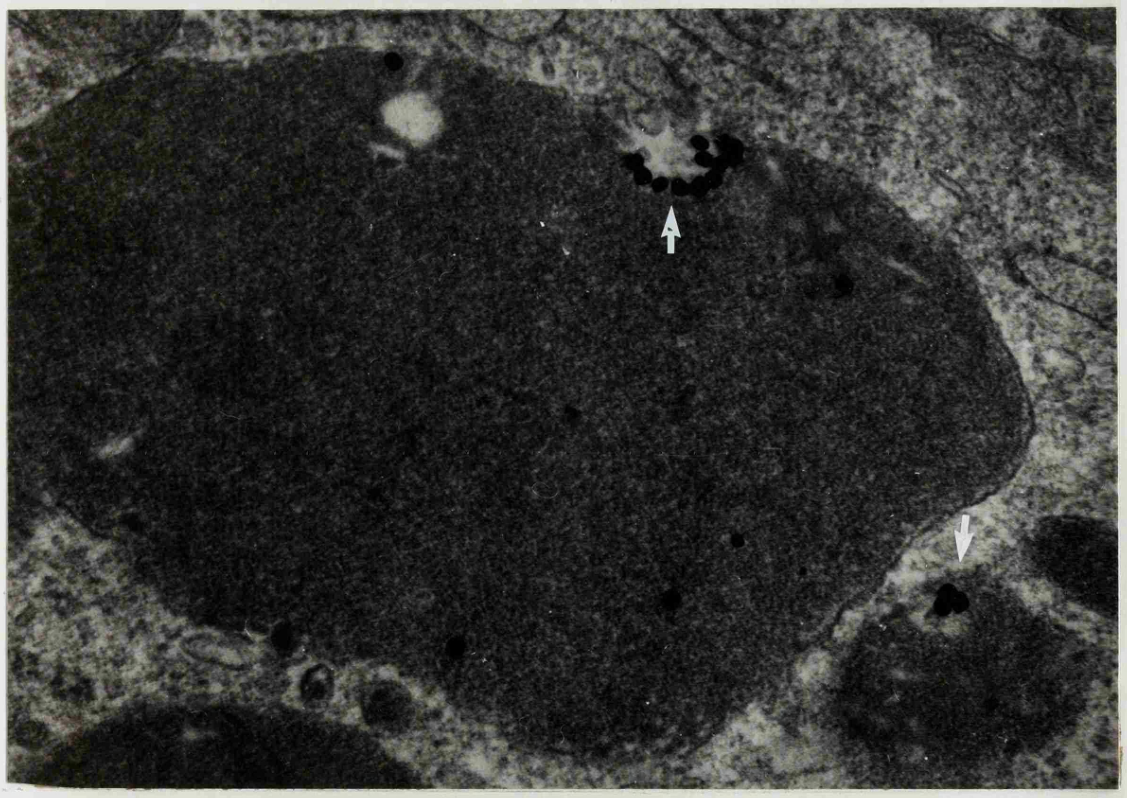


Figure 8.

Discussion

Micropinocytic uptake of macromolecules involves small vesicles of 70 - 100 nm in diameter (Allison and Davies, 1974). Electron microscopical examination of the giant yolk sac endodermal cells has shown an abundance of small coated and smooth membrane vesicles (Type I vacuoles) in association with plasma membrane at the brush border. Types II, III and IV vacuoles were also present.

In this study gold particles were first observed after 5 minutes' exposure in Type I vacuoles; they were seen in Types II, III and IV vacuoles after 10, 15 and 20 minutes respectively. By 60 minutes there was an accumulation of gold particles in both Type III and Type IV vacuoles. This suggested that macromolecules ingested by endocytosis in the giant yolk sac endodermal cells follow a pathway involving a specific sequence of vacuoles. Gold particles move from Type I to Type II, to Type III and then Type IV vacuoles. Recently Gupta and Beck (1981) have observed gold particles, endocytosed by 11½ day visceral yolk sac endodermal cells, following a similar pathway.

Straus (1963) has outlined a mechanism of endocytosis in which he suggested macromolecules are taken in by endocytosis and come to lie in small vesicles bounded by plasma membrane (micropinocytic vesicles); these fuse to form larger vacuoles (heterophagosomes) which acquire hydralytic enzymes to digest the ingested materials so fusion takes place with either primary or secondary lysosomes to create heterolysosomes (Figure 9).

A similar mechanism of endocytosis probably operates in the giant yolk sac endodermal cells in which the micropinocytic vesicles illustrated correspond to Type I vacuoles, the heterophagosomes corresponding to Types III and IV vacuoles. Previous studies have shown that material endocytosed by the rat visceral yolk sac endodermal cells is

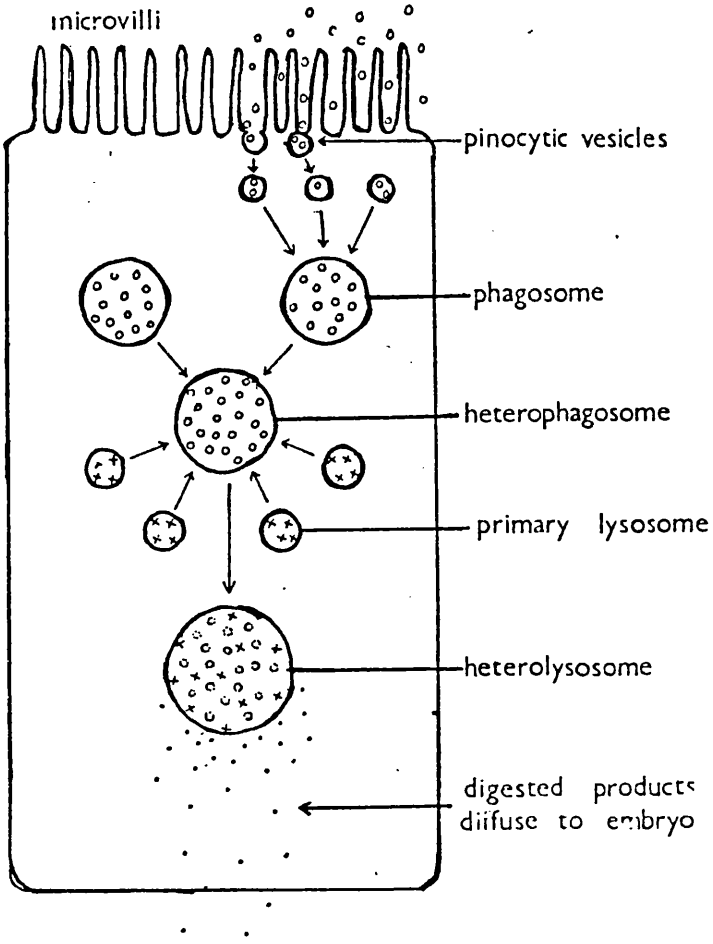


Figure 9

Diagram to illustrate the process of heterosis in its simplist form. There is evidence to indicate that heterophagosomes obtain hydrolytic enzymes by fusion with secondary lysosomes (e.g. heterolysosomes) as well as with primary lysosomes (this is now shown in the diagram). The process illustrated here is of micropinocytosis, but the uptake of macromolecules may equally well involve gross pinocytosis or even the uptake of solid particles, e.g. red blood corpuscles and endometrial cell detritus (Beck, 1976).

digested by lysosomal enzymes within the endodermal cells (Beck et al, 1967b, 1971; Beck and Lloyd, 1968). Using electron microscopical techniques Beck and Lloyd (1968) found that acid phosphatase is localised in primary lysosomes and heterolysosomes but not in the endocytic vesicles or the tubular system. They also studied the location of maternally injected horseradish peroxidase in rat visceral yolk sac epithelial cells. Using light microscopical techniques they demonstrated the presence of both horseradish peroxidase and acid phosphatase in a region close to the nucleus (i.e. the heterolysosome region) but only horseradish peroxidase near the cell apex.

In the giant yolk sac endodermal cells acid phosphatase activity was found to be localised in the supra-nuclear region and its activity was found to be equal to that in an $18\frac{1}{2}$ day visceral yolk sac grown *in vivo* (Section 5). The present study has shown the accumulation of gold particles in Types III and IV vacuoles (heterolysosomes) in the supra-nuclear region. From these observations it can be concluded that giant yolk sac endodermal cells can actively endocytose material and possibly this material is digested by lysosomal enzymes within the giant yolk sac endodermal cells.

CHAPTER V

SECTION 7

ANALYSIS OF GIANT YOLK SAC FLUID BY ELECTROPHORESIS

INTRODUCTION

Several authors (Beck, et al., 1967; New and Brent, 1972; Pyne and Deuchar, 1972; Goetze et al., 1975; Sharma and Peel, 1979) have adduced circumstantial evidence for the yolk sac having a nutritional role during rat organogenesis. Freeman et al., (1981) provided direct evidence for the role of the yolk sac in mediating embryonic nutrition; they showed that in rat conceptuses cultured in vitro, exogenous radio-labelled serum proteins are captured by pinocytosis into visceral yolk sac epithelial cells, where upon they are digested within lysosomes to amino acids which are consequently used for protein synthesis in both the visceral yolk sac and the embryo. For simplicity I have called the fluid lining the inner surface of the visceral yolk sac the yolk sac fluid, although it constitutes the fluid within the extraembryonic coelom of the rat conceptus. By 11 days of gestation the yolk sac fluid is situated between the embryo in the amniotic cavity and is surrounded by the visceral yolk sac. Such a situation persists until term (Figure 1).

The precise method by which yolk sac fluid is utilised in embryonic

nutrition is not quite clear. It contains proteins, amino acids and other solutes (Tam and Chan, 1977). Yolk sac fluid proteins are in part synthesised by the visceral yolk sac epithelium but their origin from the embryo or amnion has not been excluded. The amino acids in the fluid are largely the result of breakdown of macromolecules intralysosomally in the visceral yolk sac but some have been transported as small molecules across the yolk sac by other than the lysosomal route.

In view of the foregoing points, it is obvious that a study concerning embryonic nutrition should include a study of the yolk sac fluid which could reflect the activity and behaviour of visceral yolk sac cells.

Studies in section 2-6 of this chapter have shown that the giant yolk sac is made up of an intact membrane which is actively endocytosing macromolecules present in the incubation medium. The results indicate the probable intracellular digestion of macromolecules in the giant yolk sac endodermal cells. One of the most important applications of the giant yolk sac model is the large quantity of yolk sac fluid it can provide, enabling its biochemical contents to be characterised.

In contrast to the cultured yolk sac at $11\frac{1}{2}$ days the giant yolk sac (of an equivalent gestational age) from 12 days onwards loses its vitelline circulation with the consequent death of the embryo. Therefore, transport of amino acids and proteins across the yolk sac membranes should lead to their accumulation in the yolk sac fluid.

The aims of the studies in the following sections were to determine some characteristics of the giant yolk sac fluid and to relate these to yolk sac functions. In Section 7, the giant yolk sac fluid at the end of its culture period was analysed for its protein content by electrophoresis on gradient polyacrylamide gels. Yolk sac fluid from $18\frac{1}{2}$ days old conceptuses was used as a reference. In Section 8 the transport and accumula-

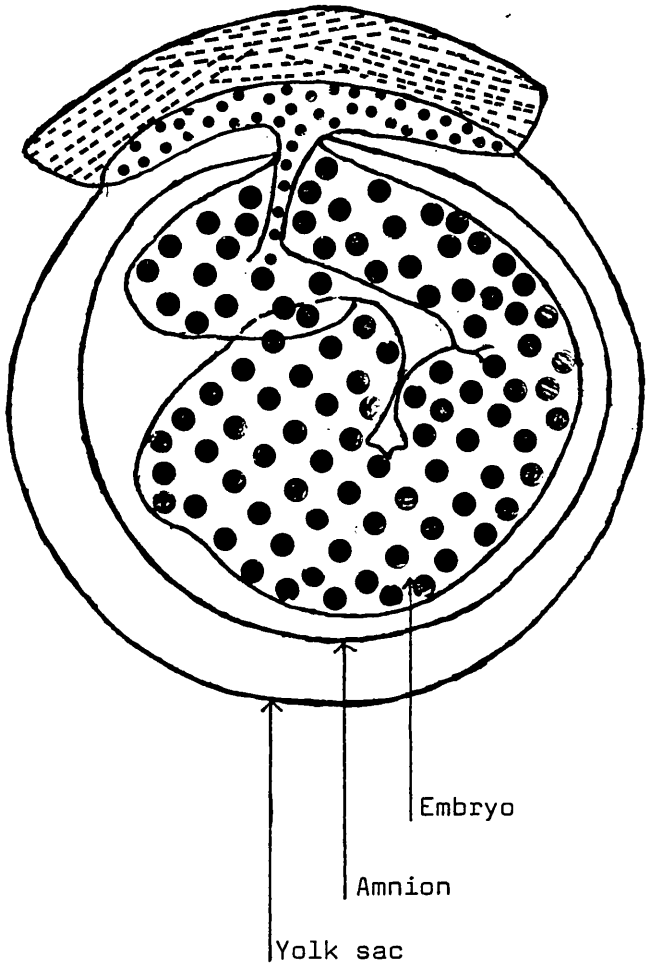


Figure 1

Diagramatic representation of rat embryo at 11½ days of gestation, illustrating the embryo and its relationship to extra-embryonic membranes (from New, 1973).

tion of C^{14}amino acids across the giant yolk sac was investigated in a timed study. Section 9 aimed to characterise giant yolk sac fluid proteins from giant yolk sac incubated in medium containing C^{14} amino acids.

In Sections 8 and 9 the 12½ day yolk sac fluid was used as a reference for the analysis of the giant yolk sac fluid. The 12½ day old conceptuses were grown from 9½ days old conceptuses cultured in serum for 72 hours. After day 11½ of gestation the conceptuses were gassed with 20% O_2, 5% CO_2, 75% N_2 which allowed the yolk sac to expand normally but resulted in the death of the embryo.

The 12½ days old conceptuses were used because: a) they do not contain a live, metabolising embryo which might complicate interpretation of the results; (b) they provide more yolk sac fluid for analysis than do the 11½ day conceptuses and (c) they might represent the functional state of an early giant yolk sac.

The work in sections 8 and 9 of this chapter was done in collaboration with Dr. M. Huxham.

MATERIALS AND METHODS

Nine and a half day old conceptuses were obtained from Wistar rats, see Chapter II, D. Pooled immediately centrifuged serum, from 20-25 rats was prepared as in Chapter II, C. Serum was always heat-inactivated prior to use.

Giant yolk sacs were prepared from $9\frac{1}{2}$ day old conceptuses as described in Section 2 of this chapter. At the end of the 9th day of culture the giant yolk sacs were harvested and washed in 3 x 30 mls of Hanks' Solution and their yolk sac fluid and yolk sac amniotic fluids were collected as in Section 2 of this chapter. Giant yolk sac fluid and amniotic fluid from 12 giant yolk sacs were pooled separately and stored at $-20^{\circ}C$. Yolk sac fluid from $18\frac{1}{2}$ days old *in vivo* rat conceptuses served as a control. Electrophoresis of the three samples (giant yolk sac fluid, amniotic fluid from giant yolk sac and yolk sac fluid from $18\frac{1}{2}$ days old *in vivo* rat conceptuses) was carried out 3 times. In the first attempt a 12.5 ul sample of giant yolk sac fluid and 25 microlitres of each of the giant yolk sac amniotic fluid and yolk sac fluid collected from $18\frac{1}{2}$ day old conceptuses were subjected to electrophoresis. In the second attempt, double the amount was used (this was to improve the staining intensity of some protein bands which stained very faintly in the first attempt). In the third attempt equal volumes (50 ul) from the above samples were subjected to electrophoresis (this was done to be used in the comparison of the staining intensity i.e. protein content, of the protein bands in each of the samples).

In all attempts ready-made polyacrylamide gradient gels 4-30 percent (obtained from Pharmacia Fine Chemicals, Sweden) were used for analysis. Electrophoresis, staining and destaining of the gels was carried out as described in Appnedix G.

Pharmacia high molecular weight protein markers were run on each gel

and used as a reference for determining the molecular weights of the protein bands in the samples. Calibration curves of the respective Rf values were plotted and the molecular weights of the proteins present in each sample calculated as in Appendix G.

RESULTS

Using a 4-30 percent gradient polyacrylamide gel the electrophoretic pattern of giant yolk sac fluid showed the presence of 14 protein bands (Figure 2); the average molecular weights of these bands are given in Table 1. Doubling the amount of samples subjected to electrophoresis did not alter the electrophoretic patterns of the sample qualitatively. There was an increase in staining intensity of all protein bands, but no new protein band appeared (Figure 3). It was obvious that certain protein bands were darker (and therefore contained more protein) than others. The degree of staining was classified as follows - very slight (+), faint (++), moderate (+++) and dark (++++). The intensity of staining was assessed when equal volumes of the 3 samples were subjected to electrophoresis on one gel.

Of the 14 bands separated in giant yolk sac fluid, 2 bands were darkly stained, 2 bands moderately stained, 6 bands faintly stained and 4 bands took very slight staining.

The electrophoretic pattern of yolk sac fluid from 18½ day old conceptuses obtained in vivo (Figure 3) showed the presence of 14 protein bands. The molecular weights of these proteins were identical to their corresponding pairs in the electrophoretic pattern of gaint yolk sac fluid (Table 1). Comparison of the electrophoretic patterns of giant yolk sac fluid with that of 18½ day conceptus yolk sac fluid showed that 7 bands had a different staining intensity. There were 4 bands in the 18½ day conceptus yolk sac fluid which stained less intensely than in giant yolk sac fluid. The molecular weights of these bands were 130OK, 90OK, 140K and 120K daltons. Three bands stained in giant yolk sac fluid less intensely than in the 18½ day conceptus yolk sac fluid. The molecular weights of these protein bands were 180K, 160K, and 110K daltons (Table 1). The electrophoretic pattern of amniotic fluid from giant yolk sac showed the presence of nine protein bands;

all had similar molecular weights to bands in giant yolk sac fluid and the average molecular weights of these proteins are shown in Table 1.

A comparison of the electrophoretic patterns of amniotic fluid and that of giant yolk sac showed the following three differences:

1. Five protein bands with molecular weights of 1,300K, 900K, 600K, 180K and 160K daltons were present in the giant yolk sac fluid and not in the giant yolk sac amniotic fluid.
2. One protein band in the range of 140K daltons stained more intensely in the giant yolk sac fluid.
3. One band in the range of 110K daltons stained more faintly in the giant yolk sac fluid.

Figure 2

Polyacrylamide-gel electrophoretogram at pH 8.3 Lane (1) 25 ul of yolk sac fluid from 18½ day old conceptus in vivo, Lane (2) 5 ul of pharmacia high molecular weight marker proteins, Lane (3) 12.5 ul of giant yolk sac fluid and Lane (4) 25 ul of amniotic fluid from giant yolk sac. (Coomassie brilliant blue stain).

Figure 3

Polyacrylamide-gel electrophoretogram, Lane (1) 50 ul of giant yolk sac fluid, Lane (2) 50 ul of yolk sac fluid from 18½ days old conceptuses in vivo, Lane (3) 50 ul of amniotic fluid from giant yolk sac and (4) 10 ul of pharmacia high molecular weight marker proteins. (Coomassie brilliant blue stain).

Figure 2.

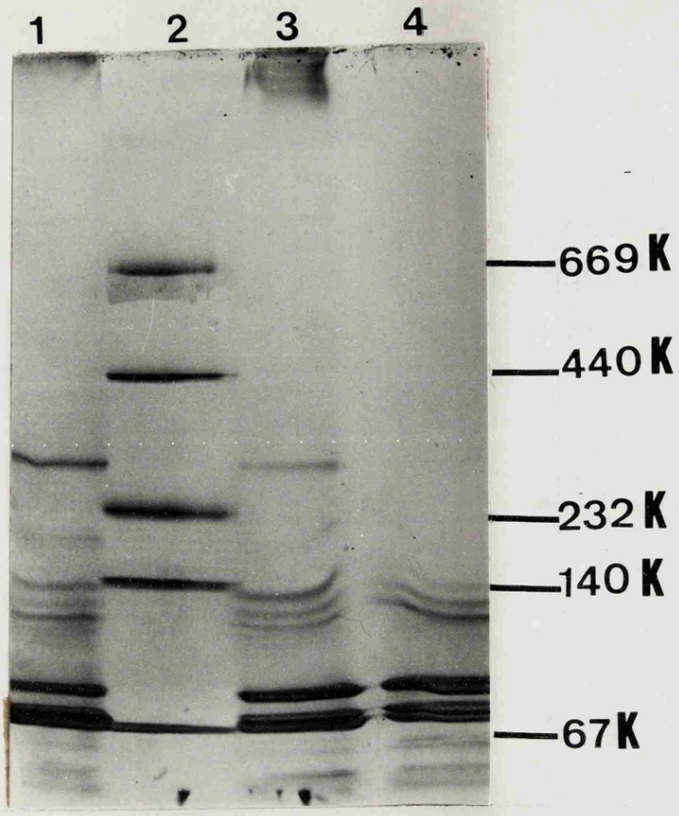


Figure 3.

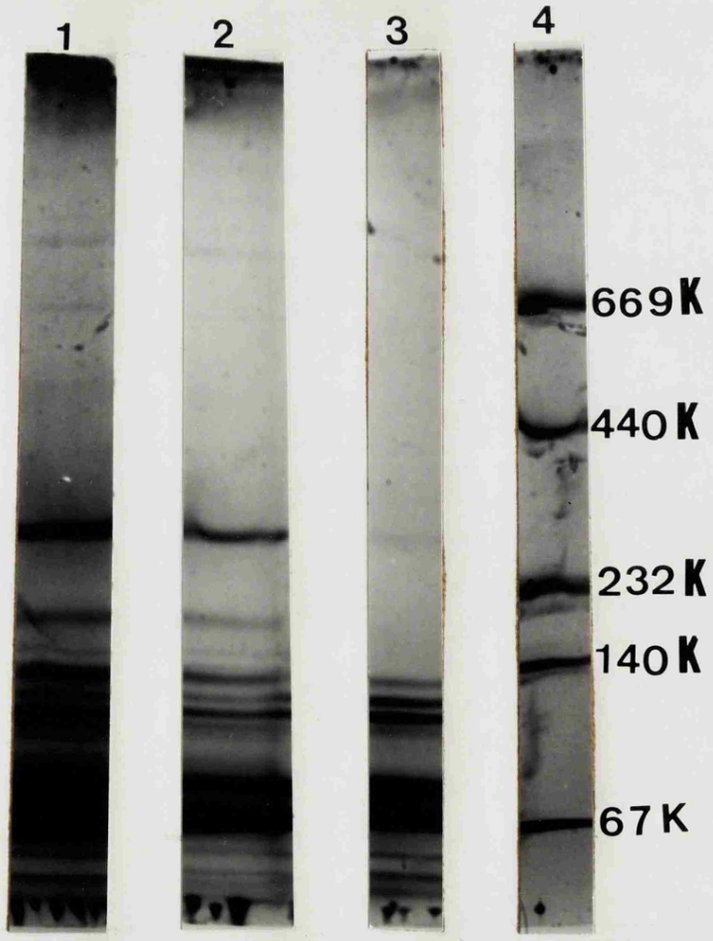


TABLE 1

Distribution of average molecular weight of proteins in giant yolk sac fluid and amniotic fluid from giant yolk sacs and a yolk sac fluid from 18½ days old conceptuses from in vivo. Separated on 4-30% gradient polyacrylamide gel, at pH 8.3. Degree of staining, (+) very slight, (++) faintly, (+++) moderate and (++++) dark.

Band No.	M.W. of proteins in giant yolk sac fluid	Staining Intensity	Band No.	M.W. of proteins in amniotic fluid from giant yolk sac	Staining Intensity	Band No.	M.W. of proteins from 18½ days yolk sac fluid	Staining Intensity
1	1,300,000	++		-	-	1	1,300,000	+
2	900,000	++		-	-	2	900,000	+
3	600,000	+		-	-	3	600,000	+
4	250,000	+	1	250,000	+	4	250,000	+
5	180,000	+++			-	5	180,000	++++
6	160,000	+			-	6	160,000	+
7	140,000	+++	2	140,000	+	7	140,000	++
8	120,000	++	3	120,000	++	8	120,000	+
9	110,000	++	4	110,000	+++	9	110,000	+++
10	94,000	+	5	94,000	+	10	94,000	+
11	80,000	++++	6	80,000	++++	11	80,000	++++
12	68,000	++++	7	68,000	++++	12	68,000	++++
13	62,000	++	8	62,000	++	13	62,000	++
14	56,000	++	9	56,000	++	14	56,000	++

DISCUSSION

This study demonstrated the presence of 14 protein bands in the electrophoretic pattern of giant yolk sac fluid. In yolk sac fluid from $18\frac{1}{2}$ days old conceptuses obtained *in vivo*, 14 protein bands of identical molecular weight to protein bands present in giant yolk sac fluid were recognised.

Of the original yolk sac fluid proteins demonstrated, some were transported across the visceral yolk sac intact, the remainder were synthesised probably by the visceral layer of the yolk sac, and possibly by the amnion.

The absence of a live embryo in the giant yolk sac suggests that at this stage the contribution made by the embryo in the synthesis of yolk sac fluid proteins is minor or absent. The qualitative similarity between proteins present in both yolk sac fluids analysed, suggest that they are similarly derived.

Comparison between electrophoretic patterns of giant yolk sac fluid and the $18\frac{1}{2}$ day yolk sac fluid using equal volumes of both fluids, showed differences in the staining intensity of 7 protein bands. Protein bands of molecular weight, 1300K, 900K, 140K and 120K daltons were stained more intensely in giant yolk sac fluid (and therefore contained more proteins) than in the $18\frac{1}{2}$ day yolk sac fluid. This is possibly due to the absence of consumption by the live embryo in the giant yolk sac system. On this assumption the presence of 3 protein bands of molecular weight 180K, 160K and 110K daltons which stained more intensely in the yolk sac fluid from $18\frac{1}{2}$ day conceptuses. This could be explained either by an embryonically contributed source or by a maternal source derived from the normal physiological changes that occur *in vivo* during pregnancy.

Electrophoresis of giant yolk sac amniotic fluid demonstrated, the presence of 9 protein bands which were similar in quality to protein bands present in the giant yolk sac fluid but differed in quantity.

From the qualitative and quantitative differences between giant yolk sac fluid and its amniotic fluid it can be concluded that the amnion allows selective passage of proteins between yolk sac fluid and amniotic fluid but not their free transport.

CHAPTER V

SECTION 8

TRANSPORT AND ACCUMULATION OF C^{14} AMINO ACIDS ACROSS THE GIANT YOLK SAC

MATERIALS AND METHODS

1. Experiment 1 - Time course study of accumulation of C^{14} amino acids in yolk sac fluid

Nine and a half days old conceptuses were obtained from Wistar rats as described in Chapter II, D. Pooled immediately centrifuged serum from 20 - 25 rats was prepared (see Chapter II, C) and heat-inactivated before use.

Preparation of giant yolk sacs and 12½ days old conceptuses

Giant yolk sacs were prepared by culturing 9½ days old rat conceptuses in fresh H.I. serum for 9 days as described in Section 2 of this chapter and, at the end of this time, they were washed twice in 30 mls Hanks' solution and rinsed in M199, both at 37°C.

As a comparison 12½ days old rat conceptuses were used. These were grown from 9½ days old embryos cultured in fresh H.I. serum for 72 hours, the first 48 hours of which were carried out as described in Chapter II, D, and concluded by the culture serum being replaced with fresh H.I. serum - again in the ratio of 1 conceptus per 1 ml of serum. The culture bottles

were gassed for 1½ minutes with 20% O_2, 5% CO_2 and 75% N_2 and incubated at 37°C for a further 24 hours. At the end of the total culture period the 12½ days old conceptuses were harvested and examined; those found to have a beating heart were discarded but usually 95% of the embryos were dead by this time. These were then washed twice in 20 mls Hanks' solution and rinsed in M199 at 37°C.

At this stage the giant yolk sacs and the 12½ days old conceptuses were ready to be cultured in the radio-active medium.

Preparation of the radio-active medium

A total of 2.5 mls radio-active amino acids (Amersham International Ltd., U.K.) containing 4884 KBq was made up to 10 mls with Medium 199 (Gibco-Europe U.K.). The labelled amino acids composition is shown in Table 1.

Incubation of the giant yolk sacs and 12½ days old conceptuses in the radio-active medium and collection of their yolk sac fluids

Two or three giant yolk sacs were cultured in one 60 ml bottle containing the radio-active medium. The bottle was gassed with a mixture of 20% O_2, 5% CO_2 and 75% N_2 and incubated at 37°C for periods of 30 minutes, 1 hour, 2 hours and 4 hours. At least three separate incubations were carried out for each of the time periods studied.

At the end of each incubation period the giant yolk sacs were washed three times in 30 mls Hanks' solution, their yolk sac fluid was collected and pooled (as in Section 2 of this chapter) and subsequently stored at -20°C.

The 12½ days old conceptuses were cultured in the radio-active medium in the same manner as for the giant yolk sacs and for the same time periods. At the end of each incubation they were washed three times in 20 mls Hanks' solution. The yolk sac membrane then being punctured with

TABLE 1

Shows the composition of the C^{14} amino acids used in 10 mls of incubation medium by activity

	Amino Acid	KBq in 10 mls Medium
1	L-Alanine	352
2 3	L-Arginine monohydrochloride	259
4	L-Aspartic Acid	370
5 6	L-Cystine hydrochloride	74
7	L-Glutamic Acid	333
8	L-Glutamine	370
9	Glycine	222
10	L-Histidine	55.5
11	L-Isoleucine	222
12	L-Leucine	463
13	L-Lysine	185
14	L-Phenylalamine	278
15	L-Proline	204
16	L-Serine	111
17	L-Threonine	204
18	DL-Tryptophan	740
19	L-Tyrosine	222
20	L-Valine	222

a finely drawn glass pipette in an area of few vitelline vessels and the fluid drawn out by suction collapsed onto the intact amnion containing fluid and the embryo. In this way up to 25 ul of yolk sac fluid per conceptus was obtained, essentially free of contamination by incubating medium and amniotic fluid.

Radio-active analysis of the yolk sac fluid

The yolk sac fluid samples analysed from each incubation period were from 6 to 10 giant yolk sacs or the same number of 12½ days old conceptuses.

To 50 ul of yolk sac fluid was added 50 ul of 10% 5-sulphosalicylic acid (SSA) (Fisons, U.K.); this was mixed and allowed to stand for 30 minutes at room temperature followed by centrifugation at 8000 X g on a Beckman microfuge for 30 seconds. To the supernatant (free amino acid fraction) was added 3.5 mls Fisofluor 1 (Fisons, U.K.) and the radioactivity counted on a Packard Tri-Carb liquid scintillation spectrometer.

The sulphosalicylic acid precipitate (protein synthetic fraction) was collected from all samples for each incubation period studied and stored at -20°C to be used later for examining the accumulation rate of proteins synthesised by the yolk sac in the yolk sac fluid.

As a control, 50 ul of the radio-active amino acid medium samples were diluted with 50 ul distilled water, mixed with 3.5 mls Fisofluor 1 and counted as before.

Experiment 2 - Analysis of yolk sac fluid for C^{14} amino acids transported across the visceral yolk sac

The fluid from giant yolk sacs and 12½ days old conceptuses used in this experiment was drawn from the pools collected in Experiment 1 of this section, where the giant yolk sacs and 12½ days old conceptuses had been incubated in M199 containing radio-active amino acids for 4 hours.

Preparation of samples for amino acid separation

To 12 ul of giant yolk sac fluid 12 ul 10% 5-sulphosalicylic acid (SSA) was added and mixed; having been allowed to stand for 30 minutes at room temperature the mixture was then centrifuged at 8000 X g on a Beckman microfuge for 30 seconds. The supernatant was then collected and dried in a water bath at 45°C after which the resultant crystalline was dissolved in 3 ul water. At this stage the samples were ready for amino acid separation.

As a control 12 ul each of the yolk sac fluid from the 12½ days old conceptuses and the radio-active medium was treated in the same way as the giant yolk sac above described.

Separation of amino acids by two-dimensional thin layer chromatography

This was carried out according to the method described by Arx & Hener. (1963), using 20 cm x 20 cm thin layer cellulose (TLC) sheets (Pierce & Warriner Ltd., U.K.)

Preparation of solvents used for the separation

Solvent I - used for separation in the first dimension.
This consisted of N-Butanol-Acetone-Diethylamine-water (10:10:2:5), the final pH of the solution was 12..

Solvent II- used for separation in the second dimension.
This was made up from Isopropanol-Formic acid-water (20:1:5) at pH 2.5.

The detecting reagent used was 5% ninhydrin spray (Pierce & Warriner Ltd., U.K.)

Separation procedure

Samples were loaded on to three separate TLC sheets for each run - all under identical conditions. Before loading the sheets were dried on a hot plate at 45°C for 2 - 3 hours and the sample was then spotted in one

corner of the sheet about 20 mm from the edges, in a total volume of 3 ul or less. The loaded sheets were then placed vertically in the solvent chamber containing Solvent I for separation in the first dimension, the solvent covering 1 cm along the lower edge of the sheets. This was allowed to travel to the top of the TLC sheets, taking between 2 - 3 hours, after which they were taken out and dried at 75°C for 1 - 1.5 hours. The solvent chamber was cleaned and 100 ml Solvent II poured in. The TLC sheets were then returned to the chamber and placed vertically at right angles to the position during the first run. Again the solvent was allowed to travel to the top of the sheets and, as before, they were dried at 75°C - this time for 2 hours. The TLC sheets were then sprayed until uniformly wetted with ninhydrin detecting reagent and subsequently allowed to dry at 45°C until the coloured spot developed. The chromatographs were photographed and the Rf value of each spot was calculated as follows:

$$Rf = \frac{\text{distance travelled by solute zone}}{\text{distance travelled by solute front}} \times 100$$

Analysis of each of the samples was repeated four times.

Detection of radio-active amino acids in chromatographs

Using X-ray film - chromatographs were exposed to X-ray film (the emulsion side adjacent to the cellulose surface of the chromatograph) in the dark for three weeks at 4°C; the films were then developed and photographed. This was carried out twice using different chromatographs from each of the samples.

RESULTS

Experiment 1 - the analysis of radioactivity in fluid collected from giant yolk sacs incubated in Medium 199, containing the radio-active agent, shows the accumulation of radio-active amino acids over a 4 hour period. The time course study revealed a linear increase in their accumulation (Figure 1). At 4 hours' incubation there was a seven-fold increase in radioactivity compared to that of the 30 minutes' incubation (Table 2). Figure 1 also shows that 12½ day yolk sac fluid accumulates amino acids at a faster rate than fluid of the giant yolk sac (1.5 times the count at 4 hours).

Experiment 2 - two dimensional thin layer chromatography separation of amino acids

Analysis of amino acids present in the giant yolk sac fluid chromatograph in Figure 2 showed the presence of 20 spots which could indicate the presence of the same number of amino acids. The relative Rf values of the spots can be seen in Table 3.

The separation of amino acids present in yolk sac fluid from 12½ days old conceptuses is shown in Figure 3; it shows the presence of 20 spots and the comparative Rf values of these were similar to those of amino acids present in the giant yolk sac fluid (Table 3).

Separation of amino acids present in the M 199 incubation medium (which contained radio-active amino acids) (Figure 4) showed the presence of 19 ninhydrin staining spots, their relative Rf values being similar to corresponding spots detected in both yolk sac fluids (Table 3).

Detection of radioactivity in the two-dimensional thin layer chromatography by X-ray films

Autoradiography of the two-dimensional thin layer chromatograph for the separation of giant yolk sac fluid amino acids (Figure 5) showed the presence of 15 radio-active spots, of which 12 were detected by ninhydrin

staining in the chromatograph of the giant yolk sac fluid (Figure 2 and Table 4). The remaining three radio-active spots - numbers 21, 22 and 23 (Figure 5) - were detected by autoradiography but did not stain with ninhydrin after development of the two-dimensional thin layer chromatograph (Figure 2 and Table 4).

Autoradiography of the two-dimensional thin layer chromatographs for the separation of $12\frac{1}{2}$ days yolk sac fluid amino acids was similar to that for the giant yolk sac fluid and contained 15 spots (Figure 6). Spots numbers 21, 22 and 23 were present as before but were not detected after ninhydrin colour development.

Autoradiography of the two-dimensional thin layer chromatographic separation of amino acids present in radio-active incubation medium (Figure 7) showed the presence of 16 spots. Again spots 21, 22 and 23 did not stain with ninhydrin (Figure 4 and Table 4).

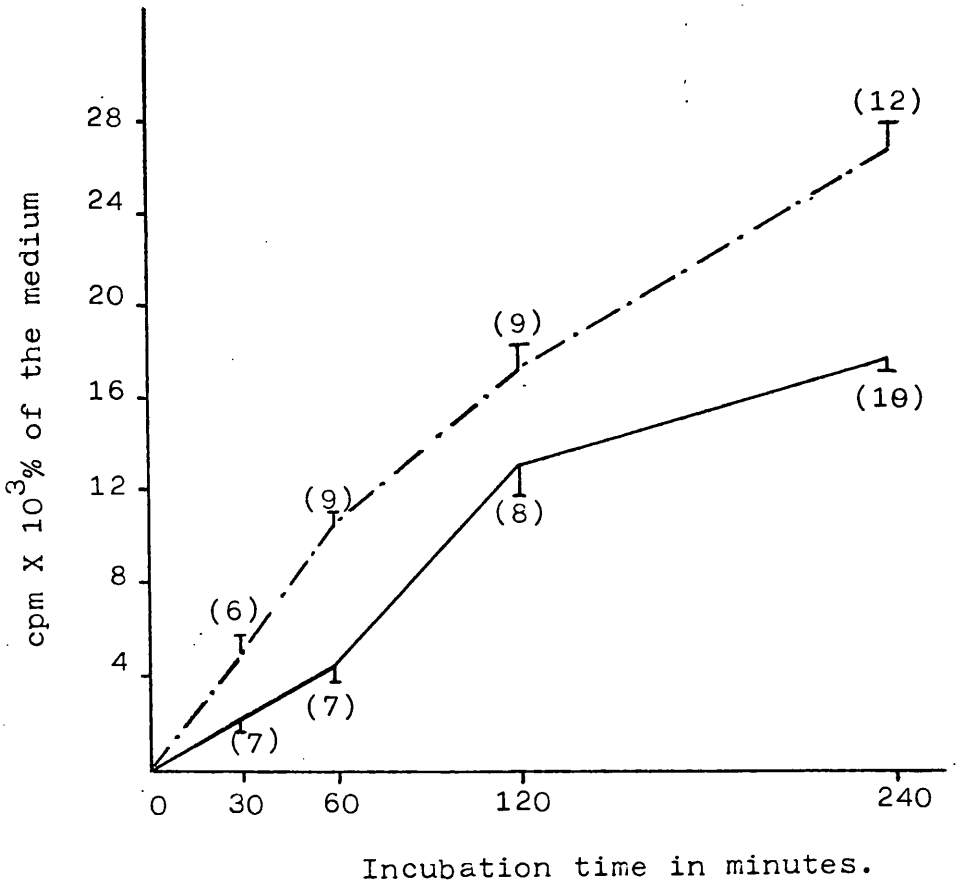


Figure 1

Timed study for accumulation of free C^{14} amino acids in giant yolk sac fluid (-) and 12½ day yolk sac fluid (-·-). Each point represents the mean ± one s.e.m. Numbers in brackets indicate the number of yolk sacs used for each condition. (See Table 2 for p values).

TABLE 2

Shows mean ± s.e.m. of cpm x 10^3% of the medium of the time course study for accumulation of C^{14} amino acids in giant yolk sac fluid and 12½ day yolk sac fluid.

Time	No. of Giant Yolk Sacs	cpm % Medium		No. of 12½ Day Yolk Sacs
		Giant Yolk Sac Flud ± s.e.	12½ Day Yolk Sac Flud ± s.e.	
30 minutes	7	2.36 ± 0.6 ($p < 0.01$)	5.25 ± 0.26 ($p < 0.001$)	6
1 hour	7	4.7 ± 0.53 ($p < 0.001$)	10.6 ± 0.94 ($p < 0.001$)	9
2 hours	8	13.19 ± 0.59 ($p < 0.02$)	17.4 ± 0.93 ($p < 0.001$)	9
4 hours	10	17.71 ± 0.7	26.69 ± 0.88	12

In the time course study for accumulation of C^{14} amino acids yolk sac fluid in both cases increases significantly with time at the p values shown.

At any time period studied the accumulation of amino acids in giant yolk sac fluid is significantly different from that in 12½ day yolk sac fluid at $p < 0.001$.

Figure 2

Two-dimensional thin layer chromatography, showing the separation of 20 amino acids present in giant yolk sac fluid. The transparancy superimposed shows diagrammatically the spots and their numbers (Ninhydrin stain).

Figure 3

Two-dimensional thin layer chromatography, showing the separation of 20 amino acids present in 12½ day yolk sac fluid. The transparency superimposed shows diagrammatically the spots and their numbers. (Ninhydrin stain).

Figure 2.

Figure 3.

Figure 4

Two-dimensional thin layer chromatography, showing the separation of 19 amino acids present in Medium 199. The transparancy superimposed shows diagrammatically the spots and their numbers. (Ninhydrin stain).

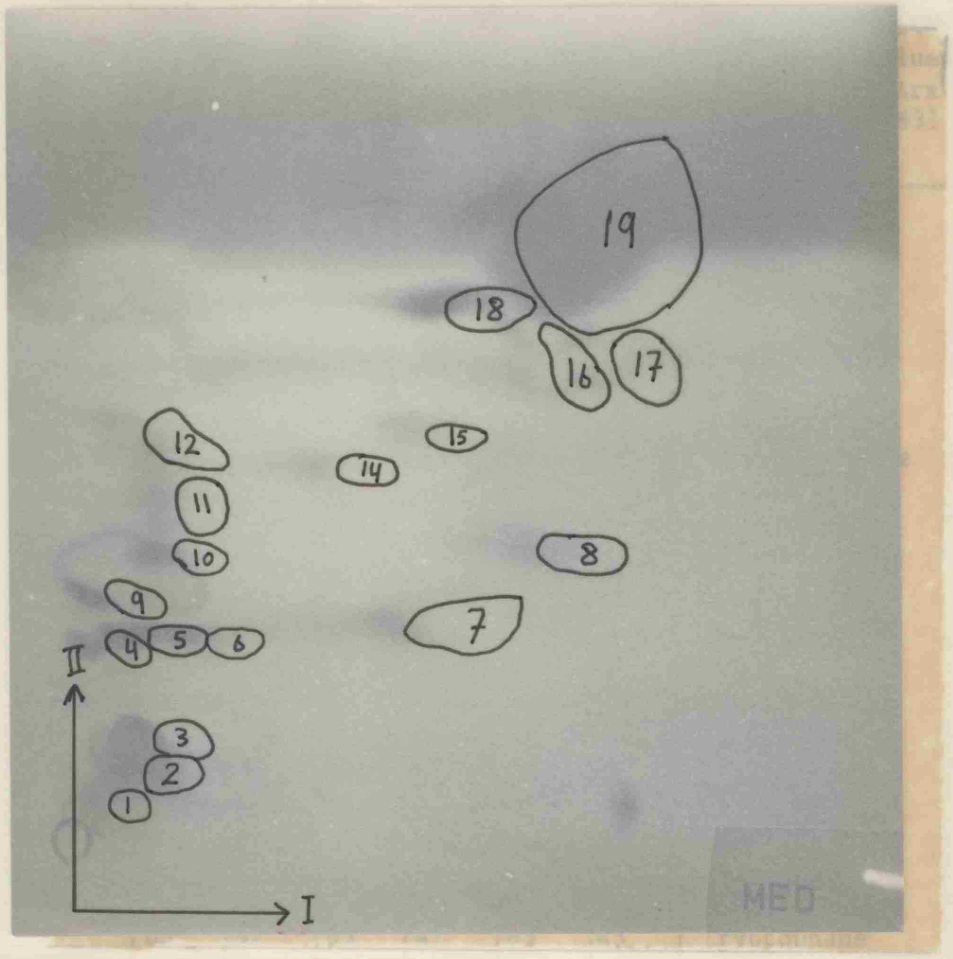


Figure 4.

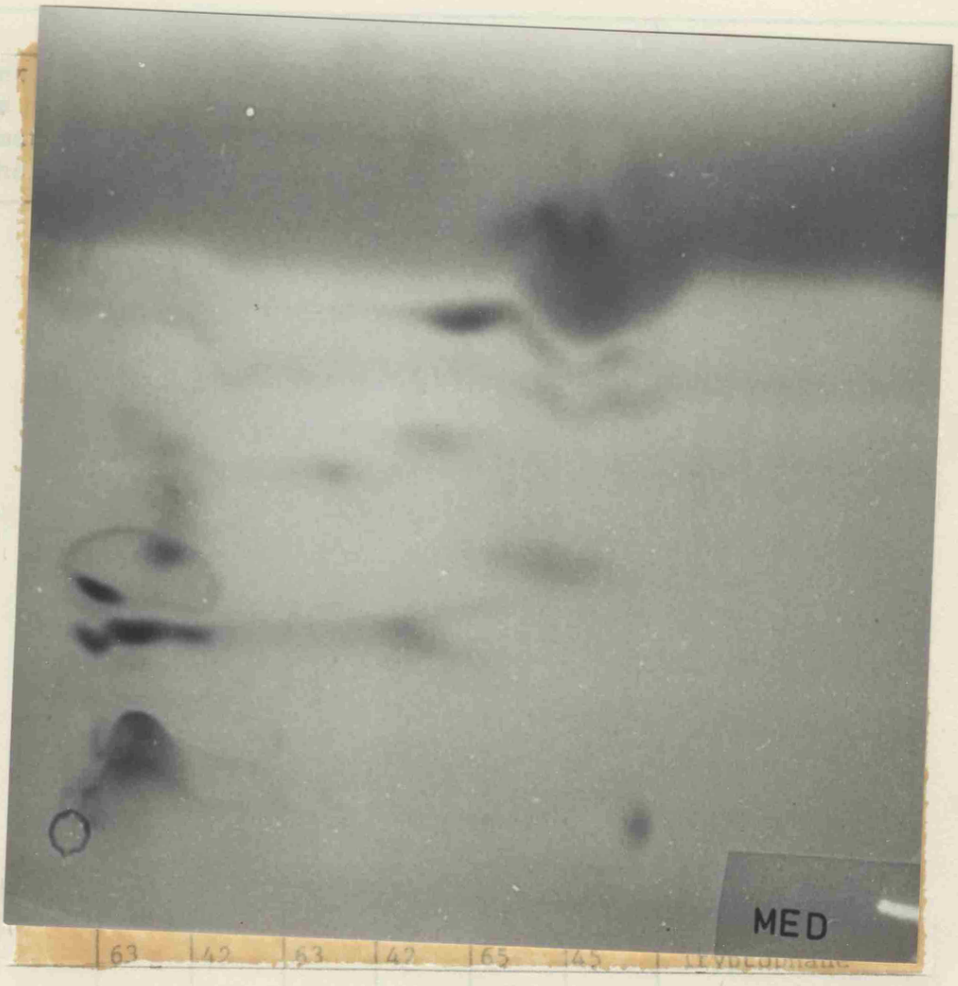


Figure 4.

TABLE 3

The relative Rf values of spots (amino acids) determined by two dimensional chromatographic analysis of amino acids present in 12½ day yolk sac fluid, giant yolk sac fluid and M199. Spots 21, 22 and 23 were detected only by autoradiography and not by the ninhydrin reagent.

Spot Number as in Chromato-graphs	12½ Day Yolk Sac		Giant Yolk Sac Fluid		M199		Amino Acids with similar Rf values described by Arx and Hener (1963)
	Rf I	Rf II	Rf I	Rf II	Rf I	Rf II	
1	5	4	5	4	5	4	-
2	2	4	2	4	2	4	-
3	9	12	9	12	9	11	Arginine
4	8	20	8	20	8	20	-
5	11	21	11	21	10	21	Aspartic Acid
6	26	21	26	21	25	21	Hydroxyproline
7	50	21	50	21	50	21	Serine
8	65	30	65	29	64.2	28	Thereonine
9	5	25	5	25	5	24	-
10	18	34	18	34	18	34	-
11	14	32	14	32	13	31	-
12	15	46	15	46	14	46	-
13	27	38	27	38	-	-	-
14	44	33	44	33	46	33	Proline
15	48	38	48	38	48	38	Tyrosine
16	63	42	63	42	65	45	Tryptophane
17	66	56	66	56	67	55	Phenylalamine
18	53	54	53	54	53	55	Valine
19	62	64	62	64	60	62	Leusine and Isoleucine
20	84	71	82	70	82	71	-
21	21	62	21	62	21	68	-
22	40	70	39	70	40	69	-
23	86	67	86	86	67	86	-

Figure 5

Autoradiograph of the two-dimensional chromatographic separation of giant yolk sac fluid amino acids, showing the presence of 15 spots (radioactive amino acids). The transparancy superimposed shows diagrammatically spots and their numbers.

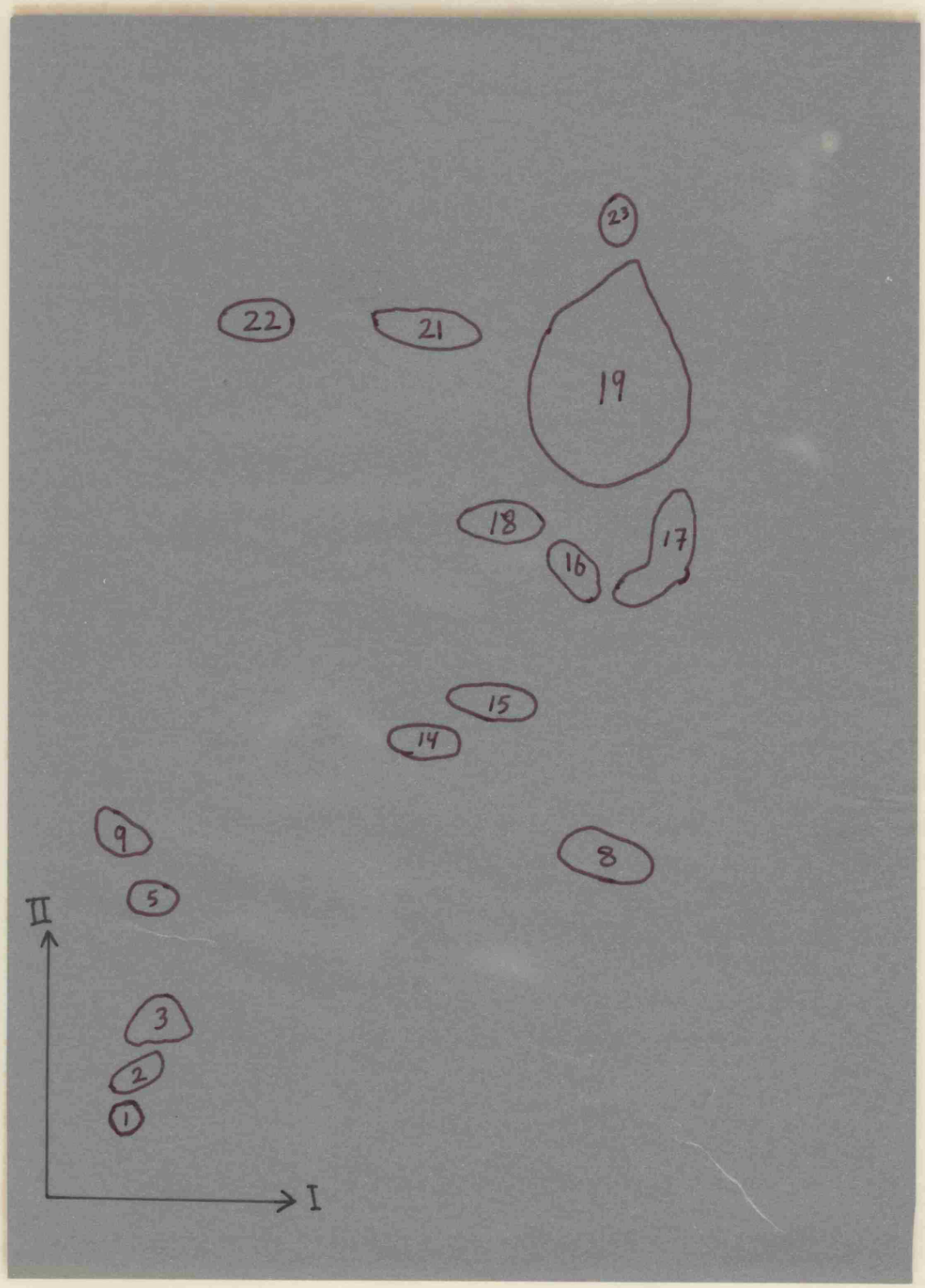


Figure 5.

Figure 5.

Figure 6

Autoradiograph of two-dimensional chromatographic separation, of 12½ day yolk sac fluid amino acids, showing the presence of 15 spots (radioactive amino acids). The transparancy superimposed shows diagrammatically spots and their numbers.

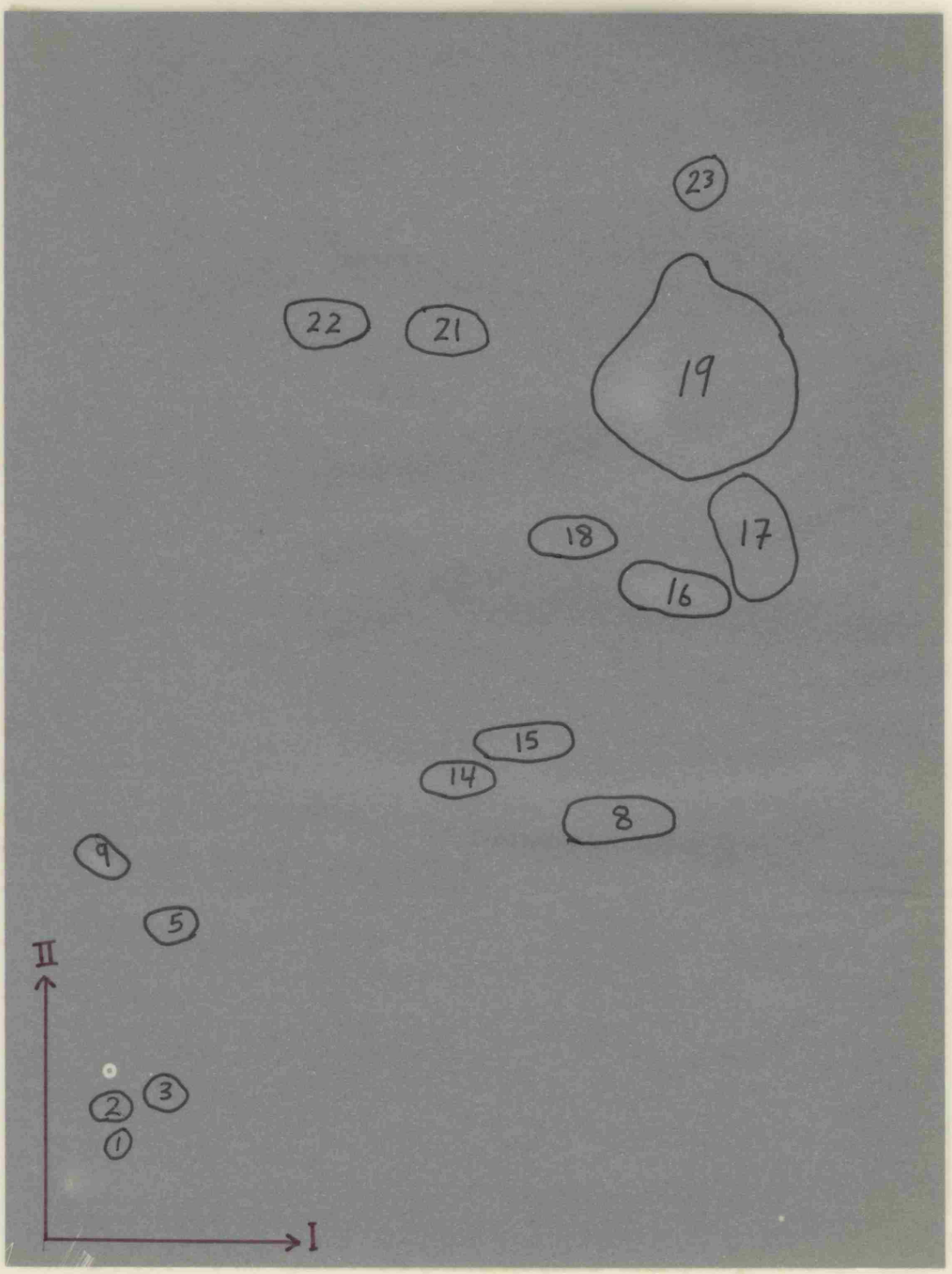


Figure 6 -

Figure 6 -

Figure 7

Autoradiograph of the two-dimensional chromatographic separation of Medium M199, showing the presence of 16 spots (radioactive amino acids). The transparancy superimposed shows diagrammatically spots and their numbers.

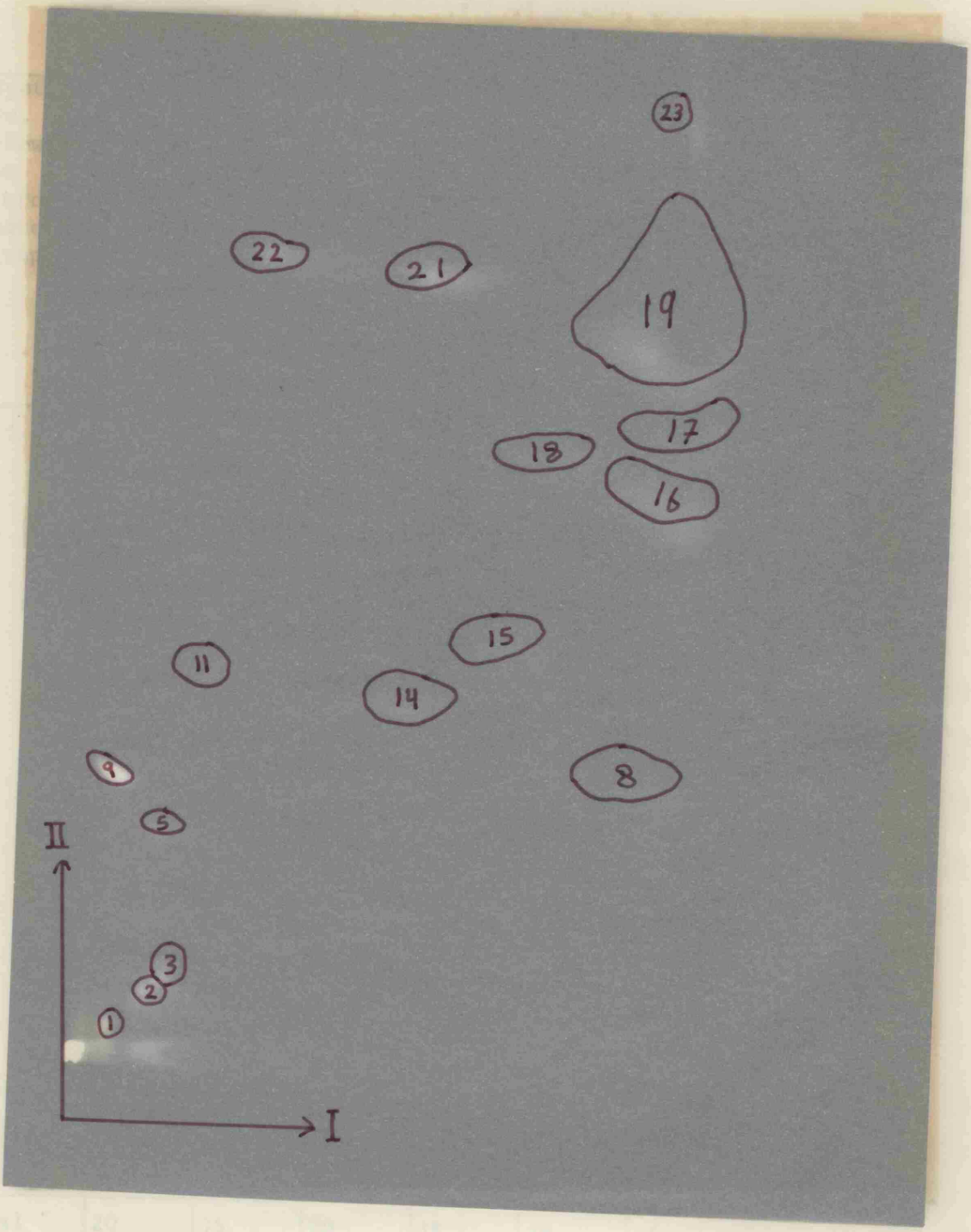


Figure 7.

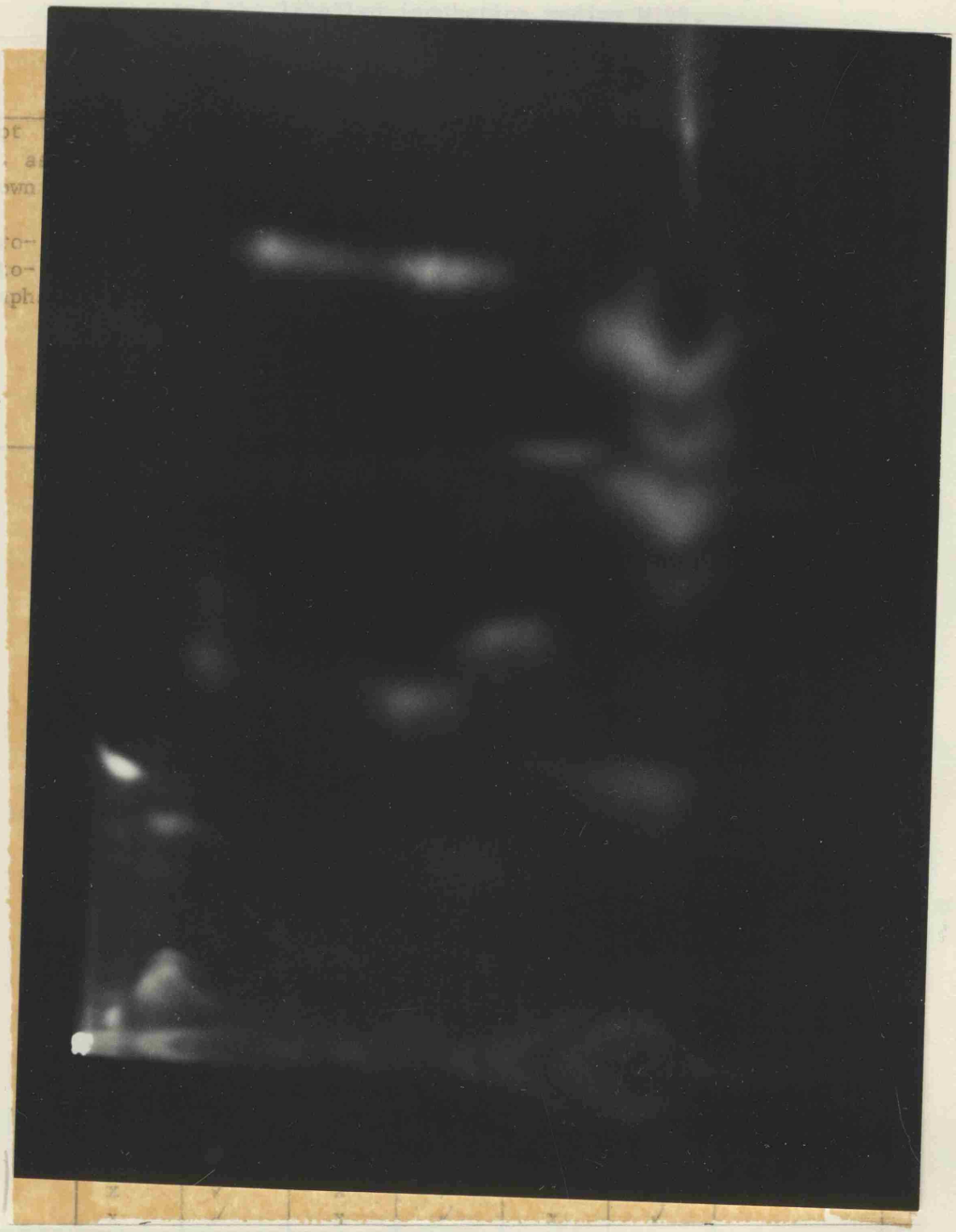


Figure 7.

TABLE 4

Comparison between results obtained from the two-dimensional thin layer chromatographic separation of amino acids present in yolk sac fluids (giant yolk sac fluid and yolk sac fluid from 12½ day conceptus) and the labelled incubation medium M199.
Spots present (✓) or absent (x).

Spot No. as shown on chromatographs	Yolk Sac Fluid of 12½ day old conceptuses		Giant Yolk Sac Fluid		M199 Containing the radioactive amino acids		Amino Acids with similar Rf values described by Arx and Hener (1963)
	Spots Present in:		Spots Present in:		Spots Present in:		
	Chromatographs Ninhydrin stain	Autoradiographs	Ninhydrin stain Chromatographs	Autoradiographs	Chromatographs Ninhydrin stain	Autoradiographs	
1	✓	✓	✓	✓	✓	✓	
2	✓	✓	✓	✓	✓	✓	Arginine
3	✓	✓	✓	✓	✓	✓	
4	✓	x	✓	x	✓	x	
5	✓	✓	✓	✓	✓	✓	Aspartic acid
6	✓	x	✓	x	✓	x	Hydroxyproline
7	✓	x	✓	x	✓	x	Serine
8	✓	✓	✓	✓	✓	✓	Threonine
9	✓	✓	✓	✓	✓	✓	
10	✓	x	✓	x	✓	x	
11	✓	x	✓	x	✓	✓	
12	✓	x	✓	x	✓	x	
13	✓	x	✓	x	x	x	
14	✓	✓	✓	✓	✓	✓	Proline
15	✓	✓	✓	✓	✓	✓	Tyrosine
16	✓	✓	✓	✓	✓	✓	Tryptophan
17	✓	✓	✓	✓	✓	✓	Phenylalamine
18	✓	✓	✓	✓	✓	✓	Valine
19	✓	✓	✓	✓	✓	✓	Leucine and Isoleucine
20	✓	x	✓	x	✓	x	
21	x	✓	x	✓	x	✓	
22	x	✓	x	✓	x	✓	
23	x	✓	x	✓	x	✓	
Total	20	15	20	15	19	16	

DISCUSSION

This study demonstrated the transport of amino acids across the visceral yolk sac and their accumulation in fluid from giant yolk sacs and 12.5 days old rat conceptuses. It also showed that the increased accumulation of radio-active amino acids in both fluids was time dependent. After four hours incubation the fiant yolk sac fluid contained 17% of the total radio-activity in the medium and yolk sac fluid from $12\frac{1}{2}$ days old conceptuses 26.6% of the total radio-activity in the medium. These figures do not indicate the total amino acid accumulation during the culture period since the culture medium contained a large amount of unlabelled amino acids and only a small percentage of C^{14} labelled amino acids. The figures do, however, represent the relative accumulation of C^{14} amino acids during the culture period.

Although at first the apparent greater accumulation of C^{14} amino acids by the $12\frac{1}{2}$ day yolk sac can be interpreted as showing a higher transport rate of amino acids, the importance of unit surface area to volume ratios cannot be ignored.

In this study the $12\frac{1}{2}$ days yolk sac has a surface area of $1mm^2$ for each $1mm^3$ of fluid, whilst the giant yolk sac has a surface area of $1mm^2$ of membrane to $3.3mm^3$ of fluid contained within it. Thus, the surface area to volume ratio of each 'sphere' differs by a factor of 3.3 in favour of the giant yolk sac. If this factor is taken into consideration, then the giant yolk sac membrane is transporting amino acids at a much higher rate than the $12\frac{1}{2}$ day yolk sac.

In experiment 2 the analysis of amino acids by two dimensional thin layer chromatography showed no qualitative differences between amino acids present in giant yolk sac fluid and the yolk sac fluid from $12\frac{1}{2}$ day old conceptuses. In both fluids 20 spots showed ninhydrin staining, whilst,

an analysis of the medium revealed the presence of only 19 spots. The ninhydrin staining spot missing from the medium but present in each of the yolk sac fluids possibly represents an amino acid resulting from the breakdown of proteins by the yolk sac membranes prior to culture in radio-active medium. It was not possible to identify all the amino acids present in the yolk sac fluid chromatographs because the two-dimensional separation technique was of poor resolution. The presence of tailing and deformation of many spots in many of the chromatographs was common and this was possibly due to interference from other compounds present in the samples (e.g. salts, vitamins and other small solutes) which were not removed prior to analysis. Therefore, a great deal of further work is necessary to remove interfering compounds prior to a full chromatographic separation of the amino acid fraction from the samples.

Nevertheless, it was possible to identify the presence of 12 amino acids by comparing the relative Rf values of the spots present in the chromatographs with Rf values of amino acids separated by Arx and Hener, 1963.

The amino acids identified were as follows: spot no. 2, Arginine, spot no. 5 Aspartic acid, spot no. 6 Hydroxyproline, spot no. 7 Serine, spot no. 8 Threonine, spot no. 14 Proline, spot no. 15 Tyrosine, spot no. 16 Tryptophane, spot no. 17 Phenylalanine, spot no. 18 Valine and spot no. 19 Leucine and Isoleucine. The radio-active spot present in M199 but not in the autoradiographs in either of the yolk sac fluids was spot no. 12 (unidentified). Since the same amino acid was detected by ninhydrin stain, the possibility that this amino acid was not transported through the yolk sac may be discounted.

It has been found that in every cell culture system, whether deriving from normal or malignant tissue, at least 13 amino acids are required for survival and growth. These are termed "essential" and are

Arginine, Cystine, Glutamine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalamine, Threonine, Tryptophane, Tyrosine and Valine. Omission of any one of these amino acids results in cell degeneration and death (see Eagle (1959) for a more detailed account). In the present study eight of these essential amino acids were shown to be present in both yolk sac fluids in addition to other non-essential ones. Failure to demonstrate the transport of other essential and non-essential amino acids present in the incubation medium does not necessarily exclude their transport across the yolk sac membranes - it merely reflects the relative detectable levels of radioactive amino acid used in the analysis.

This study was not designed to identify the transport of specific amino acids across the yolk sac, in vitro, but to use the two-dimensional thin layer chromatographic technique to compare the amino acid composition of the giant yolk sac and the 12½ day yolk sac fluids in general terms. Despite the poor resolution of chromatograms obtained in this study a number of conclusions can be drawn.

Incubation with radio-active amino acids was set up to test short term transport.characterisation of each yolk sac. The ninhydrin stained chromatograms and the autoradiographs of both types of yolk sac were similar despite the existence of 6 days' difference in age between the giant yolk sac and the 12½ days old yolk sac. Thus, the giant yolk sac maintained amino acid transporting properties during the extended culture period required for its formation.

The transport of free essential and non-essential amino acids across the yolk sac and their accumulation in the fluids from both the giant yolk sac and 12½ days old yolk sac were demonstrated and could be interpreted to suggest the importance of both essential and non-essential amino acids for the early embryonic nutrition. This suggestion is supported by the results in Chapter IV, Section 4 where it was shown that significant

improvement in embryonic development occurred only when the leupeptin treated serum was supplemented with free essential and non-essential amino acids and not with the essential amino acids alone.

In view of the difficulties experienced in the analysis of complex amino acid mixtures, in further studies concerning amino acids transport across the yolk sac, better results may be obtained when amino acids are tested singly.

CHAPTER V

SECTION 9

CHARACTERISATION OF GIANT YOLK SAC FLUID PROTEINS

MATERIALS AND METHODS

A - Experiment 1. Analysis of Yolk Sac Fluid for Proteins Synthesised by Giant Yolk Sac and accumulated in Yolk Sac Fluid

In this section I describe the characterisation of the acid insoluble fraction obtained from giant yolk sacs and 12½ day yolk sacs incubated in radioactive medium in the timed study described in Section 8, experiment 1 of this chapter. For each period studied, samples were collected from 6-10 giant yolk sacs or 12½ day yolk sacs.

Analysis of Sample Radioactivity

The sulphosalicylic acid precipitate (SSA) from 50 ul of yolk sac fluid was washed twice by re-precipitation with 5% SSA. It was then dissolved in 100 ul distilled water and mixed with 3.5 mls of Fisofloure 1 (Fisons, U.K.). The radioactivity was counted on Packard Tri-carb liquid scintillation spectrometer.

For reference 50 ul of the radioactive incubation medium (not treated with SSA) was diluted with 50 ul of distilled water, mixed with 3.5 Fisofloure 1 and counted as above.

B - Experiment 2. Analysis of Proteins Synthesised By the Yolk Sac

The giant yolk sac fluid and yolk sac fluid from $12\frac{1}{2}$ day old conceptuses were from the same pools as that collected from giant yolk sacs and $12\frac{1}{2}$ day yolk sac incubated in the radioactive medium for 4 hours as described in Section 8 of this chapter.

The gels used for electrophoresis were 7% uniform polyacrylamide gel, prepared according to the methods of Ornstein and Davies (1964), (Appendix, L). The running gel size was 10 x 7.8 x 0.27 cm, the stacking gel size was 2.5 x 7.8 x 0.27 cm.

Fifty microlitre samples of giant yolk sac fluid and $12\frac{1}{2}$ day yolk sac fluid in duplicate were applied to wells made in the stacking gel (Figure 1) and the gel was subjected to electrophoresis in a Pharmacia gel electrophoresis apparatus GE-4, using 0.14M tris-glycine electrode buffer at pH 8.3 (Appendix L).

Electrophoresis was performed at 35mA, 100 volts at $15^{o}C$ for 4 hours (until the bromphenol blue tracking dye reached the bottom of the gel).

At the end of electrophoresis, parts A and C of the gel (see Figure 1) were immediately cut. Each in turn was cut again into 2 mm horizontal strips.

Each strip was then placed in 3.5 mls of Fisofloure 1 scintillation fluid and left for 3 hours at room temperature, before its radioactivity was counted on a Packard Tri-carb liquid scintillation spectrometer.

Part B of the gel was stained and destained as described in (Appendix L). Pharmacia high molecular weight marker proteins were run on each gel as a reference for determining the molecular weights of proteins present in the samples. Calibration curves were plotted and the molecular weights of proteins present in each sample were calculated as described in Appendix G.

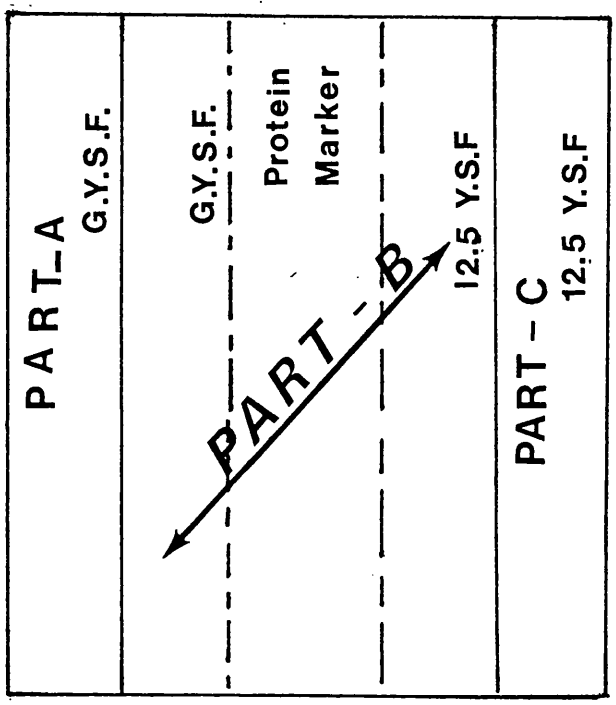


Figure 1

Diagram to show partitioning of the gel.

Parts A and C were used for radioactive analysis. Part B was stained to be used as reference for the electrophoretic pattern. Giant yolk sac fluid (G.Y.S.F.), yolk sac fluid from 12½ day old conceptuses (12½ Y.S.F.).

RESULTS

Experiment 1:- Analysis of Yolk Sac Fluid for Proteins Synthesised by Yolk Sac and accumulated in the Yolk Sac Fluid

Analysis of radioactivity in the sulphosalicylic acid precipitate showed that proteins synthesised from incorporation of C^{14} amino acids in the incubation medium by the giant yolk sac were accumulated in the giant yolk sac fluid over a 4 hour period. The timed study revealed a time dependent increase in the accumulation of the synthesised proteins (Figure 2 and Table 1). Similarly, the results in Figure 2 and Table 1 showed the $12\frac{1}{2}$ day yolk sac synthesised proteins which were accumulated in its contained sac fluid at a similar rate as in the giant yolk sac system.

Experiment 2:- Analysis of Proteins Synthesised by the Yolk Sac

Using a uniform seven percent polyacrylamide gel, the electrophoretic pattern of giant yolk sac fluid showed the presence of 14 protein bands (Figure 3); the average molecular weights of these bands are given in Table 2. The electrophoresis pattern of yolk sac fluid from $12\frac{1}{2}$ day old conceptuses showed the presence of 13 protein bands (Figure 3), these bands had similar molecular weights to the bands from giant yolk sac fluid. The difference between the electrophoretic patterns of the two fluids was the absence of a protein band of average molecular weight 600K daltons in the yolk sac fluid from $12\frac{1}{2}$ day old conceptuses. Analysis of Part A of the gel for the C^{14} amino acids incorporation into the giant yolk sac fluid proteins by the giant yolk sac revealed radioactivity in 4 gel areas in regions where the gel was stained (Part B). The molecular weights of these protein bands were 88K, 80K, 68K and 56K daltons (Figure 3).

Figure 4 showed that the protein bands of 68K and 56K daltons molecular weight contained the greatest amount of radio-labelled protein.

Analysis of C^{14} amino acid incorporation into the $12\frac{1}{2}$ day yolk sac

fluid proteins (Figure 5) showed a pattern of radioactivity distribution similar to that of the giant yolk sac fluid proteins. Again the radioactivity increased in areas where Part B of the gel showed the presence of 4 protein bands (molecular weights of 88K, 80K, 68K and 56K daltons). As in the gaint yolk sac fluid, protein bands 68K and 56K daltons contained the greatest amounts of radio-labelled protein.

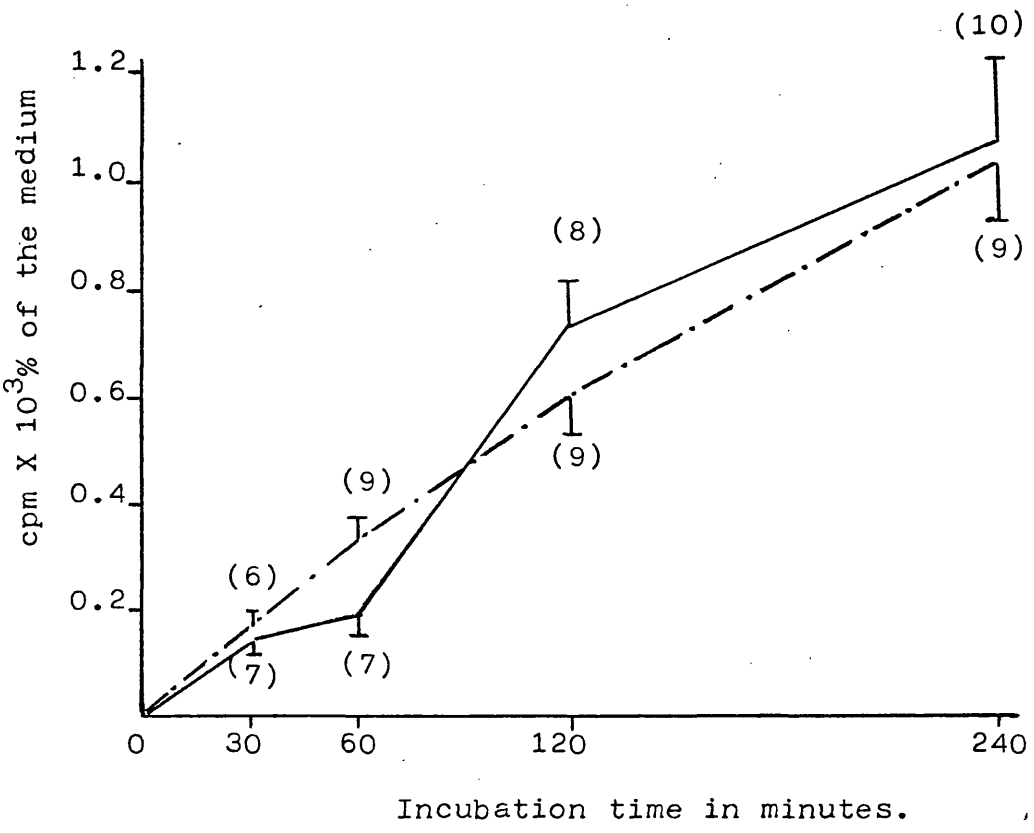


Figure 2

Timed study for the accumulation of proteins in giant yolk sac fluid (—) and 12½ day yolk sac fluid (-•-) by the incorporation of C^{14} amino acid. Each point represents the mean ± one s.e.m. Numbers in brackets indicate the number of yolk sacs used.

TABLE 1

Shows mean ± s.e.m. of c.p.m. % of the medium for the timed study for the accumulation of synthesised proteins in giant yolk sac fluid and 12½ days yolk sac fluid by the incorporation of the C^{14} amino acids.

Time	No. of Giant Yolk Sacs	c.p.m. % Medium		No. of 12½ Day Yolk Sacs
		Giant Yolk Sac Flud ± s.e.	12½ Day Yolk Sac Fluid ± s.e.	
30 minutes	7	0.15 ± 0.014	0.16 ± 0.016	6
1 hour	7	0.19 ± 0.034	0.33 ± 0.026	9
2 hours	8	0.73 ± 0.151	0.6 ± 0.069	9
4 hours	10	1.07 ± 0.156	0.93 ± 0.099	9

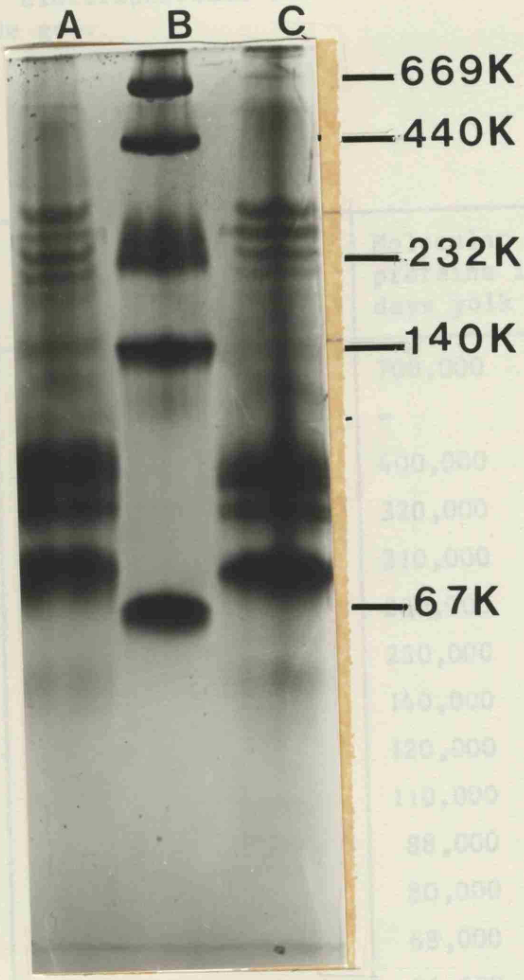


Figure 3.

Figure 3.

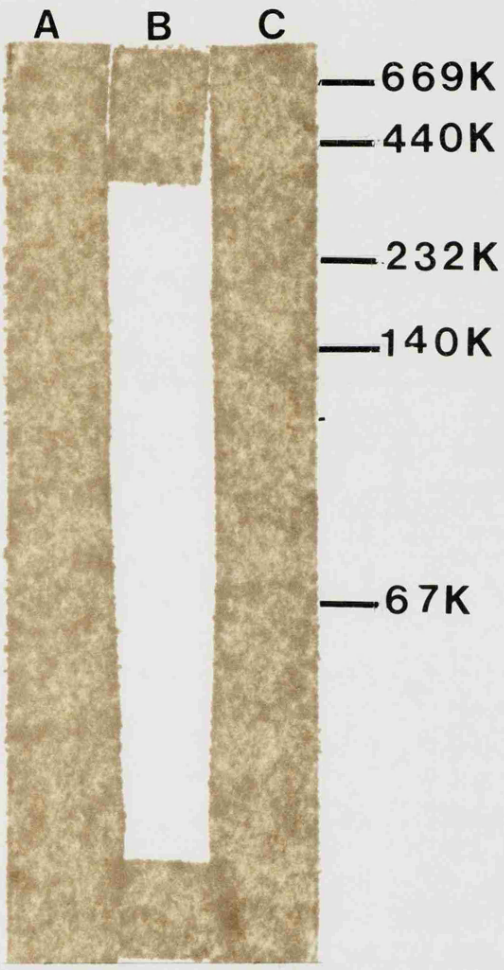


TABLE 2

Showing the approximate molecular weights of proteins present in the giant yolk sac fluid and in yolk sac fluid from 12½ days old conceptuses. Electrophoresis was carried out in a 7% uniform polyacrylamide gel.

	Molecular weight of proteins in giant yolk sac fluid	Molecular weight of proteins in 12.5 days yolk sac fluid
1	700,000	700,000
2	600,000	-
3	400,000	400,000
4	320,000	320,000
5	310,000	310,000
6	260,000	260,000
7	250,000	250,000
8	140,000	140,000
9	120,000	120,000
10	110,000	110,000
11	88,000	88,000
12	80,000	80,000
13	68,000	68,000
14	56,000	56,000

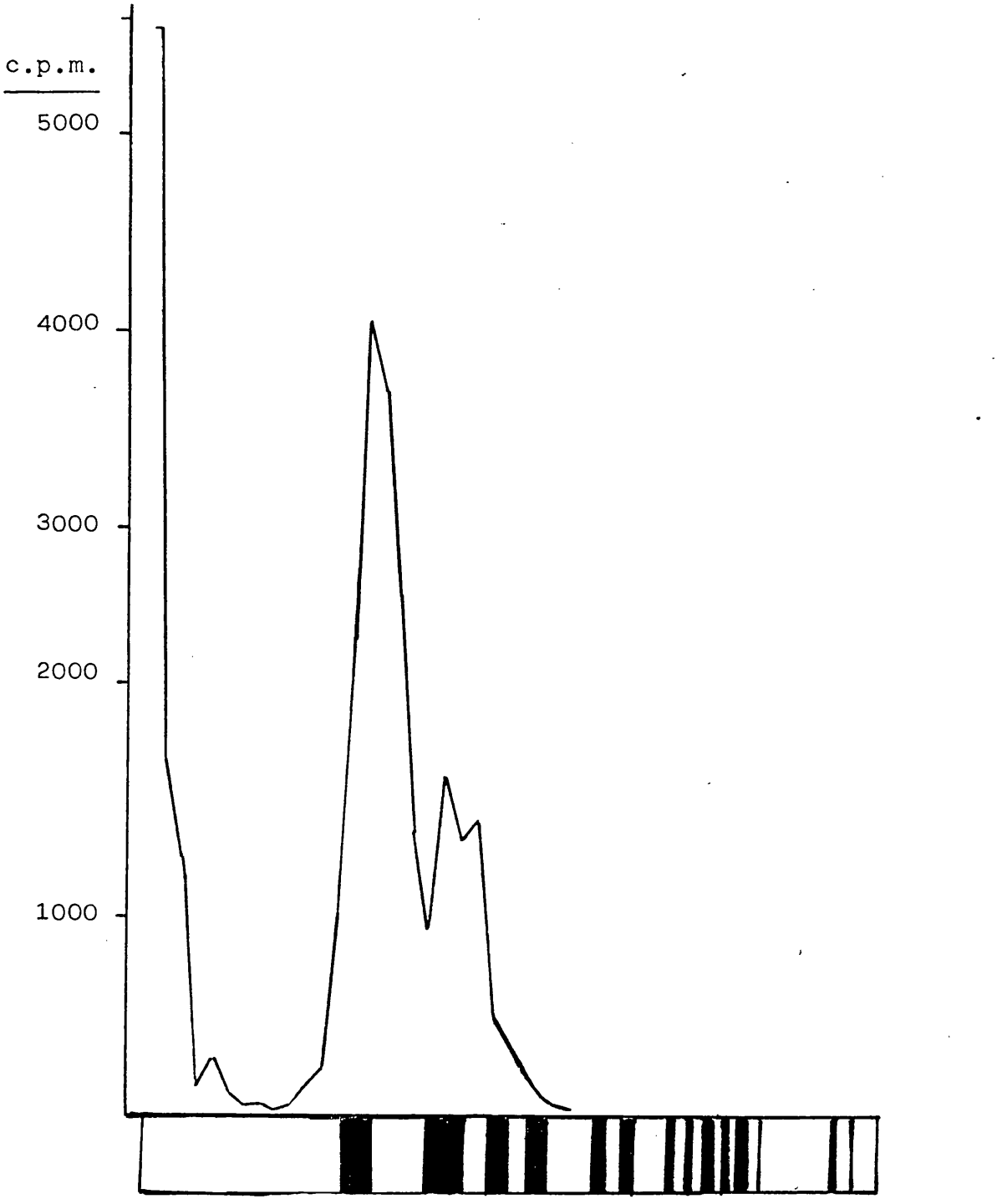


Figure 4

Analysis of radioactivity in gel strips from electrophoresis of giant yolk sac fluid. On the X axis the 14 protein bands separated by 7% polyacrylamide gel are represented. On the Y axis the radioactivity of these protein bands are given as c.p.m. Of the 14 bands only 4 bands showed measurable levels of radioactivity.

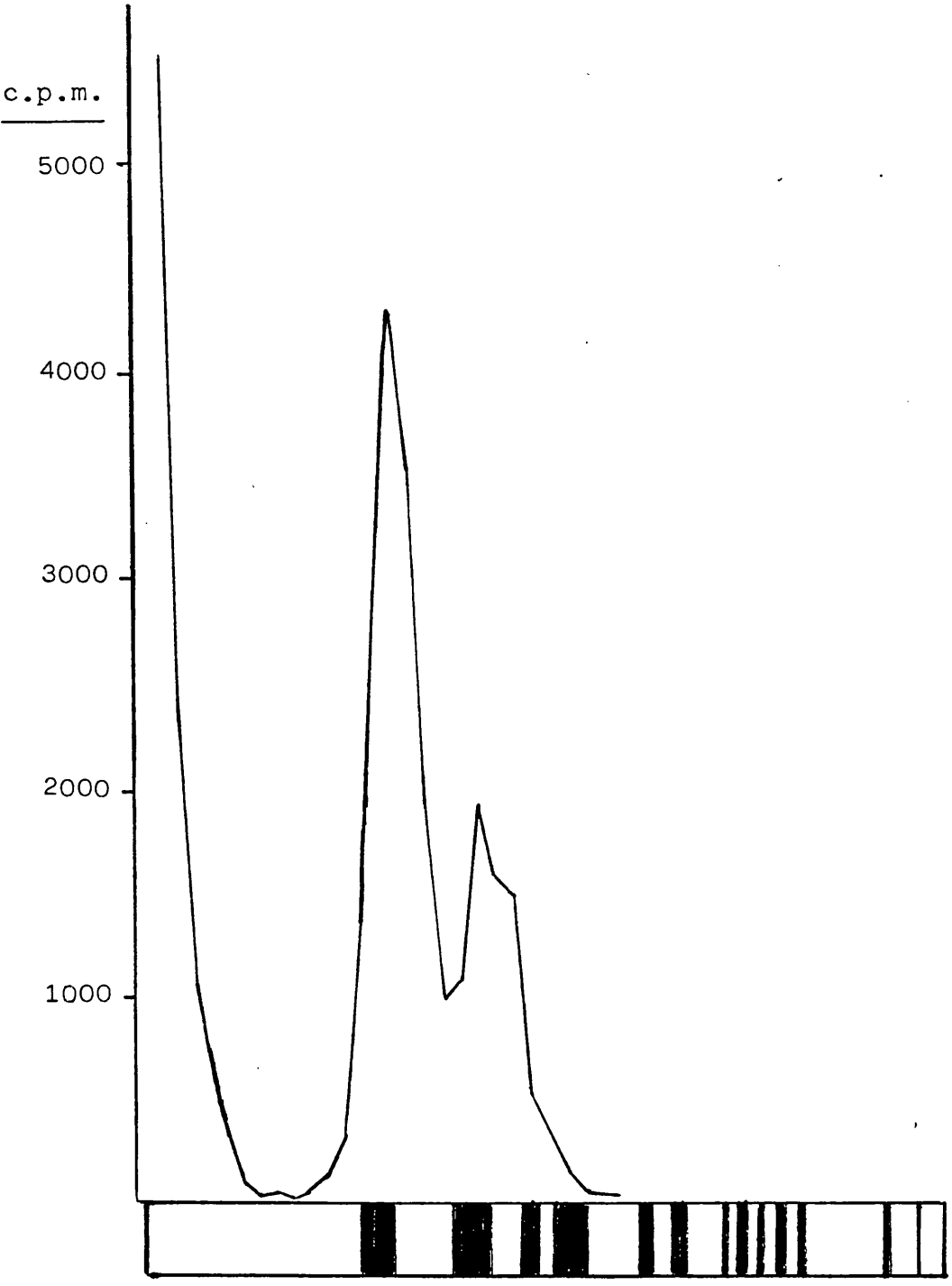


Figure 5

Analysis of radioactivity in the gel strips from electrophoresis of 12½ day yolk sac fluid. On the X axis the 13 protein bands separated by 7% polyacrylamide gel are represented. On the Y axis the radioactivity of these protein bands are given as c.p.m. Of the 13 bands only 4 bands showed measurable levels of radioactivity.

DISCUSSION

The yolk sacs of different species are known to synthesise a variety of proteins. These include albumin, pre-albumin, alphafetoprotein, alpha-antitrypsin, transferrin, embryo specific alpha-globulin and con-albumin (Gitlin and Kitzes, 1967, chick yolk sac; Gitlin and Perrcelli, 1970, human yolk sac). The mouse yolk sac has been found to be the site of synthesis of several mouse serum proteins and mouse amniotic fluid, notably alphafetoproteins, which are synthesised in the endodermal cells (Gitlin and Boesman, 1967; Gustine and Zimmerman, 1972; Dziadek and Adamson, 1978). Janzen et al., (1982) have shown that mouse visceral yolk sac synthesised transferrin in addition to alphafetoproteins. Fibronectin is another protein found to be synthesised by isolated visceral yolk sac epithelium in the mouse (Adamson and Ayers, 1979 and Hogan, 1980). Clark et al., (1982) have shown that 14.5 day rat visceral yolk sac synthesises alphafetoprotein and Type I collagen.

This study showed that both giant yolk sac and $12\frac{1}{2}$ day old yolk sac incorporated radioactive amino acids present in the incubation medium into proteins synthesised by these membranes and the accumulation rate of these synthesised proteins in giant yolk sac fluid and the $12\frac{1}{2}$ days yolk sac fluid were found to be approximately linear with the time.

Using electrophoresis on uniform 7% polyacrylamide gels, 14 protein were detected in the giant yolk sac fluid and 13 proteins in the $12\frac{1}{2}$ day yolk sac fluid, the latter being of similar molecular weight to proteins in the giant yolk sac fluid. One protein component of molecular weight 600K daltons was present in the giant yolk sac fluid but not in the $12\frac{1}{2}$ day yolk sac fluid.

This study also demonstrated, by analysis of radioactivity in gel strips, that 4 proteins of similar molecular weight present in both yolk

sac fluids were found to contain radioactivity. These 4 proteins must therefore have been synthesised by the visceral layer of both yolk sacs (or by the amnion) by incorporation of radioactive amino acids present in the incubation medium.

The molecular weights of these proteins were 88K, 80K, 68K and 56K daltons. The highest levels of radioactivity was found in a protein of molecular weight 68K daltons.

Alphafetoprotein in rat is manufactured at later stages mainly in the fetal rat liver (Gitlin and Boseman, 1967; Sell, 1973). Recently Clark et al., 1982 have shown that $14\frac{1}{2}$ day rat visceral yolk sac also synthesises alphafetoprotein.

Moreover Janzen et al. (1982) using ^{35}S-Methionine in a short term culture showed that 60% of the protein synthesised by 11.5 day mouse yolk sac was alphafetoprotein. It is likely therefore that the 68K daltons synthesised compound found in this study was alphafetoprotein.

The outcome of this study has been; firstly, giant yolk sac fluid contains 14 proteins of which 4 are found to be synthesised by the giant yolk sac and/or the amnion. The remaining proteins are probably transported unchanged by the giant yolk sac from the culture medium prior to incubation in M199. Secondly, it can be suggested that yolk sac fluid plays an important role in embryonic nutrition of the embryo by the mediation of transport and synthesises of materials, probably by the visceral yolk sac. This suggestion is based on the following findings:

(i) proteins synthesised by the giant yolk sac are accumulated in the giant yolk sac fluid.

(ii) amino acids are transported by the giant yolk sac and accumulated in the yolk sac fluid (Section 8 of this chapter).

(iii) the total protein content (Section 2) in the giant yolk sac fluid (containing dead embryos) is double that in the yolk sac fluid from 18½ day old conceptuses obtained *in vivo* and containing growing embryos.

CHAPTER V

SECTION 10

GIANT YOLK SAC FLUID AS A CULTURE MEDIUM

INTRODUCTION

The principal site of macromolecular breakdown is the visceral layer of the yolk sac and its source is the maternal plasma proteins (Everett, 1935; Cockroft, 1979; Freeman & Lloyd, 1980) (see Chapter I for review). The improved culture technique devised by (New, 1976 a) has made it possible to achieve growth and differentiation of the pre-somite head-fold stage rat embryo in vitro for a 48 hour period (New et al, 1966b). Hitherto, such good development has been possible only in media containing whole rat serum. In contrast, heterologous sera are often harmful to rat embryos (New, 1966, b). Recently, Reti et al., (1982) found growth and differentiation of rat embryos in 100% human serum was inferior to embryos cultured in whole rat serum, but good growth and differentiation occurred when the human serum was supplemented with 10% rat serum. At present, there is no chemically defined medium which can support normal growth and differentiation of rat embryos in vitro as well as whole rat serum can. However, semi-defined medium (medium consisting of rat serum extensively dialysed against balanced salt solution and supplemented with glucose and vitamins) was found to

support embryonic growth to a level comparable with that in whole rat serum (Cockroft, 1979).

Yolk sac fluid is of importance because it is situated between the embryo in its amnion and the surrounding splanchnopleure of the visceral yolk sac. It contains proteins, amino acids and other solutes, Tam and Chan (1977). Huxham (1982) demonstrated the presence of 12 proteins in the yolk sac fluid from 11.5 day old conceptuses cultured in vitro; he suggested that some of the yolk sac fluid macromolecules may be vital trophins for normal embryonic development.

Section 7 of this chapter describes the presence of 14 proteins in the giant yolk sac fluid, in Section 8 an account is given of the transport of 15 free amino acids across the giant yolk sac and their accumulation in giant yolk sac fluid. In Section 9 I have shown that the giant yolk sac fluid contains 4 proteins synthesised by the giant yolk sac and suggested that the contents of the yolk sac may result from the secretion of materials by its surrounding membrane. The aim of the present study was to test the value of the giant yolk sac fluid for supporting embryonic growth, for this reason, $9\frac{1}{2}$ day and $10\frac{1}{2}$ day old rat embryos were cultured in giant yolk sac fluid supplemented with glucose and the 8 vitamins from MEM.

As a control, embryos were cultured in yolk sac fluid from $18\frac{1}{2}$ day old conceptuses derived in vivo. Also in this study an attempt was made to culture $10\frac{1}{2}$ day old rat embryos in medium consisting of giant yolk sac fluid and exhausted dialysed serum in a 1:1 ratio. Exhausted dialysed serum supplemented with glucose and vitamins was found not to be capable of supporting good embryonic growth (see Chapter III, Section 3).

MATERIALS AND METHODS

Nine and a half and ten and a half day old conceptuses were obtained from Wistar rats as described in Chapter II, D. Pooled immediately centrifuged serum was heat-inactivated prior to use. The media required for this study were first prepared as follows:

1. Giant Yolk Sac Fluid

Nine and a half day old conceptuses were cultured for the preparation of giant yolk sacs, as in Section 2 of this chapter. At the end of the ninth day of the culture period, the giant yolk sacs were harvested and their yolk sac fluid was collected under sterile conditions as in Section 2 of this chapter. It was stored at -20^{o} in sterile containers until required.

2. Eighteen and a Half Day Yolk Sac Fluid

Yolk sac fluid from $18\frac{1}{2}$ day old rats *in vivo* was collected as laid out in Section 2 of this chapter. The yolk sac fluid was pooled and stored under sterile conditions at $-20^{o}C$.

3. Exhausted Dialysed Serum

This was prepared by repeatedly culturing $10\frac{1}{2}$ day old rat conceptuses in the same sample of Fresh H.I. serum until it stopped supporting embryonic growth (this happened after the fourth culture); see Chapter III, Section 1.

Exhausted sera from several batches were pooled and dialysed under sterile conditions against balanced salt solution for 4 days as in (Appendix E). The dialysed serum was then stored in sterile containers at $-20^{o}C$ until required.

Three experiments were carried out. In all of them 1 conceptus was cultured per one ml of medium. The pH and osmolarity of all culture media used were checked, prior to use and found to be within the normal physiological values.

In order to avoid individual differences between embryos, due to their litter of origin, conceptuses from each litter were divided between the culture bottles containing the different media.

Experiment 1

Nine and a half day old rat conceptuses were cultured for 48 hours as described in Chapter II, D in the following media.

1. Fresh H.I. serum (control).
2. Giant yolk sac fluid supplemented with 1.5 mg/ml D-glucose and 10 ul/ml of MEM vitamin concentrate (Appendix C).
3. Yolk sac fluid from $18\frac{1}{2}$ days old conceptuses supplemented with 1.5 mg/ml glucose and 10 ul/ml of MEM/vitamin concentrate.

Experiment 2

Ten and a half day old rat conceptuses were cultured for 24 hours as described in Chapter II, D in the same 3 media as in the previous experiment. Due to the very little growth and development gained by conceptuses cultured in giant yolk sac fluid and in $18\frac{1}{2}$ days old yolk sac fluid, it was thought necessary to compare their growth and development with a further control i.e. the growth of litter-mate conceptuses which had been examined and assessed immediately after being removed from the mother, were compared to the growth achieved by the experimental embryos.

Experiment 3

Ten and a half day old rat conceptuses were cultured for 24 hours as described in Chapter II, D in the following media.

1. Control serum (Fresh H.I. serum)
   This was to control the growth and differentiation of embryos cultured in experimental media.
2. Medium consisting of 50% exhausted dialysed serum and 50%

giant yolk sac fluid. This medium was supplemented with 1.5 mg/ml D-glucose and 10 ul/ml of MEM/vitamin concentrate.

3. Exhausted dialysed serum supplemented with 1.5 mg/ml D-glucose and 10 ul/ml of MEM/vitamin concentrate.
   This was the reference for the embryonic growth in this medium only.

At the end of the culture period conceptuses were examined and assessed as in Chapter II, E.

RESULTS

A - Controls

In the three experiments performed, all embryos cultured in the fresh H.I. serum (control serum) grew normally and exhibited features virtually identical to those of 11½ day old embryos *in vivo*. The results are summarised in Tables 1, 2 and 3.

B - Experimental

1. Experiment 1

The 9½ day old conceptuses cultured in giant yolk sac fluid for 48 hours, failed to grow significatnly. Every embryo was affected without excpetion; it seems that embryos died very shortly after being in culture. Examination of embryos by the end of the culture period showed that none had a visible heart beat. The yolk sac was collapsed and its diameter was reduced by 54% when compared to that of the control group. All embryos were convex anteriorly and they were under 1mm long (the crown-rump length was not included in the measurements, because the crown and the rump could not be clearly identified). No somites were visible. The results are shown in Table 1.

All 9½ day old conceptuses cultured in yolk sac fluid from 18½ day old *in vivo* conceptuses failed to grow. Their growth and development was similar to that of their litter-mates cultured in giant yolk sac fluid. The results are shown in Table 1.

2. Experiment 2

The growth and differentiation of 10½ day old conceptuses cultured in giant yolk sac fluid for 24 hours was very poor. All parameters used to assess the growth and differentiation of this group of embryos was significantly inferior ($p < 0.001$, using Student's 't' test) to those of control groups. The results are given in Table 2. By the end of the

culture period, most of the embryos were still posteriorly convex, only 25% having completed their turning. The neural tube was closed in 17% only, the defects in the remainder were: non-closure in the rhombencephalic region and/or persistance of an open posterior neuropore.

However, comparison with litter-mates examined immediately after dissection from the mother showed that some growth did occur; the mean somite number increased by an average of 9 somites; mean crown-rump length was increased by an average of 0.75 mm and the mean protein content per embryo was increased by an average of 57.5 ug/embryo. All these improvements were significant ($p < 0.001$ using Student's 't' test).

Growth and development of the $10\frac{1}{2}$ day old conceptuses cultured for 24 hours in the yolk sac fluid from the $18\frac{1}{2}$ day old conceptuses derived in vivo were very retarded. They were significantly inferior (using Student's 't' test) to those of the control group. On the other hand they were not significantly different (using Student's 't' test) from those of litter-mate conceptuses cultured in giant yolk sac fluid. The results are summarised in Table 2.

3. Experiment 3

Growth and development of $10\frac{1}{2}$ day old conceptuses cultured in medium consisting of a 1:1 mixture of giant yolk sac fluid and exhausted dialysed serum, was significantly inferior, at $p < 0.001$ (Student's 't' test) to that of conceptuses cultured in fresh H.I. serum. The results are seen in Table 3. Examination at the end of the culture period showed that all embryos had a vigorous heart beat with 82% of embryos having a vitelline circulation. Turning was completed in 63% while the remainder were anteriorly convex. The neural tube was closed in 73%, in the remaining 27% the neural tube defects were manifested as non-closure in the rhomencephalic region and/or persistance of the posterior neuropore. Fore-limb

buds were developed normally in 59% of the embryos.

Comparison of growth and differentiation parameters of this group of embryos with those of the control group, showed that the mean yolk sac diameter was reduced by an average of 0.57 mm, mean somite number was reduced by an average of 2.64 somites, mean crown-rump length was reduced by an average of 0.4 mm and the mean protein content per embryo was reduced by an average of 38.73 ug/embryo.

Growth and development of $10\frac{1}{2}$ day old conceptuses cultured in exhausted serum supplemented with glucose and vitamins was retarded; it was significantly inferior at $p < 0.001$ (Student's 't' test) to those of litter-mates cultured in medium consisting of 1:1 mixture of giant yolk sac fluid and exhausted dialysed serum. The results are seen in Table 3.

	Number of Embryos	Number of Embryos Showing:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
Control Serum Fresh H.I. serum	18	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	4.02 ±0.076	24.82 ±0.42	3.36 ±0.045	185.3 ± 8.8
Giant yolk sac fluid supplemented with glucose and vitamins	12	0 -	0 -	2 (16.6)	0 -	0 -	0 -	1 (8)	2 (17)	1.88 *±0.072	-	-	43.26 *± 4.1
18½ day yolk sac fluid supplemented with glucose and vitamins		0 -	0 -	2 (16.6)	0 -	0 -	0 -	0 -	1 (8)	2.2 *±0.09	-	-	45.81 *±3.80

Table 1. Growth and development of 9½ days rat embryos cultured for 48 hours in fresh heat-inactivated serum, giant yolk sac fluid supplemented with glucose and vitamins and 18½ day yolk sac fluid supplemented with glucose and vitamins.

Figures in parantheses denote the percentage number of normal embryos in the group.
Using Student's 't' test;

* Significant at $p < 0.001$.

	Number of Embryos	Number of Embryos Showing:-								Mean Yolk Sac Diameter (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
		Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles				
Control Serum Fresh H.I. Serum	16	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	3.97 ±0.08	25.84 ±0.53	3.38 ±0.07	197.75 ± 9.6
Giant Yolk Sac Fluid Supplemented with glucose and vitamins	12	9 (75)	0 -	12 (100)	2 (17)	3 (25)	1 (8)	9 (75)	12 (100)	2.75 *±0.093	18.6 *±0.55	2.28 *±0.072	96.2 *± 4.86
18½ Day Yolk Sac Fluid Supplemented with glucose and vitamins	10	6 (60)	0 -	10 (100)	1 (10)	1 (10)	0 -	7 (70)	10 (100)	2.56 *±0.096	17.36 *±0.64	2.2 *±0.05	84.6 *± 5.65
10½ Days Old Embryos assessed immediately after explantation	12	12 (100)	0 -	9 (75)	0 -	0 -	0 -	3 (25)	12 (100)	2.1 *±0.082	9.44 *±0.54	1.83 *±0.041	38.7 *± 3.20

Table 2 Growth and development of 10½ days rat embryos cultured for 24 hours in fresh heat-inactivated serum, giant yolk sac fluid supplemented with glucose and vitamins and 18½ day yolk sac fluid supplemented with glucose and vitamins.

Figures in parantheses denote the percentage number of normal embryos in the group. Using Student's 't' test;

* Significant at $p < 0.001$

	Number of Embryos	Number of Embryos Showing:- Presence of Heart Beat	Presence of Vitelline Circulation	Presence of Fused Allantois	Normally Closed Neural Tube	Presence of Normal Turning	Presence of Fore-Limb Buds	Presence of Normal Optic Vesicles	Presence of Normal Otic Vesicles	Mean Yolk Sac Diameter (mm) ±standard error	Mean Somite Number ±standard error	Mean Crown-Rump Length (mm) ±standard error	Mean Protein Value (ug/embryo) ±standard error
Control Serum Fresh H.I. Serum	24	24 (100)	24 (100)	24 (100)	23 (96)	23 (96)	24 (100)	24 (100)	24 (100)	4.01 ±0.10	25.77 ± 0.30	3.48 ±0.08	202.5 ± 6.57
Medium Consisting of 1;1 mixture of giant yolk sac fluid and exhausted dialysed serum + glucose + vitamins	22	22 (100)	18 (82)	22 (100)	14 (63)	16 (73)	13 (59)	22 (100)	22 (100)	3.44 ±*0.082	23.13 ±*0.34	3.08 ±*0.057	163.77 ±* 6.85
Exhausted dialysed serum, supplemented with glucose and vitamins	20	20 (100)	18 (90)	20 (100)	9 (45)	11 (55)	8 (40)	18 (90)	20 (100)	3.41 ±*0.063	21.86 ±*0.462	2.74 ±*0.072	128.3 ±* 5.43

Table 3 Growth and development of 10½ days rat embryos cultured for 24 hours in fresh heat-inactivated serum, medium consisting of 1;1 mixture of giant yolk sac fluid and exhausted dialysed serum and exhausted dialysed serum. Figures in parantheses denote the percentage number of normal embryos in the group. Using Student's 't' test; * Significant at $p < 0.001$.

DISCUSSION

The results obtained in this study show that the growth and differentiation of $9\frac{1}{2}$ day old rat embryos cultured in giant yolk sac fluid or yolk sac fluid from $18\frac{1}{2}$ day old conceptuses obtained *in vivo*, was extremely retarded. As far as their protein content was concerned both of the yolk sac fluids had the capacity to provide nutrients to $10\frac{1}{2}$ day old embryos, at least for limited embryonic growth and differentiation. The extent of growth in both yolk sac fluids was equal. Since yolk sac fluid made for culture has been supplemented with glucose and MEM/vitamins and has physiological values for pH and osmolarity, its inability to support embryonic growth might be explained in two ways. Firstly, the possibility that a deficiency or absence of specific protein(s) within the yolk sac fluid is supported by the results in Section 7 of this chapter, where analysis by electrophoresis showed that giant yolk sac fluid contained 14 proteins while that of whole rat serum (Chapter III, Section 2) contained 20 major proteins. The second possibility could be that there was an insufficient amount of total protein available for endocytosis by the yolk sac. Chapter III, Section 3 showed that normal embryonic growth and differentiation from 10.5 to 11.5 days of gestation is possible in BSS containing 20% rat serum (which would contain $\simeq$ 17 mg/ml total protein) but not in BSS containing 10% serum (which would contain $\simeq$ 8.5 mg/ml).

Results in this study show a similarity in the limited growth and differentiation achieved by the $10\frac{1}{2}$ day old embryos cultured in giant yolk sac fluid and in yolk sac fluid from $18\frac{1}{2}$ day old conceptuses obtained *in vivo*, even though the former contains a dead embryo and the latter a live one. This could mean that only materials derived from medium by the yolk sac are of nutritional value for the cultured rat embryos at this stage.

The study in Chapter III, Section 3 showed that exhausted dialysed serum supplemented with glucose and vitamins was incapable of supporting

normal growth and differentiation of 10½ day rat embryos cultured in it for 24 hours. One possible cause was thought to be a deficiency in certain nutrient materials vital for embryonic development.

The present study has shown a reasonable improvement in the growth and differentiation of embryos cultured in exhausted dialysed serum when it was diluted 1:1 with giant yolk sac fluid. Since embryos could not grow in either of the two media alone, such an improvement in embryonic growth could only have occurred if the giant yolk sac fluid had supplemented deficiencies of the exhausted serum and visa.

In conclusion, this study has demonstrated the presence of nutrient materials in the giant yolk sac fluid of vital importance for embryonic development of 10½ day old embryos cultured for 24 hours. Furthermore, it can be suggested that the nutrient materials in the giant yolk sac fluid were either derived by synthesis or by transport across the giant yolk sac.

CHAPTER V

SECTION 11

THE GIANT YOLK SAC AS A MODEL SYSTEM FOR YOLK SAC FUNCTIONS

The importance of the extra-embryonic membranes in normal mammalian fetal development has long been recognised as providing specific compartments and structures essential for the nutrition, excretion and mechanical protection of the fetus. In addition, functional roles in the selective transfer of various substances at different stages of gestation in some distinct membranous (epithelial) structures (coated vesicles) has been shown.

The multi-functional characteristics of these membranes have attracted researchers to investigate their mechanism but such work has been generally inconclusive in <u>in vivo</u> studies due to maternal interference. However, a great deal of information has been provided when embryos and their extra-embryonic membranes have been grown <u>in vitro</u> during organogenesis (New & Stein, 1964; New et al., 1976 a, b).

The small size of the visceral yolk sac at 11½ days' gestation (3.5mm in diameter) and the amount of yolk sac fluid which can be

collected (≃ 8 ul) from each conceptus are insufficient for detailed analysis. Therefore several authors have cultured either pieces, or the whole visceral yolk sac opened at the ecto-placental cone end, from older embryos (Fridhandler & Zipper, 1964 - use $17\frac{1}{2}$ days rat visceral yolk sac; Padykula et al., 1966 - used pieces from $13\frac{1}{2}$ days rat visceral yolk sac; Williams et al., 1975 a & b - used whole $17\frac{1}{2}$ days rat visceral yolk sac). Since visceral yolk sacs in the above studies were continuously exposed to the incubation medium on both sides of the membrane the actual processes of diffusion, transport and excretion of the materials across the visceral yolk sac could not be carried out effectively.

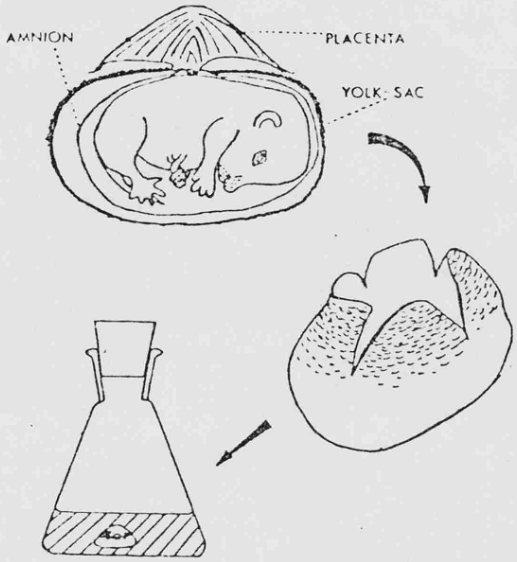
In this respect Chan and Wong (1978) devised a technique whereby they mounted the $17\frac{1}{2}$ days rat visceral yolk sac on a perspex chamber (Figure 1) in order to study the Na^+ transport. The drawbacks to this method were the mechanical stress exerted on the membrane and the absence of the yolk sac fluid from the exocoelomic side of the visceral yolk sac. Similarly, creation of an environment on the exocoelomic side of the visceral yolk sac which is very different from that found *in vivo* and *in vitro* could make interpretation of any results difficult. The model devised in this thesis (the giant yolk sac system) has been tested and there is good evidence indicating that its function as an extra-embryonic membrane is similar to that of the visceral yolk sac both *in vivo* and *in vitro*.

The advantages of the giant yolk sac system over and above those outlined previously are as follows:

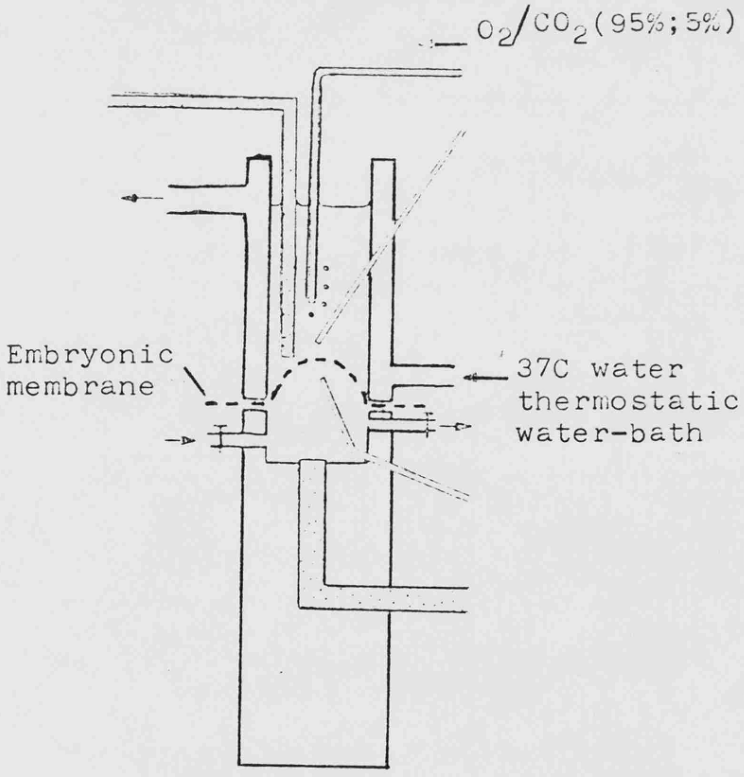
(i) it provides large quantities of yolk sac tissue (surface area = ≃ 3 cm^2) and yolk sac fluid (≃ 600 ul) for detailed biochemical analysis;

(ii) it provides an intact yolk sac epithelium which maintains the integrity of the products of

Figure 1



A Dissection of a 17.5 day rat yolk sac for organ culture (Williams et al, 1975a).



B Diagram of chambers devised by Chan and Wong, 1978. The visceral yolk sac is clamped horizontally between two perspex chambers with the maternal side of the membrane facing upwards.

histiotrophic nutrition (i.e. yolk sac fluid);

(iii) it provides a system which survives for some time in culture.

Thus, the following studies can be carried out using the giant yolk sac system:

1. transport, diffusion and excretion of materials across the visceral yolk sac;
2. biochemical analysis of proteins synthesised by the visceral yolk sac;
3. studies concerning membrane receptors, especially those associated with coated vesicles and their contents;
4. as a model system for lysosomal studies where materials thought to have an effect on lysosomal functions can be added to the incubation medium and their effects followed up by an analysis of, for example, fractionated lysosomes.

The giant yolk sac system can also be used for cell multiplication kinetics and for the study of teratological problems where teratogenic action has been associated with a secondary manifestation of its direct effect on the visceral yolk sac. Such effects could be mediated by disturbing the digestive function of the yolk sac and could be followed by the analysis of the passage of critical substances to the embryo.

CHAPTER SIX

CHAPTER VI

CONCLUSION

The studies in Chapter III showed that the serum has a finite ability to support embryonic growth and development. After four successive 24-hour cultures of $10\frac{1}{2}$ days old rat embryos whole rat serum was unable to support embryonic growth. After further analysis of the factors involved this observation was explained in two ways:

(i) specific macromolecular components or growth factors present in the serum could have been depleted by repeated culture of embryos;

(ii) the accumulation of both micro and macromolecular toxic materials during the exhaustion of serum could have resulted from the metabolic activities of the embryos and/or its extra-embryinic membranes.

Following on from this Chapter IV was designed to test the nutritional value of free amino acids as a substitute for some proteins. The addition of leupeptin (which is known to inihbit lysosomal cathepsinic enzymes) to the culture serum artificially deprived the yolk sac of its ability to completely digest pinocytosed macromolecules. Since supplemen-

tation of serum containing leupeptin with essential and non-essential amino acids resulted in a significant improvement in growth and development of embryos in culture (as an extension of the work of Beck & Lowy, 1981), three important conclusions can be drawn:

(i) amino acids derived from digested pinocytosed proteins are used by the embryo (and this supports the work of Freeman et al., 1981);

(ii) the embryo also utilizes free amino acids present in the culture medium;

(iii) both essential and non-essential amino acids are required for embryonic growth.

However, the provision of amino acids is only one (albeit major) feature of histiotrophic nutrition carried out by the visceral yolk sac and analysis of the other features (e.g. protein transport, protein synthesis, small solutes transport) requires a system which must provide great flexibility.

In Chapter V a model system (the giant yolk sac) for the rat visceral yolk sac was developed. The major advantage of this system lies in its capacity to produce the products of histiotrophic digestion in large quantities for biochemical analysis. The giant yolk sac functions as an extra-embryonic membrane in a way similar to that of the visceral yolk sac *in vivo* and *in vitro* and it was further demonstrated that the processed histiotroph of the giant yolk sac contains synthesised proteins and amino acids. The direct passage of amino acids from the culture medium across the giant yolk sac membrane to the fluid contained within it confirmed the observations and conclusions of Chapter IV.

Finally, giant yolk sac fluid as a whole was found partially to restore the ability of dialysed exhausted serum to support embryonic growth. This observation alone suggests that, as a product of histiotrophic

nutrition, giant yolk sac fluid can to a certain extent replace the vital nutritional factors missing from exhausted serum (see Chapter III).

The work in this thesis goes some way towards defining the nutritional requirement of rat embryos during a major part of organogenesis. It also characterises some of the mechanisms involved in histiotrophic nutrition, developing a very useful model system for further study in this field and for the study of the effects of pharmacologically active agents on these mechanisms.

APPENDICES

APPENDIX A

PROTEIN ESTIMATION OF EMBRYOS

The relative BSA equivalent total protein value was determined for individual embryos using a modification of the original method of Lowry et al. (1951). Neutralization of the alkali required to digest the embryonic protein was necessary since colour development is sensitive to pH changes. Preparation of stock solutions and reagents is shown in Tables 1 and 2.

Method

1. One millilitre of IN NaoH was added to each embryo in a seated plastic tube and incubated at 37°C for 2 hours, vortexing every 15 minutes.

2. One hundred and forty five micromillilitres of 3N HC1 was added to each tube prior to further vortexing.

3. Two 0.5 ml aliquots were pipetted from each tube into two 9 ml test tubes.

4. Two and a half millilitres of Folin A was added to each of the smaller tubes before they were left to stand for 20 minutes at room temperature.

5. Two hundred and fifty micromillilitres of Folin B was then added to each tube as it was placed on the whirly-mixer. This was carried out in order to achieve immediate mixing, since at the pertaining pH, Folin B was quickly inactivated.

6. The tubes were then allowed to stand for 45 minutes at room temperature.

7. The absorption of the reaction product was read using a digital

TABLE 1

Stock Solutions and Their Preparation

	Stock Solution	
a.	1N NaoH	40 gm NaoH made up in 1 litre distilled water
b.	3N Hcl	3.67 ml of concentrated Hcl made up to 11 ml with distilled water.
c.	Sodium Carbonate	2 gms anhydrous Na_2 CO_3 per 100 ml distilled water.
d.	Copper Sulphate	1 gm cus04 $5H_2O$ per 100 ml distilled water.
e.	Sodium Tartarte	2 gm (cH (oH). COO Na)$_2$ $2H_2O$ per 100 ml distilled water.
f.	Bovine Serum Albumin	0.458 gm B.S.A. per 100 ml 1N NaoH stored at -20°C.
	Folin Ciocalteus Reagent	B.D.H. (stored at 4°C).

TABLE 2

Reagents and Their Preparation

	Reagents	
1.	Folin A prepared daily	100 ml of stock solution c + 1 ml of stock solution d + 1 ml of stock solution e. Mix.
2.	Folin B prepared immediately before use	Folin stock solution diluted 1:1 with distilled water.

spectrophotometer (Cecil Instruments) with a tungsten filament at a wave-length setting of 750 nm. The apparatus was zeroed using distilled water.

Six standards of 0, 10, 20, 30, 40 and 50 µl Bovine serum albumin (BSA) stock solution were placed in tubes, each made up to 1 ml with 1N NaoH and then processed simultaneously with the embryos from stage 2 onwards. All absorbance values for the samples were normalized by subtraction of the mean absorbance value for 0ul of BSA. A gradient value representing the standard line was calculated and used to determine the total amount of protein per embryo using the formula,

$$\frac{\Delta a}{b} \times 2.29 = \text{ug protein in embryo.}$$

Where Δa = mean normalised absorbance value for the sample.

b = gradient (usually around 0.05).

$\times 2.29$ = 0.5 ml sample from 1.145 ml total volume at the start.

APPENDIX B

MEASUREMENT OF OSMOTIC PRESSURE

This was carried out by using a Knauer electronic semi-micro osmometer, type M, the sample volume was 0.15 ml and the results were expressed in milliosmol/kg.

EAGLE'S MINIMUM ESSENTIAL MEDIUM (MEM) VITAMINS:-

There are 8 vitamins, which were found by Eagle (1959) to be essential for growth and survival of mammalian cells in culture. The vitamin mixture was obtained as a liquid concentrate from Flow Laboratories Limited (Irvine, U.K.). Table 1 shows each vitamin and its concentration in the liquid concentrate.

In this work, culture media were supplemented with 10u of the MEM vitamin concentrate per ml of medium when stated.

Table 1. MEM vitamins and their concentration in the liquid concentrate used.

Vitamin	mg/ 1 ml
D-Ca pantothenate	1.0
Choline Chloride	1.0
Folic Acid	1.0
I-insoitol	2.0
Nicotinamide	1.0
Pyridoxal Hcl	1.0
Riboflavin	0.10
Thiamin Hcl	1.0

BALANCED SALT SOLUTION (BSS):

The BSS used was based on a combination of Earle's and Tyrode's salts and the formula is shown in Table 1.

Cockroft (1979) found that this solution gave better and more consistent results of growth and differentiation of embryos cultured in serum dialysed against this solution compared to either Earle's or Tyrode's salt solutions alone.

Table 1. Formula of the balanced salt solution (BSS).

Salt	Concentration (gm/litre)
NaCl	6.9
KCl	0.3
$MgSO_4.7H_2O$	0.1
$Mgcl_2.6H_2O$	0.05
$NaH_2PO_4.2H_2O$	0.1
$NaHCO_3$	2.0
$Cacl_2$	0.2

APPENDIX E

DIALYSIS OF SERUM

The dialysis tubing (Visking Scientific Instrument Centre Ltd.) was rinsed in sterile water for a period of 24 hours prior to use. Serum batches of 10-20 ml were dialysed against 200-400 ml glucose-free sterile balanced salt solution (BSS) (See Appendix D) constantly stirring at $4^{o}c$ for 4 days; the BSS was changed daily and the whole procedure was carried out under sterile conditions.

APPENDIX F

ANTIBIOTIC 1

This was obtained from Flow Laboratories Limited (Irvine, U.K.) in liquid form. It contained, Penicillin (5000 1u/ml) and Streptomycin (5000 ug/ml). To inhibit bacterial growth, all culture media used in this work contained 20 ul of this solution per ml of medium.

ELECTROPHORESIS

Apparatus Used For Electrophoresis

1. Pharmacia gel electrophoresis apparatus GE-4 11.
2. Electrophoresis power supply Vokam (Shandon-Southern).
3. Pharmacia gel destainer GD-4 11.
4. Destainer power supply battery charger.
5. Pharmacia gel slab drier GSD-4.

Gels used - are ready made Pharmacia polyacrylamide linear gradients of two designations - PAA 2/16 and PAA 4/30.

The characteristics of these gels are shown below:

	PAA 4/30	PAA 2/16
Molecular weight range for globular proteins	50,000 - 2,000,000	100,000 - 5,000,000
Gel dimensions (mm)	78 x 78 x 2.7	78 x 78 x 2.7
Polyacrylamide linear gradient concentration (%)	4 - 30	2 - 16

Staining Solution

Coomassie Brilliant Blue R250	0.115 gms
Destaining Solution	100 mls

Destaining Solution

Ethanol	500 mls
Acetic Acid	160 mls
Water	1,340 mls

Electrode Buffer (reservoir buffer)- pH 8.3.

TRIS	43 gms
Boric Acid	20.16 gms
Na_2EDTA	3.72 gms
Water	make up to 4 litres

Procedure

After the gel cassette was fitted into the apparatus and the sample applicator inserted into the gel cassette, the electrophoresis tank was filled with reservoir buffer and a complete buffer circulation started. The cooling system of the chamber was connected to the mains water supply and any air bubbles entering the sample applicator were carefully displaced.

Gels were pre-equilibrated (run) at 125 volts for 20 minutes.

In order to load the samples the power pack was disconnected and the upper reservoir opened. The pump continued to circulate buffer only in the lower reservoir. samples and marker proteins were run (pre-electrophoresis) for 15 minutes at 70 volts (constant). Thereafter 125 volts (constant) was maintained for the main run at 14-15^{o}C for 15-18 hours. At the end of this time the gel was removed from the cassette and immersed in the staining solution for 2 hours at room temperature. Gels were differentiated in destaining solution for 3-4 hours, in the Pharmacia GD4 apparatus at 12 volts prior to photography. For storage, the gels were partially dehydrated by immersion in aqueous acetone and then placed between two pieces of moist cellophane overnight in the Pharmacia GSD4 gel drying apparatus under partial vacume to complete the process.

Molecular weight determination

This was carried out using the curve established with the high molecular weight calibration kits (Pharmacia) assuming that the unknown proteins have the same relation-

ship of molecular size and weight as do the globular standard proteins.

A. Preparation of the calibration curve

1. The migration distance of the standard protein was measured with a ruler on the gel photograph.
2. The relative migration values (Rf) for the standard proteins were calculated , as follows:

$$Rf = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to referance point}}$$

The end of the gradient gel was used as the point for calculating Rf values of both standard and unknown proteins.

3. A calibration curve was constracted by plotting the Rf values of standard proteins and the logs. of their corresponding molecular weights.

B. Molecular weight estimation of unknown proteins

1. The migration distance of the unknown protein was measured as in A(1) above.
2. The Rf values of the unknown protein was calculated as in A(2) above.
3. The point corresponding to the Rf value of the unknown protein was located on the calibration curve and the value on the log. scale which relates to this point gives the estimated molecular weight of the protein.

Protein Estimation

This was carried out using the original method of Lowry et al. (1951). Preparation of stock solution and reagents is shown in Tables 1 and 2.

Preparation of Working Standards

A duplicate set of standards was run with each set of samples measured and six standards were prepared - 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of BSA stock solution - and each was made up to a final volume of 0.5 ml with distilled water.

Assay Procedure

To the working standards and 0.5 ml of the samples:

1. 2.5 ml of alkaline copper reagent was added, mixed and allowed to stand for 10 minutes at room temperature;
2. 0.25 ml diluted Folin's reagent was added to each tube, mixed immediately on the whirl-mixer and allowed to stand for 30 minutes at room temperature;
3. The absorption in each tube was measured against the distilled water control at E750 on a digital spectrophotometer (Cecil Instruments);
4. The standard curve absorption vs. concentration was plotted from the resultant quantities of standard protein ranging from 0-150 ug BSA.

The protein content of the unknown sample was then calculated.

TABLE 1

Stock Solutions and Their Preparation

	Stock Solutions	
1.	Copper Stock	0.5% $CusO_4$-$5H_2O$
2.	Alkali Stock Solution	10% anhydrous Na_2 CO_3 in 2% NaoH
3.	Bovine Serum Albumin Stock Solution	30 mg B.S.A. dissolved in 100/ml 1N Nao H for 1 hour at 37°C and was stored at -20°C
4.	Folin Ciocalteus Reagent	BDH (stored at 4°C).

TABLE 2

Reagents and Their Preparation

	Reagents	
	Alkaline Copper Reagent Prepared freshly	1 ml of copper stock solution is made up to 80 ml with distilled water and then 20 ml of alkali stock solution is added.
	Folins Reagent prepared immediately before use	Folin stock solution diluted with distilled water 1:2.

Appendix I

Veronal acetate buffer

This was made by dissolving 17.4 gm sodium acetate and 29.4 mg sodium barbital in distilled water to a final volume of one litre.

Appendix J

Zetterquist salt solution

This was made by dissolving 8 gm sodium chloride, 4 gm potassium chloride and 2 gm calcium chloride in distilled water to a final volume of one litre.

APPENDIX K

TYRODES SOLUTION

The solution was made up in distilled water.

SALTS	gm/Litre
NaCl	8.00
Kcl	0.20
$CaCl_2$	0.20
$MgCl_2.6H_2O$	0.10
$NaH_2PO_4.2H_2O$	0.05
$NaHCO_3$	1.00
Glucose	1.00

APPENDIX L

SEVEN PERCENT UNIFORM POLYACRYLAMIDE GEL SYSTEM

This was made according to the system described by Ornstein and Davies (1964).

The 7% uniform polyacrylamide gels were cast in the pharmacia GSC8 apparatus between 8 x 13 cm glass plates using 2mm plastic spacers.

Buffers:

Small pore buffer (SPB) pH 8.9 (running gel)	1NHCl	48 mls	Make up to 100 mls with water
	TRIS	36 gms	
	TEMED	0.23 ml	
Large pore buffer (LPB) pH 6.7 (stacking gel)	1NHCl	48 mls	Make up to 100 mls with water
	TRIS	5.98 gms	
	TEMED	0.46 ml	
Electrode buffer pH 8.3	Glycine	28.8 gms	
	TRIS	6.0 gms	
	Water	4 litres	
Acrylamide stock	Acrylamide 38.8 gms Methyl-bis Acrylamide 1.2 gms Water - make up to 100 mls.		

		1 gel	2 gels	3 gels	4 gels	
Running gel	SPB	6.25	9.3	12.5	18.6	
	Acryl	8.75	13.5	17.5	27	(mls)
	Water	35	52	70	104	
		Degas				
	ApS 10%	150 ul	225 ul	300 ul	450 ul	
Stacking gel	LPB	2	2.5	3	3.5	
	Acryl	2	2.5	3	3.5	(mls)
	Water	12	15	18	21	
	Sucrose	3.5 gms	4.5	4.8	5.4	
		Degas				
	ApS 10%	50 ul	70 ul	90 ul	110 ul	

Stain 0.115 gms/100 mls destain Coomassie Brilliant Blue R250

Destain 25 mls ethanol
8 mls acetic acid
67 mls water

Procedure

Small pore buffer, acrylamide stock and water were mixed and degassed for 3 minutes. To this was added the required volume of ammonium persulphate stock (10%) and the mixture poured down the angled glass plates. This was overlaid with a small amount of water until the gel had polymerised. The gel was then re-equilibrated with 1/8 th diluted small pore buffer overnight at 4^{o}C.

The stacking gel was prepared in the same way (but with large pore buffer and sucrose) and allowed to polymerise around a sample applicator comb inserted into the unpolymerised gel or overlaid with water as before.

Samples usually contained 40% sucrose and 0.1% bromphenol blue. Gels were electrophoresed at 35 mA per gel in the pharmacia GE4 electrophoresis apparatus with tap water cooling, until the bromphenol blue marker dye had reached the bottom of the gel. After electrophoresis gels

were stained with 0.11% Coomassie Brilliant Blue R250 overnight at room temperature and destained using the Pharmacia GD4 apparatus. They were photographed and the molecular weight of the proteins were estimated as in Appendix G.

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STATEMENT

The accompanying thesis/~~dissertation~~* submitted for the degree of Ph.D. entitled THE INVESTIGATION OF SOME NUTRITIONAL REQUIREMENTS OF RAT EMBRYOS UNDERGOING ORGANOGENESIS IN VITRO is based on work conducted by the author in the Department of ANATOMY of the University of Leicester mainly during the period between OCTOBER 1978 and MAY 1983

All the work recorded in this thesis/~~dissertation~~* is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other university/~~part of this work has been submitted for another degree as indicated below in the University of~~*

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