THE NUTRITIONAL FUNCTION OF THE VISCERAL YOLK SAC AND
ITS SUSCEPTIBILITY TO MODIFICATION BY TERATOGENS DURING
ORGANOGENESIS IN THE RAT: A QUANTITATIVE IN VITRO STUDY

by

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ABSTRACT

The New technique for the in vitro roller culture of 9.5-day rat conceptuses in homologous serum has been used to provide direct quantitative biochemical evidence for the long-suspected nutritional function of the visceral yolk sac during early organogenesis.

Conceptuses, cultured in the presence of ^{125}I-labelled PVP, $[^3H]$ dextran, ^{125}I-dBSA, $[^3H]$ leucine-labelled serum proteins or $[^3H]$ leucine-labelled homologous haemoglobin showed no uptake of intact macromolecules into the embryo. All five however were taken by pinocytosis into the visceral yolk sac. The indigestible macromolecules ^{125}I-labelled PVP and $[^3H]$ dextran accumulated in the yolk sac lysosomes, but the radio-labelled proteins were digested there to yield either $[^{125}I]$ iodo-tyrosine or $[^3H]$ leucine. $[^{125}I]$ Iodotyrosine (non-metabolisable) was released into the culture medium but $[^3H]$ leucine was used for protein synthesis in both the yolk sac and the embryo. The digestive capacity of the yolk sac was further demonstrated by direct assay of lysosomal acid hydrolase activity in yolk sac homogenates. SDS Polyacrylamide gel electrophoresis showed that $[^3H]$ leucine, derived from the digestion of $[^3H]$ leucine-labelled haemoglobin, became incorporated into a large number of yolk sac and embryo proteins. Although free non-radiolabelled leucine was shown to decrease the extent to which $[^3H]$ leucine from radiolabelled protein was incorporated into the proteins of the conceptus, it is argued that, in vivo, yolk sac degradation of pinocytosed protein is of paramount importance in the provision of amino acids for protein synthesis in the conceptus.

The lysosomotropic teratogens trypan blue, suramin, sodium aurothio-malate, anti-visceral yolk sac antiserum and leupeptin, present in culture with radiolabelled macromolecules, inhibit either pinocytosis by the yolk sac (trypan blue, suramin, antiserum), normal fusion of pinosomes and lysosomes (suramin) or proteolysis in the yolk sac (aurothiomalate, leupeptin). It is suggested that the mechanism of teratogenic action of these compounds might involve an inhibition of yolk sac-mediated histiotrophic nutrition.

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ABBREVIATIONS

BSA   Bovine serum albumin

dBSA   Formaldehyde-denatured bovine serum albumin

PVP   Polyvinylpyrrolidone

SDS   Sodium dodecyl sulphate

TEMED   Tetramethylethylenediamine

TES   N-Tris(hydroxymethyl)methyl-z-aminoethane
                     sulphuric acid

TCA   Trichloroacetic acid

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Embryotrophic Nutrition

1.1.1 Current Status of Knowledge

A placenta is defined by Mossman (quoted by Beck, 1976) as "an intimate apposition or fusion of fetal organs to maternal tissues for physiological interchange". Usually, reference to a placenta implies a chorioallantoic placenta, a highly vascularised organ which has assumed the principal role in maternal-fetal exchange in the Eutheria. The effectiveness of the chorioallantoic placenta depends on a well-developed embryonic cardiovascular system and, clearly therefore, organogenesis is well advanced at the onset of function of this placenta. In rodents chorioallantoic placentation does not occur until mid-gestation, by which time many organs are well differentiated. The question therefore arises as to how the nutritional demands of the rodent embryo are satisfied during the early stage of organogenesis - what mechanisms exist to nourish the embryo during this time?

In the absence of a chorioallantoic placenta, the growing embryo must be sustained by nutrients present in its immediate environment. Such nutrients, termed histiotroph by early embryologists, are considered to consist largely of maternal macromolecules, chiefly the breakdown products of proliferated endometrial cells, uterine secretions and extravasated maternal blood (Beck, 1970). It is uncertain however whether the largely macromolecular nature of histiotroph is in itself important. It could simply be that the nutritional demands of the embryo are satisfied by micromolecular nutrients which diffuse into, and are assimilated by, the cells of the embryo, there being no need of a specialised mechanism to channel nutrients to the embryo.

To gain a better understanding of histiotrophic nutrition is not of mere academic interest. Teratologists have long recognised that the developing embryo is maximally sensitive to teratogenic insult during the period of early organogenesis (Wilson, 1973). A large number of studies (summarised by Wilson, 1973) has conclusively proved that before and following organogenesis the embryo is almost completely refractory to teratogenesis, whereas during organogenesis it is highly susceptible to the action of teratogens. Chemical teratogens act on biochemical mechanisms of the embryo, and it is important to understand what these biochemical mechanisms are.

Only in rodents, and in particular the rat, has histiotrophic nutrition been studied in any detail. Mounting evidence, albeit indirect, implicates the visceral yolk sac in serving a nutritional function during early organogenesis. This conspicuous extra-embryonic membrane lies adjacent to the embryo at the primitive streak stage, and during early organogenesis comes to surround it completely, persisting like this till term (Beck, 1970, 1976).

Brunschwig (1927) inferred a placental function of yolk sac from investigations of iron uptake by the early post-implantation embryo. He observed that the yolk sac provided the only route of access for the iron during its passage to the embryo. Several workers ((Goldman, 1909; Wislocki, 1921; Everett, 1935) noted that the visceral yolk sac was capable of "absorbing" the dye trypan blue, and suggested a placental role for the yolk sac. Payne and Deuchar (1972) found that the removal of the visceral yolk sac from 10-day rat embryos grown in culture for 24 hours led to poor growth and development. Relative to controls, these embryos showed a much lower total protein content and a reduced ability to incorporate $[^3H]$ leucine,

present in the culture medium, into embryonic tissue.

Morphological studies have revealed that the endodermal cells of the visceral yolk sac (see section 1.2), which are exposed to histio-troph present in the yolk sac cavity during early organogenesis, bear numerous microvilli on their apical surfaces, and have a well-developed vacuolar system (Lampson, 1966; Beck et al., 1967a; Krzyzo.wska-Gruca and Schiebler, 1967; Jollie and Triche, 1971). These features are characteristic of cells engaged in pinocytosis (see section 1.3) and the use of suitable histochemical markers such as horseradish peroxidase (HRP) (Beck et al., 1967a; Seibel, 1974) and electron-dense compounds such as ferritin and iron dextran (Gupta et al., 1979) have confirmed that pinocytosis of macromolecules, followed by inclusion within intracellular vacuoles, is a property of these cells during early organogenesis. Following their studies with HRP, Beck et al. (1967a) proposed a mechanism whereby the visceral yolk sac could act as a placenta. They observed that soon after parenteral administration of HRP, the enzyme became enclosed within apical vacuoles of the yolk sac endoderm. With time the HRP, still entrapped within vacuoles, was seen deeper in the cell, ultimately localising in regions of the cytoplasm that gave a strong reaction for acid phosphatase, an enzyme known to be located exclusively in lysosomes, digestive organelles of the cell. Later still, the intralysosomal concentration of HRP was observed to fall. Interestingly, at no stage were there present significant quantities of marker enzyme in the embryonic tissues. They hypothesised that although the HRP was unable to penetrate intact to the embryo tissues, the cells of the yolk sac endoderm pinocytosed the HRP and digested it within their lysosomes. This implied that protein, and perhaps

other macromolecular nutrients, was taken up and degraded by the visceral yolk sac endoderm, yielding digestion products, amino acids, which were subsequently available to the embryo for its own anabolic purposes. Proteins were not transmitted intact directly across the yolk sac to any significant extent. Seibel (1974) has since confirmed that in the rat horseradish peroxidase does not reach the embryo during organogenesis, while Sharma and Peel (1979) have made similar observations with fluoroscein-labelled serum proteins. In the early post-implantation mouse conceptus, Sherman and Atienza-Samols (1979) have reported that a maternally-derived esterase is present in the yolk sac layer but not in the embryo and, as far back as 1959, Anderson (Anderson, 1959) observed a very low rate of transfer of intact gamma-globulin across the yolk sac and suggested that this was due to degradation of the protein in this layer.

However, the Beck and Lloyd hypothesis is open to alternative explanation. For example, HRP was measured enzymatically and the low level of its activity in lysosomes may simply reflect denaturation of the enzyme in the acidic milieu of the lysosome. Furthermore, there is absolutely no evidence for the utilisation by the embryo of amino acids derived from any protein digestion in the yolk sac. Most importantly of all, there is no clue as to the possible significance of the contributions of this process, if it exists, to embryotrophic nutrition as a whole. As already stated, it is conceivable that amino acids exogenous to the conceptus are able to satisfy the obvious needs of the embryo for protein building blocks, or that specific embryonic proteins are synthesised in maternal tissues and are transferred directly to the embryo, perhaps

gaining access to the latter by a specific mechanism of transport through the yolk sac.

Since for the formulation of this hypothesis, Beck and Lloyd and their associates have sought more direct evidence of the yolk sac's ability to pinocytose and digest macromolecules. However, owing to the minute amount of tissue available at early organogenesis, quantitative biochemical studies have been difficult. Accordingly, yolk sac from later in gestation - 17.5 days - has been studied in an organ culture system. Of course _in vivo_, any placental function of the yolk sac is likely to have been taken over by the chorio-allantoic placenta at this stage, and consequently there is some risk in extrapolating back to early organogenesis the interpretations of observations made with the 17.5 day tissue. Nevertheless, ultra-structural appearance of the tissue is similar at both organogenesis and 17.5 days (Williams et al., 1976), and the distribution of HRP in the near-term conceptus is essentially identical to that described above for the early organogenetic conceptus (Beck et al., 1967a).

Using the 17.5-day rat yolk sac culture system, a wealth of data has been accumulated which clearly demonstrates the tissue's ability to pinocytose and degrade macromolecules.(Williams et al., 1975a,b; Roberts et al., 1976, 1977; Moore et al., 1977; Duncan and Lloyd, 1978; Pratten et al., 1978, 1980; Duncan et al., 1979, 1981; Ibbotson and Williams, 1979; Livesey and Williams, 1979). Of specific relevance to the alleged role of the yolk sac in histio-trophic nutrition are results showing that proteins are taken pinocytically into the yolk sac cells and are digested intralysosomally

to the amino acid level (Williams et al., 1975b; Livesey and Williams, 1979). These amino acids are quickly released from the tissue back into the culture medium. It is clear from such a quantitative study that the (17.5-day) yolk sac has the capability to perform the nutritional function suggested for it. Whether or not the tissue behaves similarly during early organogenesis is still questionable. And of course, the 17.5 day yolk sac in culture does not, and inevitably cannot, reveal whether amino acids derived from yolk sac proteolysis are utilised by the embryo. Certainly, a considerable advance towards resolving this issue would be made if similar quantitative experiments could be performed with the early rat conceptus in vitro. A convenient method now exists for the culture of the early post-implantation rat conceptus complete with visceral yolk sac (New et al., 1973, 1976a). Conceptuses of 9.5 days gestational age can be grown continuously for 48 hours, during which time development in vitro parallels that seen in vivo. This 48 hour period represents the principal organogenetic phase of development and includes the period immediately prior to the formation of a functional chorioallantoic placenta. The technique thus presents an ideal opportunity to establish the extent to which if any, the visceral yolk sac is involved in histiotrophic nutrition, an area of study for which the technique has not before been used.

1.1.2 A Target for Teratogens

Reference has already been made to the observations of some decades ago (Goldman, 1909; Everett, 1935) that the bisazo dye, trypan blue, when administered to the pregnant rat, localises in the visceral yolk sac, specifically the lysosomes (Beck et al., 1967a), and does not

reach the embryo save for trace quantities in the gut endoderm (Dencker, 1977). It has also been known for many years that trypan blue is teratogenic in the rat when administered during organogenesis (Gillman et al., 1948). The dye therefore, is ideally placed to affect any nutritional function in which the yolk sac lysosomes might be involved. It was regarded as rather significant, therefore, when trypan blue was observed to inhibit a number of lysosomal enzymes (Beck et al., 1967b; Lloyd et al., 1968). Interestingly, trypan blue if applied later than day 10 of gestation in the rat, abruptly ceases to be teratogenic (Wilson et al., 1959). This is about the time when the chorioallantoic placenta becomes operative and presumably takes over the major nutritional mechanism. In the light of these observations, a hypothesis to account for the teratogenic action of trypan blue was advanced (Beck et al., 1967b; Lloyd and Beck, 1967). Briefly, this was as follows:

Trypan blue, bound to plasma albumin, is pinocytosed by the visceral yolk sac endoderm and comes to lie within the lysosomes of these cells. Free trypan blue is generated here by digestion of the associated albumin, and the dye, unable to diffuse out of the lysosomes, accumulates, eventually reaching a concentration inhibitory to the lysosomal enzymes. The hypothesis goes on to say that as a consequence of lysosomal enzyme inhibition, histiotroph remains undigested and the supply of nutrients to the embryo at this most critical stage of development is interrupted, with irreparable consequences for the embryo. Once the chorioallantoic placenta becomes functional, the yolk sac's role in providing for the embryo becomes greatly diminished and any inhibition of its function seems to cause no permanent damage to the embryo.

The 17.5-day yolk sac culture system has again provided useful quantitative information regarding the mechanism of action of trypan blue. Formaldehyde-denatured ^{125}I-labelled bovine serum albumin is a substrate that is normally rapidly pinocytosed and digested by 17.5-day yolk sac, the radiolabelled digestion products, chiefly $\left[^{125}I\right]$ iodotyrosine, being released back into the culture medium (Williams et al., 1975b). The addition of trypan blue to the culture medium at concentrations above 100 μg/ml inhibits the uptake and degradation of the radiolabelled protein. Closer analysis of this inhibitory action of the dye showed that pinocytosis rather than intralysosomal digestion was being affected (Williams et al., 1976). Pinocytosis by the yolk sac and not, as was at first thought, intralysosomal digestion was therefore considered to be the primary target of trypan blue's action. The original hypothesis was therefore modified (Williams et al., 1976) in accordance with these observations. However, the postulated consequence of trypan blue's teratogenic action, a reduced flux of amino acids to the embryo, remained unaltered.

Further evidence for an inhibition by trypan blue of yolk sac pinocytosis has come from a morphological study of conceptuses from trypan blue-treated mice (Batten and Haar, 1979). The dye was observed to cause a marked reduction in the number of microvilli on the apical borders of the yolk sac endodermal cells. Compatible with both the original and modified hypotheses are observations made by Berry (1970), confirming earlier work (Jensh and Brent, 1967), who showed that embryos from rats treated with trypan blue on day 7 of pregnancy contained less protein than controls up to the 35-somite stage. Following the establishment of a chorioallantoic placenta however, the difference between control and treated embryos disappeared.

Furthermore, within the experimental group, abnormal embryos were observed to have a lower protein content than morphologically normal embryos. Zawoiski (1975) showed that when the diets of trypan blue-treated pregnant mice were supplemented with either protein or glutamic acid, a significant reduction in the incidence of certain malformations occurred. A similar action has since been described for supplement of threonine (Zawoiski, 1980).

It has already been emphasised that the postulated role of the visceral yolk sac in mediating the nutritional supply of the early rat embryo lacks definitive evidence. Its candidacy as a target for teratogenic action is, therefore, likewise in need of further investigation.

1.2   Normal Development of the Rat Conceptus between 9.5 and 11.5 days of Gestation

It is appropriate here to consider some of the more notable developmental events occurring during early organogenesis in the rat. Description will be confined to the embryo itself, and to the visceral yolk sac. The parietal yolk sac and overlying Reichert's membrane (Figure 1.1) are not described. Observations made in vivo suggest that these two structures do not retard the access of potentially nutritive protein to the visceral yolk sac endoderm (Beck et al., 1967a; Seibel, 1974; Sharma and Peel, 1979).

At 9.5 days, the head-fold stage rat embryo (Figures 1.1 and 1.2) is relatively undifferentiated. It is estimated to contain about 3,000 cells (Köhler et al., 1972) and less than 5 µg of protein (Cockroft, 1977). The somites, blocks of mesoderm lying in two longitudinal strips either side of the neural tube, have not yet

appeared. The embryo at this stage is dorsally concave, the gut endoderm being contiguous with the visceral yolk sac endoderm and lining the inner face of the yolk sac cavity. Also present at 9.5 days are the allantoic bud, neural plate and cardiogenic primordium (Beaudoin, 1980). During the period between 9.5 and 10.5 days, considerable growth and differentiation occurs (Figures 1.2 - 1.4). The cell number has now risen to about 100,000 (Köhler et al., 1972) and protein content to between 30 and 50 μg (Cockroft, 1977). At 10.5 days embryonic somites number 7 - 15. A neural tube has formed, and fused along a considerable portion of its length, though both anterior and posterior neuropores are still unclosed (Edwards, 1968). Three brain vesicles are present, as are optic and otic vesicles (Nishimura and Shiota, 1977). An S-shaped heart has now formed and has begun contracting. The allantois has fused with the chorion and the development of the chorioallantoic placenta is nearing completion (New, 1978).

By 11.5 days (Figure 1.5), cell number has risen to several million (Köhler et al., 1972) and protein content is now between 200 and 500 μg (Cockroft, 1977). Perhaps the most conspicuous feature of development during this time is the axial rotation of the embryo to the dorsally convex position and its detachment from the visceral yolk sac. This turning of the embryo to the characteristic fetal position has been described by Deuchar (1971). Another easily observable feature of the conceptus by this stage is the vitelline circulation, connecting the yolk sac with the embryo. This is a significant event for the embryo since it increases the efficiency of delivery of nutrients derived from degradation of histiotroph in the yolk sac, as well as enabling more rapid excretion of waste from

the embryo. The heart is now a four-chambered structure and is beating strongly. Both posterior and anterior neuropores have now closed and the brain has developed five vesicles (Beaudoin, 1980). The morphogenesis of the neural tube has recently been investigated (Morriss and New, 1979). The fore-limb buds are also detectable by 11.5 days. Table 1.1 summarises the early organogenetic development of the rat embryo.

The rodent visceral yolk sac has been the subject of a great deal of morphological study over the past two decades (Anderson, 1959; Padykula and Wislocki, 1961; Padykula et al., 1966; Beck et al., 1967a; Krzyzowska-Gruca and Schiebler, 1967; Lloyd et al., 1968; Carpenter and Ferm, 1969; King and Enders, 1970; Haar, 1971; Jollie and Triche, 1971; Seibel, 1974; Franke et al., 1975, 1976; Schluter, 1978; Baeckland and Heinen, 1979; Batten and Haar, 1979; Carpenter and Dishaw, 1979; Gulamhusein et al., 1979; Gupta et al., 1979; Sharma and Peel, 1979).

Three distinct morphological zones can be identified. The first of these, the major component, is the endodermal cell layer, which lines the inner face of the yolk sac cavity. The columnar endodermal cells, as already pointed out, display all the characteristics of a pinocytosing cell. These include a well developed microvillous apical border and a subapical canalicular system. A variety of electron-dense and electron-lucent vesicles are also detectable in the supranuclear region of the cell. Gupta et al. (1979) have classified four distinct types of vesicle within the cell, all of which are implicated in the pinocytic process. A large nucleus is basally located and all the common cell organelles are present. A schematic diagram of an endodermal cell is presented in Figure 1.6.

The endodermal layer is delimited by a basement membrane below which lies a mesenchymal layer, the second distinct region of the visceral yolk sac. Within this layer are observed loosely distributed connective tissue macrophages. Further, it is in the mesenchyme that the vitelline capillaries develop, complete with lining endothelial cells. A serosal basement membrane separates the mesenchymal layer from a narrow basophilic layer of mesothelium, the third morphological zone, which lines the exocoelom.

The most overt changes that occur during early organogenesis are the initiation of the vitelline circulation (described in the mouse by Haar and Ackerman, 1971a,b), and the occlusion of the yolk stalk following rotation and detachment of the embryo so that the yolk sac completely envelops the embryo. Another interesting change has recently been discovered by Gupta and associates (Gupta et al., 1981). A stereological study has shown that the ratio of volume of pinocytic vesicles relative to the rest of the cell decreases between 9.5 and 11.5 days of gestation in culture, suggesting that the endoderm becomes less pinocytic at about the time the chorioallantoic placenta begins to function.

1.3   Pinocytosis and lysosomal catabolism

Central to the theory of histiotrophic nutrition by the visceral yolk sac are the processes by which locally available macromolecules are taken up into the endodermal cells (pinocytosis) and subsequently degraded (lysosomal catabolism). It is relevant here, therefore, to outline some of the features of these processes, with specific reference to yolk sac.

Pinocytosis describes the entrapment of small droplets of extracellular

fluid by plasma membrane, and is distinct from phagocytosis which refers to the ingestion of micro-particulate matter such as bacteria and erythrocytes. Phagocytosis is not observed in yolk sac cells and the largest molecule which can enter the yolk sac by pinocytosis is of the order of 7×10^6 molecular weight (Duncan et al., 1981). Pratten et al. (1980) have classified the sequence of events which characterise pinocytosis, into three groups (Figure 1.7). The first of these involves the internalisation of the plasma membrane, which may or may not be stimulated by the attachment of some substance to the membrane. Following invagination, the vesicle or pinosome containing fluid is 'pinched off' from the plasma membrane and is translocated deeper into the cell. During translocation a number of fusion events occur. Initially pinosomes fuse with each other to produce larger vesicles which fuse ultimately with primary lysosomes, giving rise to secondary lysosomes. Formation of a secondary lysosome thus exposes the pinocytosed material to the formidable battery of lysosomal hydrolases and results in the degradation of the exogenous substrate. The third feature of pinocytosis may be called lysosomal regression. Low-molecular weight catabolites diffuse out of the lysosome into the cytosol and this is accompanied by a shrinkage of the lysosome and its ultimate disappearance from the cell. Non-digestible material is not degraded within secondary lysosomes and is accumulated. Regurgitation of such undigested material is not a general property of mammalian cells.

Examination of the internalisation step of pinocytosis has led Jacques (1969) to describe two basic mechanisms of substrate capture (Figure 1.8). Substances with no affinity for the internalising

plasma membrane are taken into the cell entirely in solution, so-called fluid-phase pinocytosis. In contrast to this, certain molecules bind strongly to the plasma membrane and are pinocytosed with almost total exclusion of fluid; adsorptive or receptor-mediated pinocytosis. Between these two extremes, a spectrum of mixed uptake modes exist, which vary in the extent to which the substrate binds to the plasma membrane and the volume of fluid incorporated into the pinosome.

Active interest in pinocytosis over the last decade or so has produced a great deal of information on the process. For example, it is known that pinocytic uptake is energy-requiring in a number of different cell types (reviewed by Silverstein et al., 1977) and this is also true of the cells of the yolk sac endoderm (Duncan and Lloyd, 1978). There is a requirement too for cytoskeletal involvement in pinocytosis (Duncan and Lloyd, 1978). However, identification of any control mechanism for pinocytosis has not thus far been achieved. Calcium and cyclic AMP have been proposed as regulators of pinocytosis, but definitive evidence is lacking (Pratten et al., 1980). Clues to the nature of the control mechanism might be provided by the discovery of substances that modify the rate of pinocytic uptake. Both poly-anions (Cohn, 1966) and polycations (Ryser and Hancock, 1965; Seljelid et al., 1973; Pratten et al., 1978; Shen and Ryser, 1978) have been observed to enhance pinocytic uptake in various cells. An important distinction is to be made here however. A stimulation of pinocytosis may be due to either an increased rate of formation of pinosomes, or to an increased affinity of the reference substrate for the internalising membrane. Conceivably, an altogether different pinocytic mechanism may be induced to operate by these

apparent stimulators. It is significant that none of the enhanced
pinocytic rates observed with polyanions and polycations have been
attributed to accelerated pinosome formation and that all are
explicable by other means. Concanavalin A has been reported to
stimulate the uptake of markers, normally pinocytosed in the fluid-
phase, by macrophages (Edelson and Cohn, 1974), though it must be
said that this is part of a wider effect that includes an inhibition
of pinosome-lysosome fusion and the induction of lysosomal enzyme
synthesis. The rat yolk sac has proved obstinately refractory to
stimulation and the pinocytic rate, at least during the latter
third of gestation, appears to be a constitutive phenomenon
(Ibbotson, 1977).

The uptake step is rate-limiting in the overall process of uptake,
digestion and catabolite release of protein by rat yolk sac. The
evidence for this is as follows. It has been repeatedly observed
that the yolk sac accumulates non-digestible substances such as
^{125}I-labelled polyvinylpyrrolidone, colloidal $[^{198}Au]$ gold and
$[^{14}C]$ sucrose from the culture medium. In contrast, the yolk
sac-associated radioactivity measured when a digestible protein
substrate (^{125}I-labelled bovine serum albumin) is present in the
medium, quickly reaches a steady state. Simultaneously, ^{125}I-labelled
catabolites are released back into the culture medium at a constant
rate (Moore et al., 1977; Lloyd and Williams, 1978). This pattern
indicates that pinocytosis, rather than digestion, is the rate-
limiting step. The formation of secondary lysosomes results in
the confrontation between pinocytosed material and the hydrolytic
enzymes of the lysosome. Lysosomes are known to contain hydrolases
whose activity is directed against many classes of biological

macromolecule. Included is a great variety of proteinases, some with quite specific cofactor requirements. Lysosomal enzymes have in common a low pH optimum, and the acidic milieu of the lysosome not only maximizes the catalytic activity of the enzymes but also serves to partially denature macromolecular substrate, particularly protein, increasing its susceptibility to attack.

Two major types of lysosomal proteinase are recognised. The cysteine proteinases (recently reviewed by Kirschke et al., 1980), such as cathepsins B, H, L and N, require the presence of a thiol group of a cysteine residue in the active site, and the aspartic proteinases, most notable among which is cathepsin D, require the carboxyl group of an aspartic acid residue for activity (Barrett, 1980). Voluminous literature describes the properties and characteristics of the lysosomal proteinases, and this has been lucidly summarised (Heath and Barrett, 1977; Barrett and Macdonald, 1980).

Alas little information exists on the lysosomal proteinases of the yolk sac. Beck et al. (1967b) studied the inhibitory action of trypan blue on the activities of several enzymes, including cathepsin D, of a lysosomal fraction of rat yolk sac homogenate. Using rabbit yolk sac, Jones and Hemmings (1971) reported considerable proteolytic activity of a lysosomal fraction against a number of specific peptides and serum proteins, hydrolysis of the latter being attributed to the action of cathepsin D. More recently Sherman and Atienza-Samols (1979) have measured the activities of a number of lysosomal enzymes (though not proteinases) in a crude homogenate of mouse yolk sac. Additionally, histochemistry has identified several lysosomal enzymes in rat yolk sac (Padykula, 1958; Bulmer, 1963, 1965; Christie, 1967).

The proposed mechanism of histiotrophic nutrition by the visceral yolk sac requires, amongst other things, that the yolk sac cells are able to pinocytose and intralysosomally digest macromolecules. The evidence presented here demonstrates that the yolk sac is capable of both functions.

1.4   Aims of this Thesis

It is clear from the foregoing discussion (Section 1.1) that much remains to be learnt about the precise nature of any involvement of the visceral yolk sac in the mediation of the nutritional supply to the developing rat embryo, and the broad objective of this study is to further this understanding.

Subsequent chapters describe experiments specifically designed to answer questions posed by the gap in our understanding of this proposed process. These questions are as follows:

1. Do macromolecules reach the embryo directly without the involvement of the visceral yolk sac, or does pinocytosis by the yolk sac provide the only possible route of access to the embryo for exogenous macromolecules?

2. If the latter is the case, are macromolecules taken up by the yolk sac then delivered directly to the embryonic tissues intact, or are they immobilised in the yolk sac?

3. What is the digestive capacity of the visceral yolk sac? And are degradable compounds pinocytosed by the yolk sac catabolised there also?

4. Assuming that macromolecules are taken up and degraded by the yolk sac, are the products of digestion assimilated by the rapidly growing embryo?

5. Is the embryo dependent on macromolecular sources of nutrient,

   or are micromolecular nutrients capable of sustaining it during

   the early organogenetic phase? In other words, is the postulated

   mechanism of histiotrophic nutrition of quantitative significance?

Answers to these questions, provided from observations of the early

rat conceptus *in vitro*, should dispel some of the uncertainty

surrounding the visceral yolk sac's function, with respect to

histiotrophic nutrition, during early organogenesis.

With the data from these experiments in hand, further experiments

investigate the claim that the aetiology of certain teratogen-induced

congenital malformations may be a disturbance of the nutritional aspect

of yolk sac function. This is achieved by studying the effects of

five compounds of proven or suspected teratogenicity, all of which

are known to be lysosomotropic and therefore dependent upon pinocytosis

for entry into cells, on the established properties of yolk sac

nutritional function.

Ectoplacental cone

Ectochorionic cyst

Yolk sac cavity

Extraembryonic coelom

Amnion

Allantois

Visceral yolk sac

Head fold
Parietal yolk sac

Cardiogenic primordium

Reichert's membrane

Amniotic cavity

Notochord

Decidual
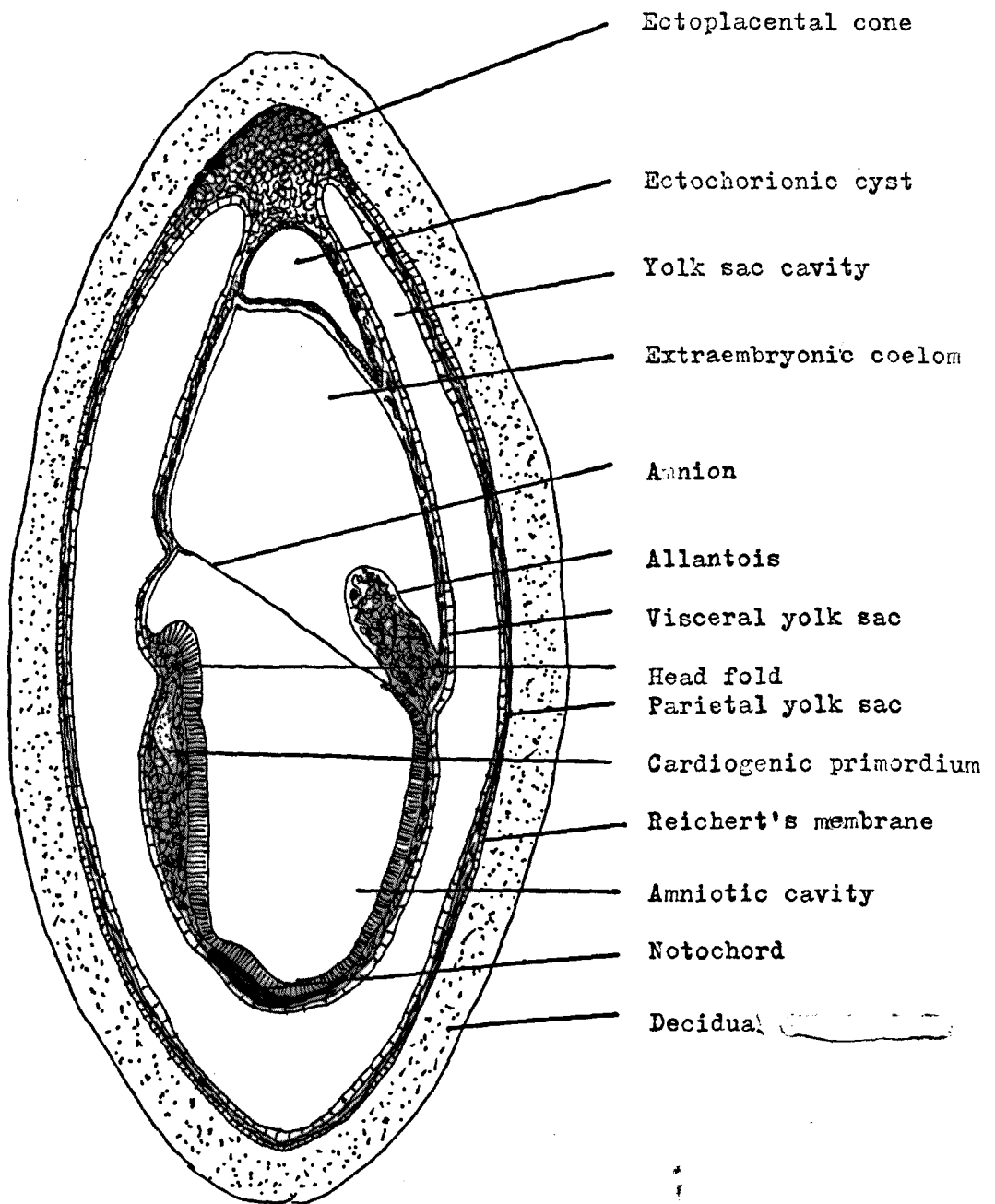
Figure 1.1   Schematic diagram of head-fold stage rat egg cylinder
at 9.5 days of gestation

Figure 1.2 Rat conceptuses explanted from the mother at 9.5 days
of gestation. The head-fold stage embryo is indicated by an arrow.
Other details of morphology are given in Figure 1.1 and Table 1.1.
The conceptus on the left hand side has been divested of Reichert's
membrane and underlying parietal endoderm. These two membranes
stand clear of the conceptus at the embryonic pole as can be seen
in the right hand conceptus. Scale x30.

Figure 1.3 9.5-Day rat conceptus cultured for 12 hours (equivalent
to gestational day-10). Considerable growth and development of the
embryo has occurred. Conceptus is now more spherical. Other details
given in Figure 1.1 and Table 1.1. Scale x30.

Figure 1.4 9.5-Day rat conceptus cultured for 24 hours (equivalent
to gestational day-10.5). Embryo has begun to rotate to the
characteristic dorsally-convex fetal position, detaching from the
visceral yolk sac which now envelops the embryo. Other details
given in Table 1.1. Scale x30.
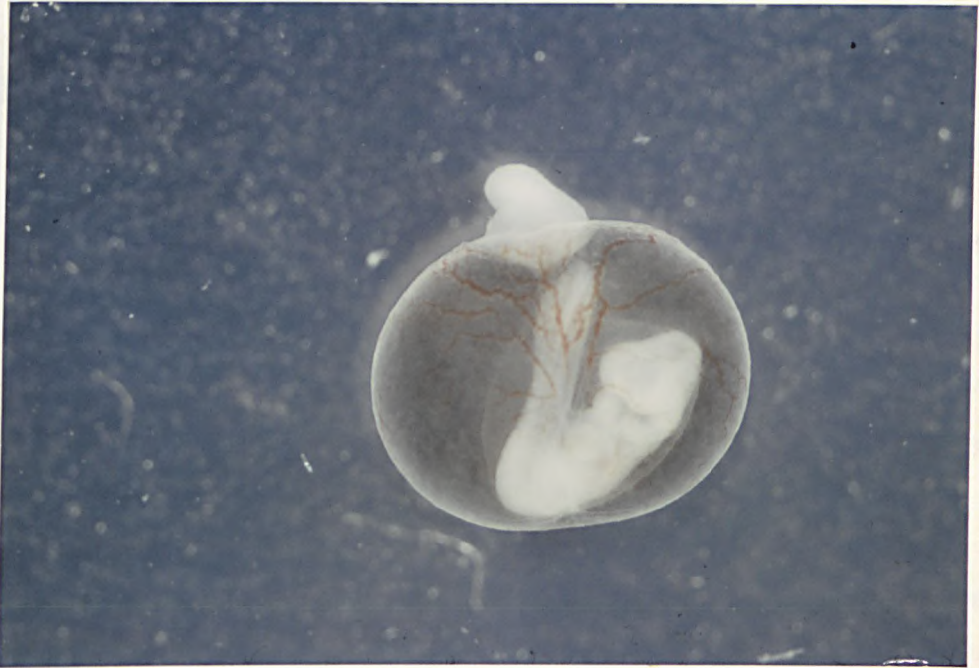
Figure 1.5 9.5-Day rat conceptus cultured for 48 hours (equivalent to gestational day-11.5). The embryo has now completely rotated. The choriovitelline circulation is well established. Other details given in Table 1.1. Scale x12.5.

Yolk sac cavity

Microvilli

"Fuzz-lined" caveoli
"Fuzz-lined" vesicle

Apical canalicular systems

Junctional complex

Lateral plasma membrane

Apical vacuole

Mitochondrion

Golgi apparatus

Smooth ⎫
        ⎬ endoplasmic reticulum
Rough ⎭

Nucleus

Glycogen deposits

Lipid droplets

Visceral basement membrane
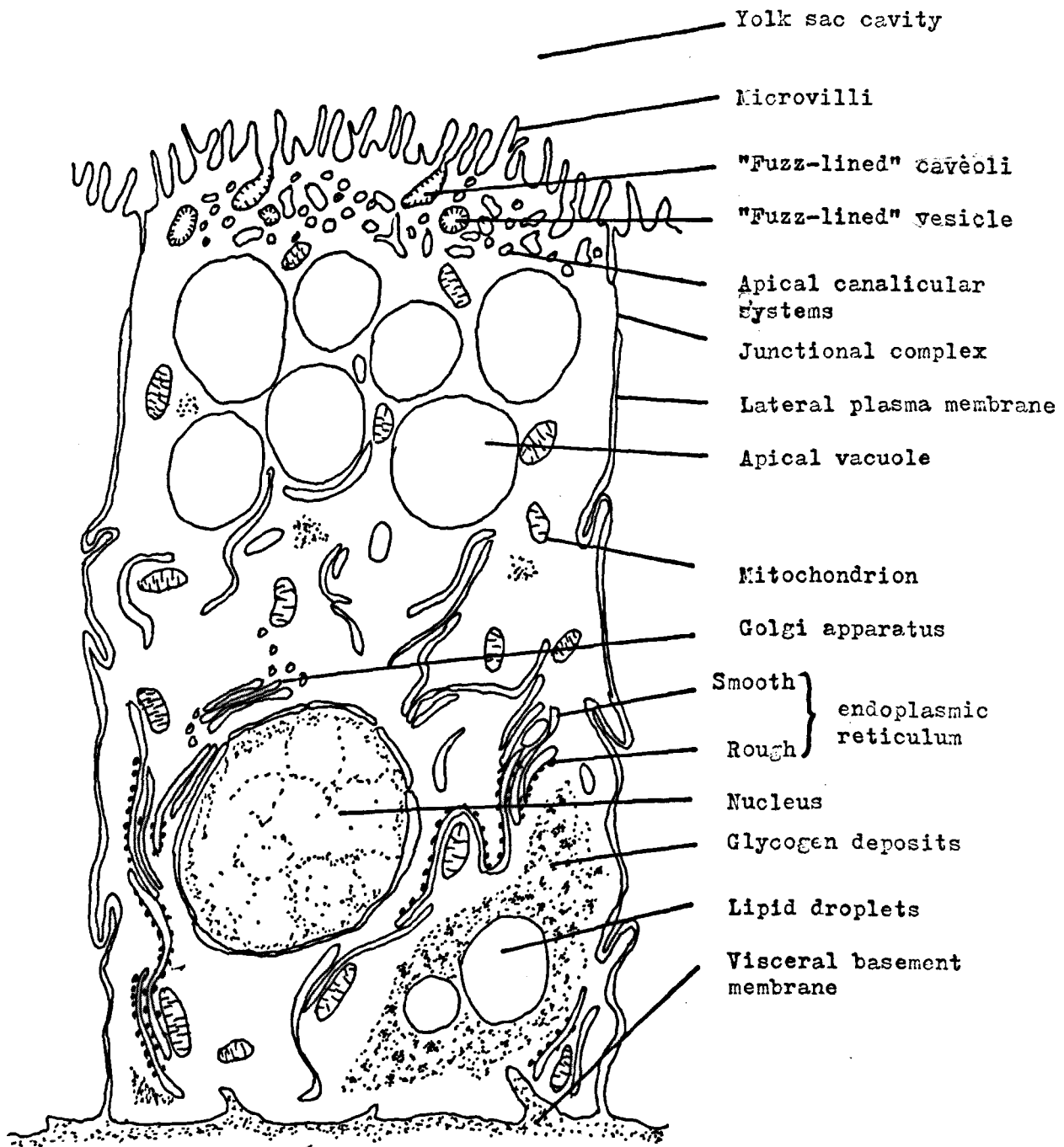
Figure 1.6   Schematic diagram of visceral yolk sac
              endodermal cell

Figure 1.7 Diagram to show the pathway of pinocytosis, leading
to degradation of pinocytosed substrates within
secondary lysosomes and the release of catabolites
into the cell cytoplasm

Figure 1.8   Types of pinocytosis (after Jaques, 1969)

Cell exterior

invaginating plasma membrane

1. Fluid-phase uptake

2. Adsorptive uptake

3. Mixed-type uptake

<u>Table 1.1 Normal Development of the Rat Embryo up to 11.5 days of</u>
<u>Gestation</u>

<u>External Appearance</u>

Day 9.5-10.5   Somites 1-15 are identifiable; embryo is bent
                dorsally; first branchial arch appears.

Day 10.5-11.5   Somites number 22-28; embryo rotates to dorsally
                convex position; second and third branchial arches
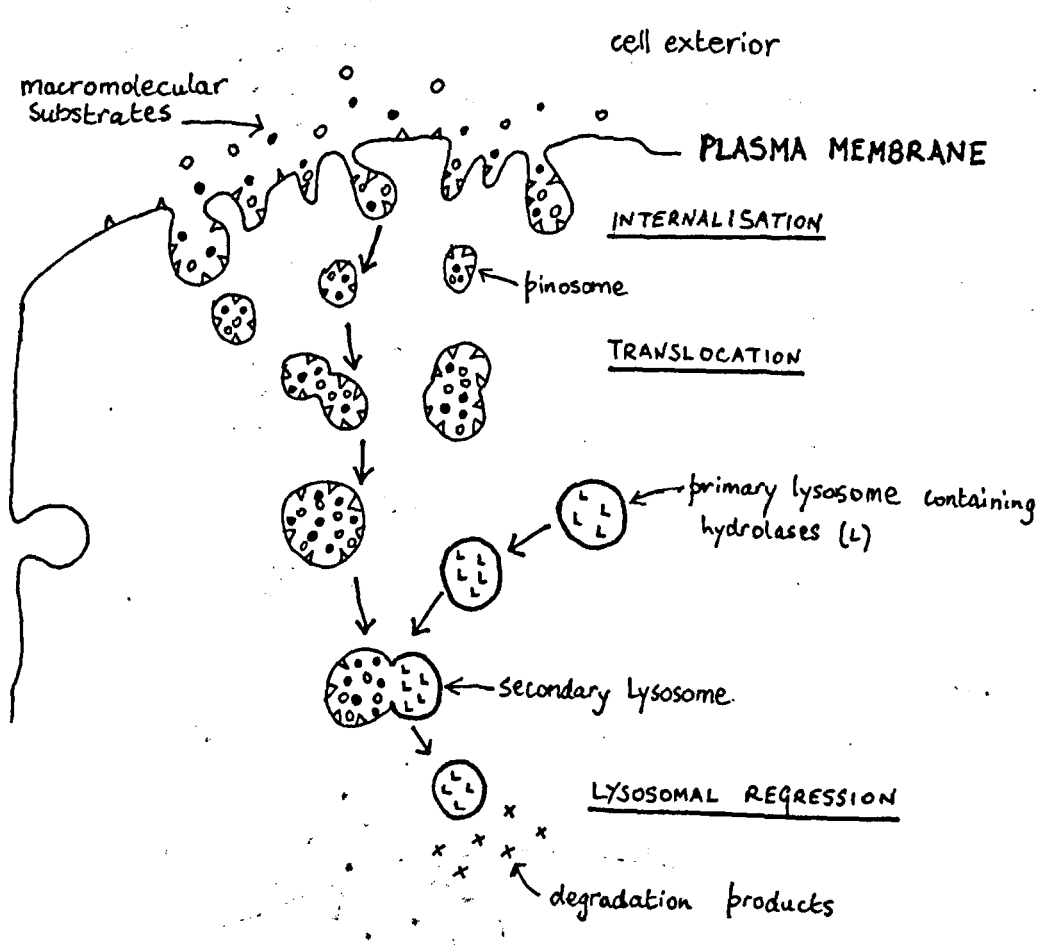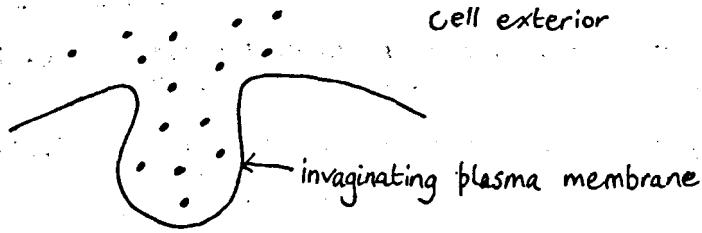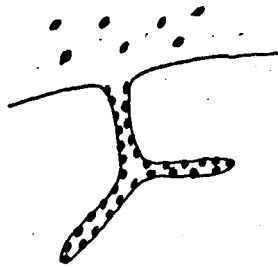   appear; tail fold and lateral body wall folds
                form; maxillary processes appear; anterior limb
                buds appear.

<u>Nervous System</u>

Day 8-9   Neural plate appears.

Day 9-10   Neural folds form and begin fusing; neural crest
                formed at junction of neural folds and surface
                ectoderm; optic evaginations appear from
                prosencephalon.

Day 10-11   Three brain vesicles present, prosencephalon,
                mesencephalon and rhombencephalon; infundibulum
                originates in floor of prosenchephalon; otic placodes
                form; cranial ganglia begin formation; optic vesicles
                contact surface ectoderm; neural tube closed but for
                anterior and posterior neuropores; Rathke's pouch
                begins outgrowth from roof of future mouth.

Day 11-11.5   Five brain vesicles present, telen-, dien-, mesen-,
                meten- and myelencephalon; closure of anterior and
                posterior neuropores; optic cups begin to form two
                layers; otic vesicles close; olfactory placodes
                appear.

<u>Circulatory System</u>

Day 8-9   Cardiogenic primordia appear.

Day 9-10   Fusion of cardiogenic primordia with formation of
                simple tubular heart which begins sporadic
                contractions; aortic arch I is forming.

Day 10-11   S-shaped tubular heart forms; aortic arches II and
                III appear; cardinal, umbilical and vitelline veins
                can be recognised; atrium, ventricle and sinus
                venosus formed; embryonic circulation is established.

Day 11-11.5   Aortic arches III and IV are well developed while
                arches I and II regress; interatrial septum begins
                to form.

Digestive System

Day 9-10   Foregut begins to form.

Day 10-11   Oral membrane ruptures; liver diverticular formed;
                  first pharyngeal pouch is present; thyroid primordia
                  appear in pharynx; hindgut begins formation.

Day 11-11.5   Laryngo-tracheal groove appears; primordium of dorsal
                  pancreas present; stomach recognisable.

Respiratory System

Day 11.5   Paired lung buds formed.

Urogenital System

Day 8-9   Intermediate mesoderm (precursor of urogenital system)
                  forms.

Day 9-10   Germ cells observed in yolk sac epithelium.

Day 10-11   Nephrogenic ridges appear in intermediate mesoderm;
                  (pro)nephric ducts begin formation; germ cells
                  migrate from yolk sac, entering hind gut and
                  mesenteries.

Day 11-11.5   First mesonephric tubules develop; gonadal ridges
                  appear.

(After Christie, 1964; Edwards, 1968; Nishimura and Shiota, 1977,
Beaudoin, 1980).

CHAPTER TWO

THE UPTAKE OF NON-DIGESTIBLE SUBSTRATES BY EARLY
ORGANOGENESIS-STAGE RAT CONCEPTUSES

2.1 Introduction

The question of whether or not uptake by the yolk sac is an obligatory
step in the passage of macromolecules to the embryo is fundamental to
the proposed role of the yolk sac in mediating histiotrophic nutrition,
and must be resolved at the outset. This can be done simply, by the
use of the whole-embryo culture technique. Experiments are described
in this chapter in which non-digestible macromolecules, ^{125}I-PVP and
$\left[^3\text{H}\right]$ dextran, were added to the culture medium in which conceptuses
were growing. The distribution of radioactivity in the yolk sac
and embryo after a given time interval was determined.

From the average molecular weights of these two compounds (^{125}I-PVP
$\approx$ 30,000 - 40,000, $\left[^3\text{H}\right]$ dextran $=$ 70,000) it may safely be assumed
that any uptake will be by pinocytosis. Both macromolecules have been
shown to be accumulated by the 17.5-day rat yolk sac in culture
Williams et al., 1975a; Roberts et al., 1976). Accumulation of
^{125}I-PVP has also been observed in the villous epithelial cells
of the small intestine of the neonatal rat (Clarke and Hardy, 1969),
the rat peritoneal macrophage (Pratten et al., 1978) and everted rat
intestinal sacs in vitro (Bridges and Woodley, 1978). Dextran
accumulation has been demonstrated in mouse liver parenchymal and
Kupffer cells (De Man et al., 1960; Daems and van Rijssel, 1961),
proximal kidney tubule cells (Maunsbach, 1969), spleen red pulp
macrophages (Bowers, 1969), J7742 mouse macrophages (Berlin and
Oliver, 1980; Walter et al., 1980), mouse peritoneal macrophages
and BHK21 cells (Geisow et al., 1981) and rat peritoneal macrophages
(Pratten, unpublished data).

Studies of pinocytosis are simplified by the use of non-digestible substrates. The difficulties of measuring uptake of digestible macromolecules, where digestion products may be re-utilised in cellular biosynthetic processes, are avoided and since, as has already been mentioned, pinocytosed macro-molecules are not significantly released from the cell, uptake can be measured as an accumulation of substrate.

2.2   Materials

Culture Equipment

Roller incubator	BTC Engineering Ltd., Cambridge
Culture bottles	Baird and Tatlock Ltd., London
Silicone rubber bottle stoppers	Gallenkamp & Co. Ltd., Brimingham

Counters

Packard auto-gamma scintillation spectrometer 5136	Packard Instruments Ltd., Caversham,
Packard 2425 liquid scintillation spectrometer	Berks.

Other Equipment

CE 272 linear readout spectro-photometer	Cecil Instruments Ltd., Cambridge
Super minor bench centrifuge	MSE Ltd., Loughborough, Leics.
Coolspin centrifuge	MSE Ltd., Loughborough, Leics.
Disposable syringes	Sterilin Products Ltd, Middlesex
Disposable syringe needes	Sterilin Products Ltd, Middlesex
Sterile conical test tubes	Sterilin Products Ltd, Middlesex
Sterile plastic 30ml universal containers	Sterilin Products Ltd, Middlesex
Plastic disposable 3ml tubes	Luckham Ltd, Burgess Hill, Sussex

Radiochemicals

^{125}I-PVP (preparation IM.33P)	Radiochemical Centre, Amersham, Berks
$[^3H]$ dextran (preparation TRA.382)	Radiochemical Centre, Amersham, Berks

Other Reagents

Hanks balanced salt solution	Flow Laboratories Ltd, Irvine, Scotland
Antibiotic mixture	Flow Laboratories Ltd, Irvine, Scotland
Lumagel scintillation fluid	Lumac Systems Ltd, Basle, Switzerland
Folin Ciocalteau reagent	Fisons Ltd, Loughborough, Leics.
Bovine serum albumin (product A.3478)	Sigma Chemical Co. Ltd, Poole, Dorset

Gas Mixtures

5% O_2/5% CO_2/90% N_2	British Oxygen Corporation, Special
20% O_2/5% CO_2/75% N_2	Gases Division, Deer Park Road,
40% O_2/5% CO_2/55% N_2	London.

2.3   Methods

2.3.1   Whole-Embryo Culture

2.3.1.1 Preparation of Culture Medium

Homologous serum from either male or female rats was used as a culture medium. The procedure for serum preparation was as follows:

The animal was firstly anaesthetised with diethyl ether. It was then laid on the bench and a beaker of ether-soaked cotton wool was placed over its head to maintain anaesthesia. Care was taken that the animal did not die from ether overdose before the blood was removed. A large opening was then made in the ventral body wall using clean

instruments, and the intestines displaced to one side, except for the descending colon which was clamped with a haemostat and moved in the opposite direction, tearing away the mesentery that attached it to the rest of the intestine and to the posterior body wall. To expose the dorsal aorta the mesentery (peritoneum) covering the posterior body wall was gently rubbed away with surgical swabs. Blood was withdrawn from the aorta with a 10 ml syringe and a 40 mm x 8/10 needle. The needle was inserted into the aorta at the point of bifurcation into the iliac vessels, and carefully pushed up the aorta for an inch or so. The heart usually stopped beating after about 8-10 ml of blood had been collected. The syringe was then removed, the needle discarded and the blood transferred to a sterile 15 ml plastic conical test-tube, which was immediately centrifuged at 2,000 g for 10 minutes. Centrifugation precipitated the blood cells and a fibrin clot formed in the supernatant plasma. After allowing at least an hour for the clot to form completely, the clot was squeezed with forceps to remove the contained serum, and the blood recentrifuged at 2,000 g for 10 minutes.

The clear serum, which constitutes about half the volume of the original blood, from all rats bled in a session was pooled in 30ml sterile plastic universal containers, antibiotics added and stored at -20°C until required. The antibiotics added were a mixture of penicillin (5,000 i.u./ml) and streptomycin (5,000 μg/ml) at a ratio of 0.2ml antibiotic solution to 10ml serum.

Before use for embryo culture, the serum was thawed, heat-denatured at 56°C for 30 minutes to inactivate the complement, and centrifuged at 2,000 g for 5 minutes to precipitate any particulate matter. The clear serum was then decanted into a fresh vessel and maintained at 37°C until culture commenced.

2.3.1.2 Explantation of the Conceptuses

Day 0 was considered to be that day on the morning of which a vaginal plug was found, and day 0.5 noon of the same day.

Pregnant female rats of 9.5 days gestation were killed by a blow to

the head. The ventral body wall was quickly opened and the uterus

explanted into a petri dish containing Hanks balanced salt solution.

Further dissection was carried out in a sterile laminar flow cabinet.

First, the uterus was cut into portions each of which contained a

single swelling. Then, each portion was stripped of uterus with

a pair of fine watchmakers' forceps to reveal the pear-shaped

decidual mass, containing the egg cylinder. In fresh Hanks and

under a dissecting microscope, the decidua was carefully teased

away and the egg cylinder dissected free. The outermost extra-

embryonic membranes, the parietal yolk sac endoderm and the adherent

overlying Reichert's membrane were excised. Thus the egg cylinder,

with the visceral yolk sac endoderm layer exposed, was ready for

culture.

2.3.1.3 Culture of Rat Egg Cylinders

The egg cylinders explanted from all the pregnant females in an

experiment, were shared evenly between culture vessels to minimise

variation between experimental groups. For each egg cylinder

cultured, 1 ml of culture serum was added. Culture took place in

either 60 or 30 ml bottles depending on the number of conceptuses

to be cultured. Initially culture bottles were gassed

for two minutes with a gas mixture comprising 5% O_2/5% CO_2/90% N_2.

At 24 hours of culture, bottles were re-gassed with 20% O_2/5% CO_2/

75% N_2 and at 32 hours of culture with 40% O_2/5% CO_2/55% N_2. After

initial gassing, the culture bottles were placed on horizontal rollers,

rotating at between 30 and 60 rpm, which were encased in a purpose-built

thermostatted perspex box at 37°C.

Culture was maintained for 48 hours, during which time the development of the conceptus, from egg cylinder to advanced organogenesis, is apparently identical to in utero development during the same period. In order to assess development at the end of culture, the following criteria of normal development were routinely applied to all conceptuses. Immediately upon removal from culture and while still warm, the presence of an embryonic heart beat and a yolk sac circulation was checked. The yolk sac diameter normally 3-4 mm, was measured under the microscope using an eyepiece graticule. Further checks included normal axial rotation of the embryo to the dorsally convex position, the presence of the forelimb buds, the fusion of the allantois with the chorion, closure of the neural tube, somite number (22-28) and embryonic protein content (120-240 μg). (Protein was estimated as described below, 2.3.4). Conceptuses failing to satisfy any of these criteria, no more than 5% of all cultured, were discarded.

2.3.2 Studies on the Uptake on Non-digestible Radiolabelled Macromolecules by Rat Conceptuses

Cultures were established as already outlined. At different time points between 24 and 1 hour before harvesting, ^{125}I-PVP was added to the culture medium at a final concentration of either 1.35, 2.8 or 5.6 μg/ml or $[^3H]$ dextran at a final concentration of 2.0, 5.0 or 10.0 μg/ml. All cultures were maintained for a total of 48 hours so that radiolabel was present in the culture medium, for different periods up to 24 hours.

At harvesting, conceptuses were washed in Hanks solution, yolk sacs and embryos dissected apart and washed separately in a further three

changes of Hanks solution. Each tissue was then solubilised in 0.5 ml of 0.25 M NaOH at 37°C for 1 hour. This solution was then neutralised by adding 0.5 ml of 0.25 M HNO_3 and the volume brought to 1.1 ml by addition of distilled water. Protein assay (see Section 2.3.4) was performed on duplicate 0.05 ml portions. The remaining 1.0 ml was assayed for radioactivity (Section 2.3.5). Duplicate samples of culture medium, 1.0 ml with ^{125}I-PVP and 0.5 ml with $[^3H]$ dextran, were assayed for radioactivity.

2.3.3 Studies on the Growth of Conceptuses during the Culture Period

In order to interpret quantitative data on uptake of macromolecules, it is essential that the growth characteristics of conceptuses during the culture period are understood. A study was therefore made of the increase in protein content of yolk sac and embryo between 24 and 48 hours of culture, the period during which uptake is being followed. Groups of conceptuses were removed from culture at different times during the second 24 hours of the culture period. At harvesting conceptuses were washed, dissected and solubilised as outlined above. Protein assay was performed on duplicate 0.05 ml portions of the tissue solutions.

2.3.4 Estimation of Protein

Protein content of tissues was measured using the method of Lowry et al. (1951). To 0.05 ml portions of either yolk sac or embryo solution (see Section 2.3.2) was added 0.2 ml of M NaOH and 0.25 ml of distilled water. To this solution 2.5 ml of Folin A reagent (prepared by adding 100 ml of 2% anhydrous sodium carbonate to 1.0 ml of 1% copper sulphate solution and 1.0 ml of 2% potassium sodium tartrate solution) was added and the mixture left to stand at room temperature for 20 minutes.

Following this, 0.25 ml of Folin B reagent (prepared by diluting Folin Ciocalteau solution with an equal volume of distilled water) was added with immediate mixing, and the colour allowed to develop at room temperature for a minimum of 45 minutes. The absorbances of the solutions were then measured at a wavelength of 750 nm.

In order to convert absorbance into protein concentration, a series of protein standards were prepared using bovine serum albumin (BSA) as reference protein. The standards were made up with different dilutions of a 0.1 mg/ml solution of BSA as outlined below.

Protein (μg)	BSA Soln. (ml)	Distilled Water (ml)	M NaOH (ml)
0	0	0.3	0.2
2	0.02	0.28	0.2
5	0.05	0.25	0.2
8	0.08	0.22	0.2
10	0.10	0.20	0.2

All standards were in triplicate. Details of the addition of Folin A and Folin B reagents were as described above. Absorbances at 750 nm of standard protein solutions are directly proportional to protein concentration, giving a straight calibration line. Estimation of tissue protein levels are therefore easily obtained by reference to this line. Since 0.05 ml of tissue solution is used for protein estimation out of a total volume of 1.1 ml, multiplication of the observed figure by 22 gives the total protein content of the tissue.

2.3.5 Measurement of Radioactivity

^{125}I-PVP radioactivity was measured directly on a gamma scintillation counter. Either 1.0 ml of tissue solution or culture serum was placed in a 3 ml plastic disposable tube and counted. The only correction

necessary for a gamma emitting sample, such as ^{125}I, is to subtract background counts from the observed count. Tissue solutions were counted for 5 minutes and medium samples for 30 seconds. The tissue counts were corrected for background and multiplied by 1.1 (since radioactivity was measured in 1.0 ml of a solution, whose total volume was 1.1 ml) to give a value for the radioactivity of the whole tissue. [3H] Dextran radioactivity was measured in a liquid scintillation spectrometer. Of the 1.0 ml of tissue solution remaining after protein estimation, 0.5 ml was added to 4.5 ml of Lumagel scintillation fluid and counted for 20 minutes. Duplicate 0.5 ml samples of culture medium were prepared similarly and counted for 4 minutes. Background radiation was determined by counting duplicate samples of 0.5 ml of distilled water in 4.5 ml of Lumagel. The observed count was corrected for quenching and background, and, in the case of tissue samples, multiplied by 2.2 to give a value for the radioactivity of the whole tissue.

A series of quenching standards comprising 0.5 ml of different dilutions of serum containing a standard quantity of [3H] dextran (giving approximately 10^5 c.p.m.) in 4.5 ml of Lumagel scintillant were counted, and the external standard number was plotted against the percentage counting efficiency, relative to water, for each standard. The resulting plot, or quench curve, was a straight line. Regression analysis of the quench curve established an equation of the type $y = mx + c$, where y = counting efficiency and x = the external standard number, to describe the curve. In order to determine the counting efficiency, y, of a given experimental sample, the external standard number generated from that sample, x, was substituted into the empirically determined quench equation. Thus the observed count of a sample that was counted with an efficiency of 70%, would be divided by 0.7 to give a quench-corrected count.

2.3.6 Quantitation of Uptake

To determine the uptake of radioactivity into the yolk sac and embryo, the following expression, described by Williams et al. (1975a), was used:

$$U = \frac{Y \text{ (or E)}}{M \times Py \text{ (or Pe)}}$$

where U = the uptake of radioactivity by either yolk

sac or embryo in a given time,

Y = the total radioactivity in the whole yolk

sac in counts per minute, corrected for

background, (and, in the case of $\left[^{3}H\right]$ dextran,

quenching),

E = the total radioactivity in the whole embryo

in c.p.m., corrected for background (and,

in the case of $\left[^{3}H\right]$ dextran, quenching),

M = the radioactivity per microlitre of medium

in c.p.m., corrected for background (and,

in the case of $\left[^{3}H\right]$ dextran, quenching),

Py = the protein content in milligrams of the

whole yolk sac,

and Pe = the protein content in milligrams of the

whole embryo.

Uptake thus expressed is a clearance, as the volume of culture medium whose contained substrate is internalised per milligram of yolk sac (or embryo) protein and has the units of microlitres of culture medium per milligram of protein.

The expression of uptake in this form has several advantages. First, the effect of a variable quantity of tissue protein is eliminated, as

is the effect of radioactive decay of the isotope on the specific radioactivity of the substrate. This is particularly relevant to substrates containing ^{125}I which has a relatively short half-life of 60 days. Secondly, reproducibility of results between experiments can be assessed.

The rates of accumulation of both ^{125}I-PVP and $[^{3}\text{H}]$ dextran by conceptuses were such as to cause no significant depletion of the substrates in the culture medium. The figure M in the uptake equation therefore refers to the radioactivity in the culture medium at the end of culture.

2.4   Results

2.4.1 Development of conceptuses in culture

Conceptuses grew and developed progressively throughout the 48 hour culture period. Fewer than 5% of conceptuses failed to satisfy the criteria for normal development (Section 2.3.1.3) and these were discarded.

Figure 2.1 shows the protein content of yolk sacs and embryos from 24 hours to 48 hours of culture. It can be seen that the increase in protein content of both tissues was small initially
but accelerated during the later stages of culture.

2.4.2 Uptake of ^{125}I-PVP by Yolk Sacs and Embryos

Figures 2.2, 2.3 and 2.4 show how the amount of radioactivity found in the embryo and yolk sac at 48 hours varied with the time at which ^{125}I-PVP, at final concentrations in the medium of 1.35, 2.8 and 5.6 μg/ml respectively, was added to the culture medium. It may be seen that no radioactivity entered the embryo. In contrast the yolk sacs contained significant amounts of radioactivity; the amount found
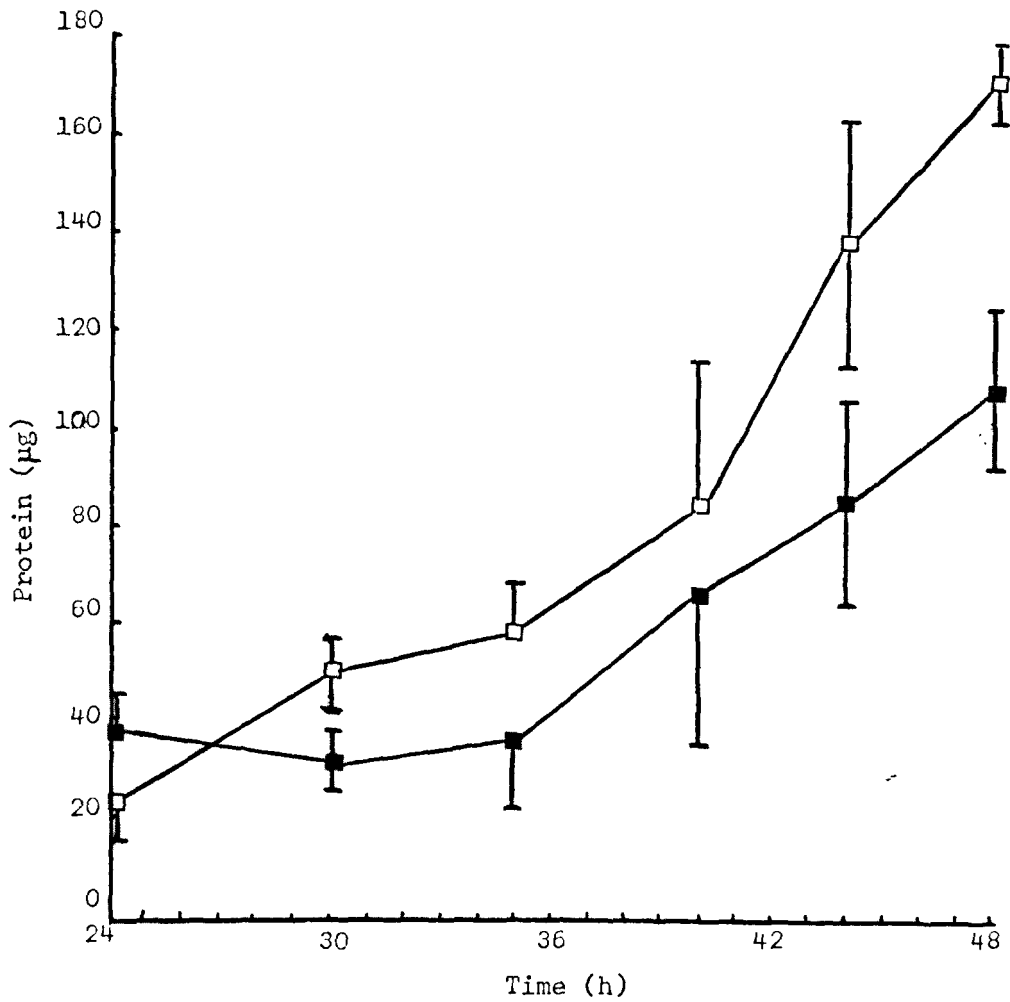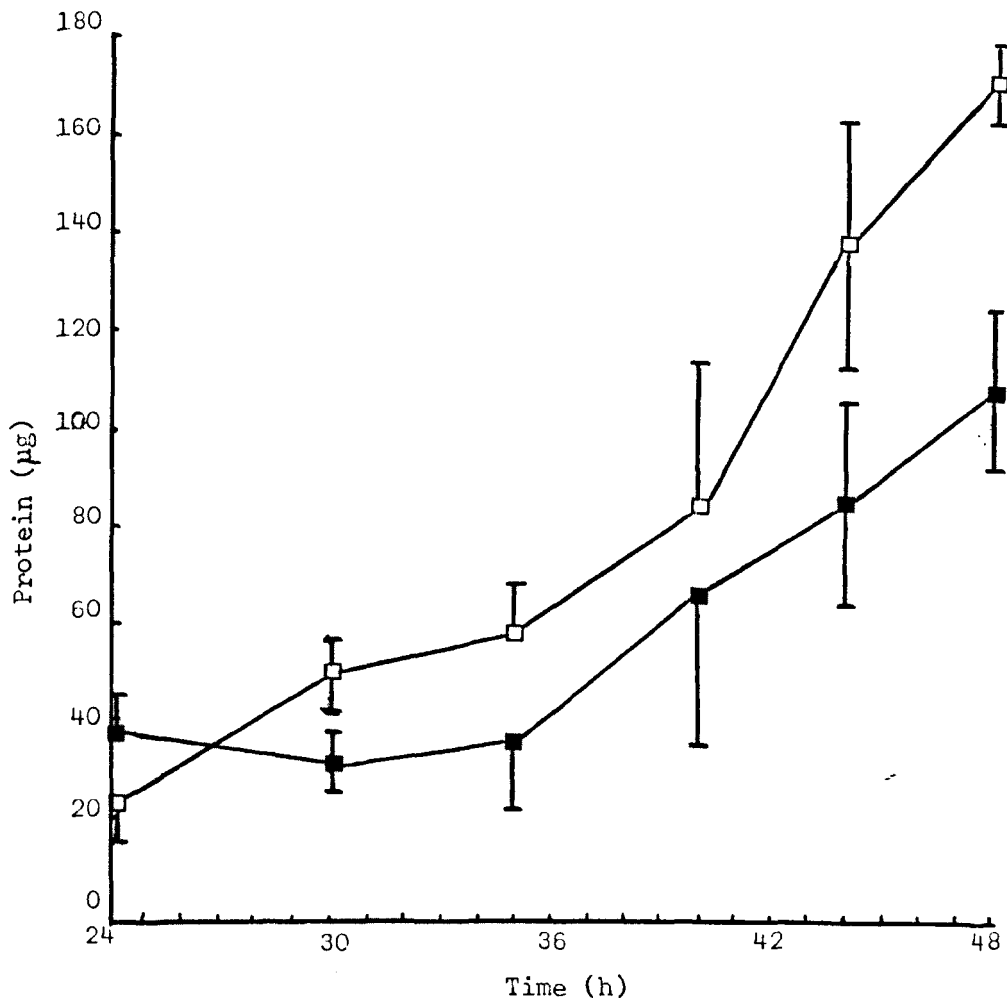
Figure 2.1   Increase in protein content of yolk sac (■), and embryo (□) of conceptuses cultured in vitro. Each point represents mean (+ standard deviat S.D.) of at least 6 determinations.

Figure 2.1 Increase in protein content of yolk sac (■), and embryo (□) of conceptuses cultured in vitro. Each point represents mean (+ standard deviation S.D.) of at least 6 determinations.
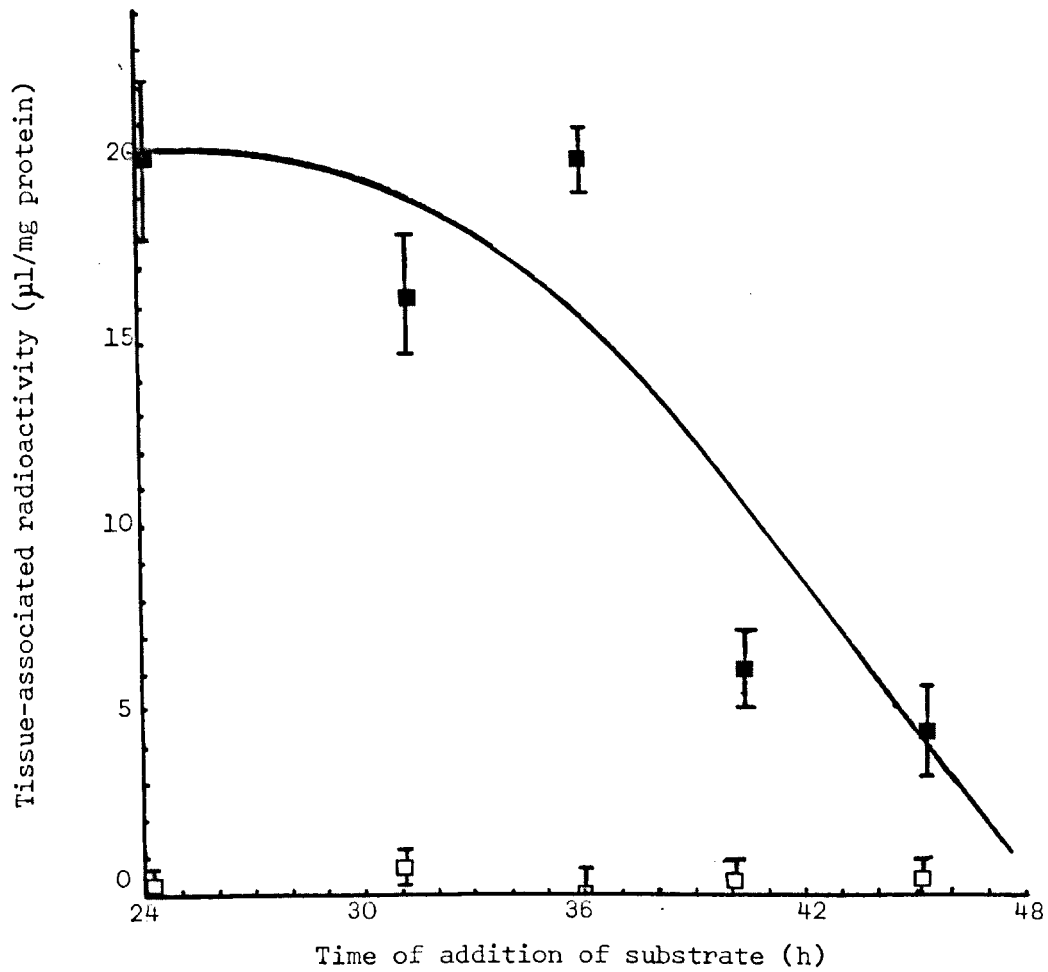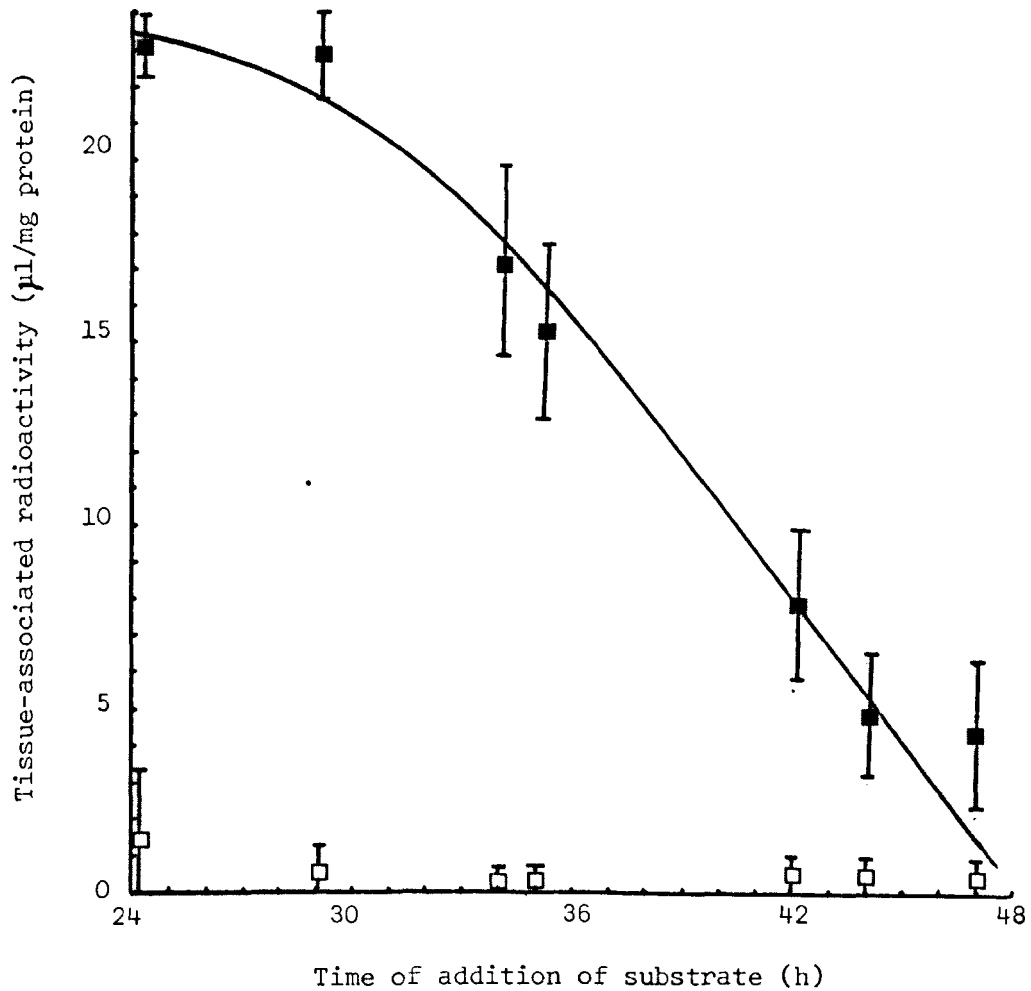
Figure 2.2 Radioactivity associated with yolk sac(■), and embryo (□)
after incubation of conceptuses in the presence of ^{125}I-PVP (1.35 μg/ml).
Each point represents mean (+ S.D.) of at least 6 determinations.

Figure 2.3 Radioactivity associated with yolk sac (■), and embryo (□)
after incubation of conceptuses in the presence of ^{125}I-PVP (2.8 μg/ml).
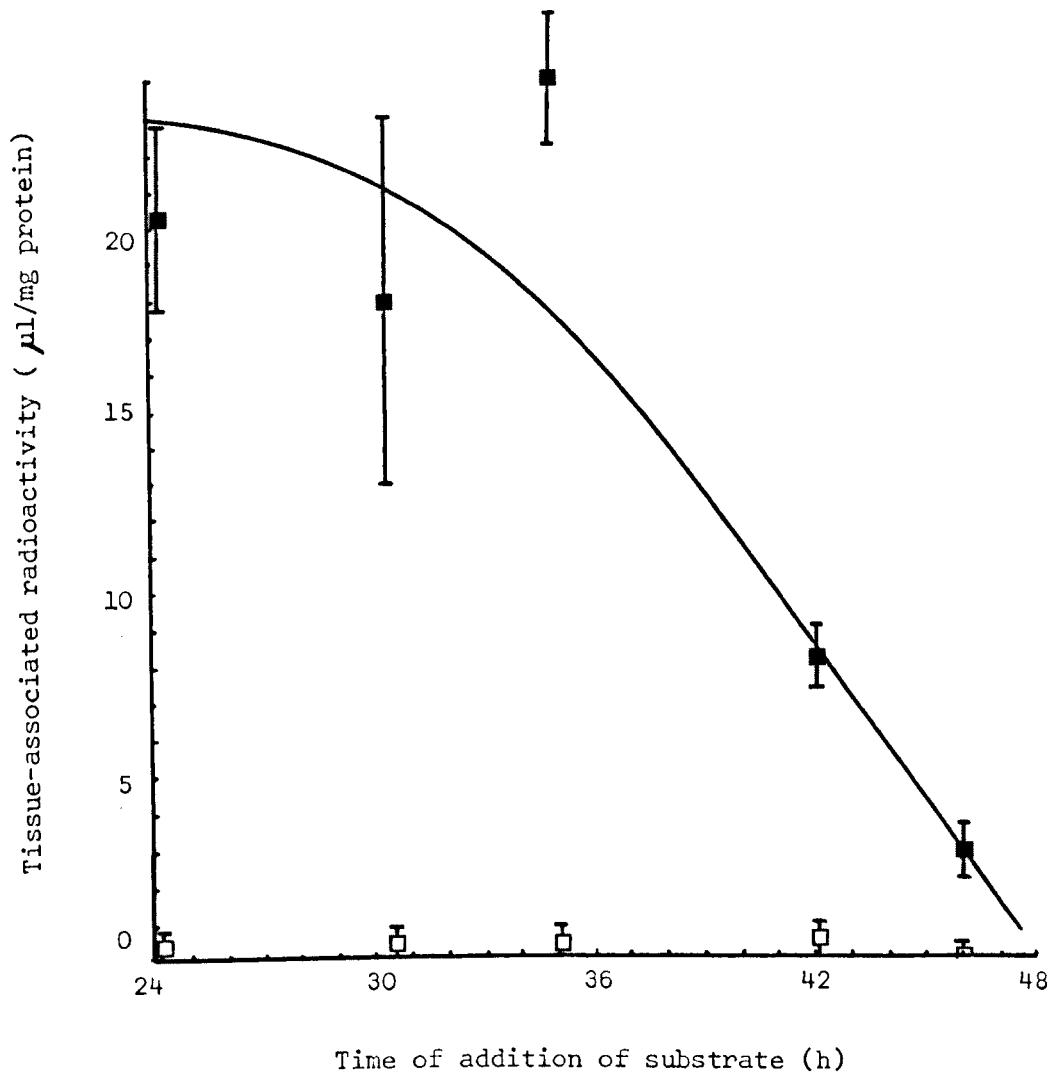Each point represents mean (± S.D.) of at least 6 determinations.

Figure 2.4 Radioactivity associated with yolk sac (■), and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (5.6 µg/ml). Each point represents mean (± S.D.) of at least 6 determinations

Figure 2.5 Radioactivity associated with yolk sac (■), and embryo(□) after incubation of conceptuses in the presence of $\left[^3H\right]$ dextran (2 μg/ml) Each point represents mean (± S.D.) of at least 6 determinations.
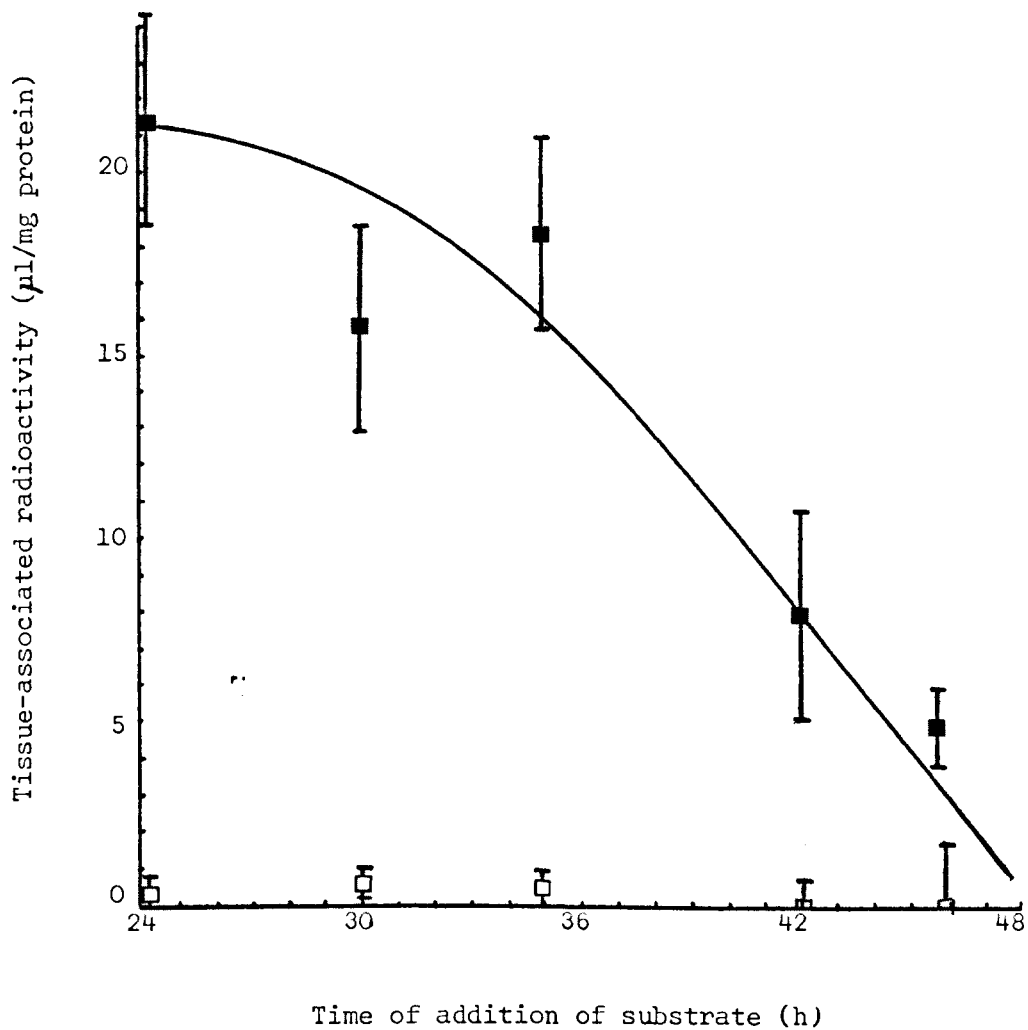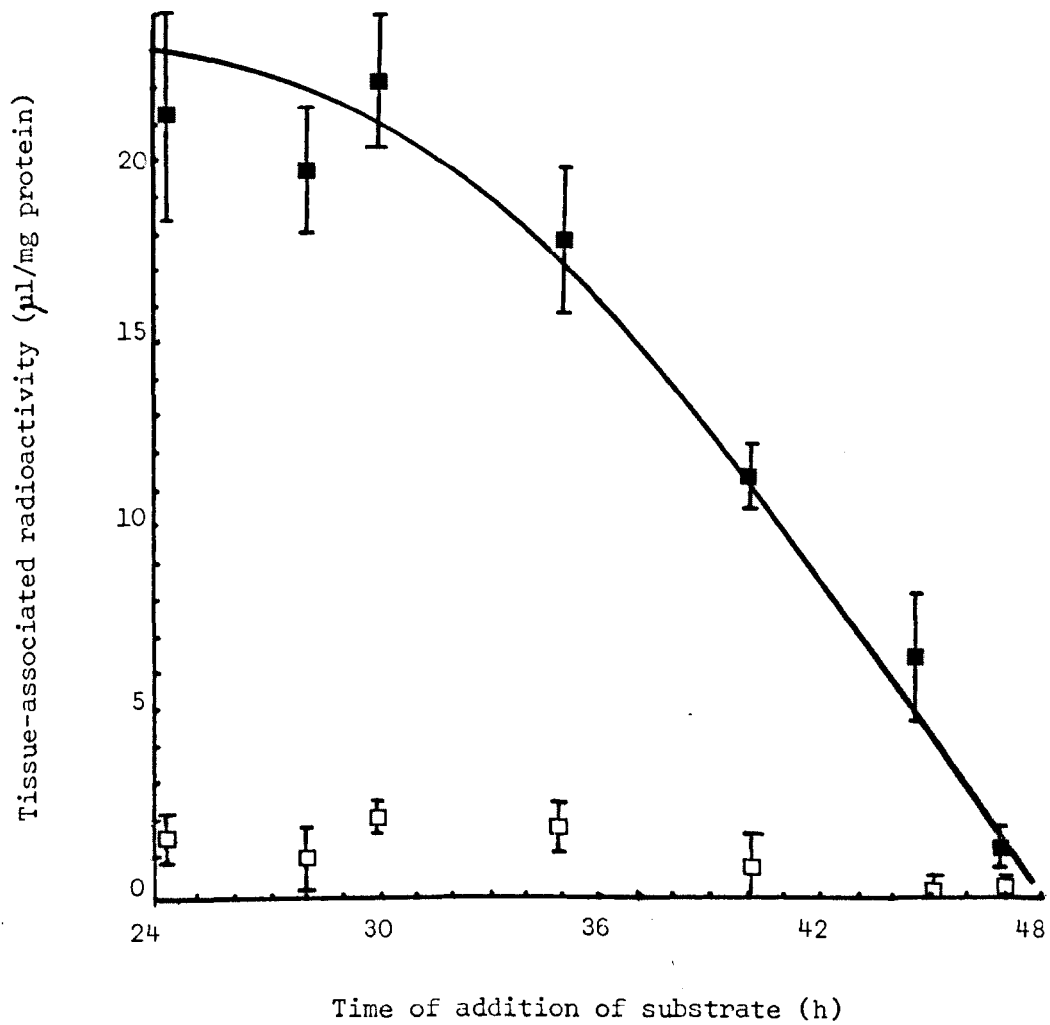
Figure 2.6 Radioactivity associated with yolk sac (■), and embryo (□) after incubation of conceptuses in the presence of $[^3H]$dextran (5 μg/ml). Each point represents mean ($\pm$ S.D.) of at least 6 determinations.

Figure 2.7 Radioactivity associated with yolk sac (■), and embryo (□) after incubation of conceptuses in the presence of [^{3}H]dextran (10 μg/ml). Each point represents mean (± S.D.) of at least 6 determinations.
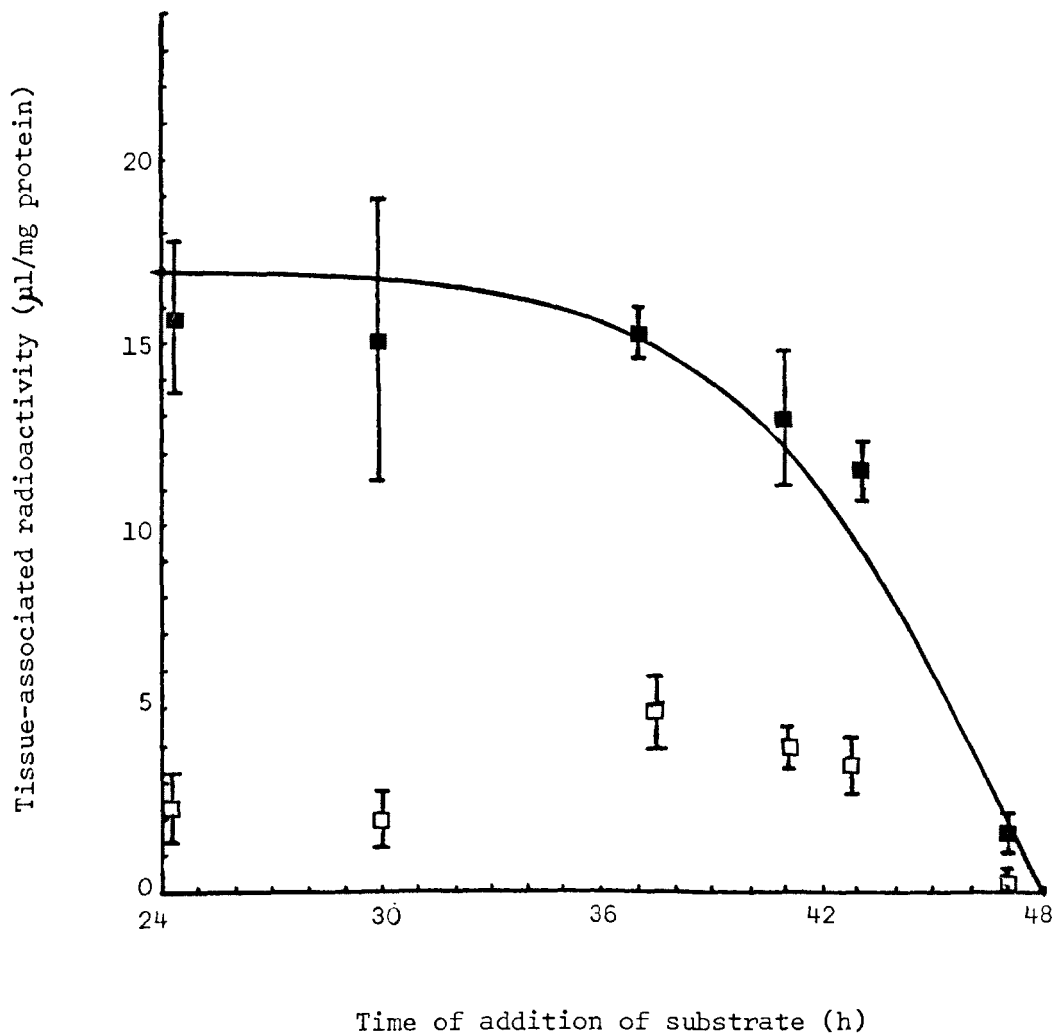
was roughly proportional to the duration of exposure to ^{125}I-PVP, with the exception that little difference was seen between tissues incubated with radiolabel for 24 hours and those in which radiolabel was added at 30 hours.

Essentially identical data were obtained at each of the three different concentrations of ^{125}I-PVP used.

2.4.3 Uptake of [^{3}H] dextran by Embryos and Yolk Sacs

Figures 2.5, 2.6 and 2.7 show how the amount of radioactivity found in the embryo and yolk sac at 48 hours varied with the time at which [^{3}H] dextran, at final concentrations of 2, 5 and 10 μg/ml respectively, was added to the culture medium. The pattern of uptake was essentially the same as that observed with ^{125}I-PVP as substrate. Accumulation of radio-activity in the yolk sac was slight between 24 hours and about 30 hours of culture and became apparently linear with time after about 30 hours of culture until harvesting. However, in contrast to the ^{125}I-PVP data, some radioactivity, but not progressive accumulation, was detected in embryos, at the two highest concentrations of [^{3}H] dextran employed. Excluding the unlikely possibility of contamination of embryos with yolk sac that had been incompletely separated following culture, two reasons for this observation suggest themselves. Either the [^{3}H] dextran preparation supplied contained a small percentage of [^{3}H] glucose, or the yolk sac is able to digest [^{3}H] dextran at a low rate, releasing [^{3}H] glucose which is able to diffuse into the embryonic tissues.

2.5 Discussion

The accumulation of ^{125}I-PVP by the yolk sac demonstrates the pino-cytic capacity of the early organogenesis-stage yolk sac. The inability

of this substrate to penetrate into the embryo clearly indicates
that the embryonic cells do not capture the macromolecule directly
and also that indigestible macromolecules captured by the yolk sac
are immobilised within this tissue, presumably within lysosomes,
and are not transferred into the embryo.

Yolk sac accumulation of $[^3H]$ dextran follows a distinctly similar
pattern to that observed with ^{125}I-PVP, once more demonstrating that
the yolk sac at this early stage in organogenesis has the ability to
ingest very large molecules pinocytically. It is most unlikely,
though not entirely impossible, that the low and irregular level of
embryonic radioactivity observed in figures 2.6 and 2.7 is the
result of a direct penetration of $[^3H]$ dextran.

These observations have several implications for histiotrophic
nutrition. If the early organogenetic embryo is dependent to any
major extent on macromolecular nutrient, then the yolk sac clearly
has an important role to play in mediating the nutritional supply.
The postulated mechanism of histiotrophic nutrition by the visceral
yolk sac (Beck et al., 1967a; Williams et al., 1976) requires that
this tissue is capable of pinocytosis. This has repeatedly been shown
to be so with the 17.5-day rat yolk sac in culture (Williams et al.,
1975a; Roberts et al., 1977; Duncan and Lloyd, 1978), where ^{125}I-PVP
uptake by yolk sac was linear with time, but has not hitherto been
demonstrated in the yolk sac from early organogenesis when histio-
trophic nutrition is presumed to be of most importance to the embryo.
The data reported here indicate that the early organogenesis-stage
yolk sac is capable of pinocytosis.

An equal, if not more crucial, requirement of the histiotrophic
nutritional mechanism is that macromolecules are not taken directly

into the embryonic tissues. The data show that this is indeed the case for ^{125}I-PVP and $[^3H]$ dextran and furthermore, ^{125}I-PVP and $[^3H]$ dextran pinocytosed by the yolk sac, are not transferred intact into the embryo. These data in themselves do not constitute proof that histiotrophic nutrition according to the mechanism of Beck et al. actually takes place. Rather, they dispel some of the scepticism that has surrounded the hypothesis, and as such provide direct biochemical support for the process.

An examination of the kinetics of the uptake of ^{125}I-PVP and $[^3H]$ dextran by the yolk sac reveals some interesting facts. The time course of uptake of either substrate at all the concentrations used is almost identical. The amount of radioactivity present in the yolk sac at harvesting depended on the length of time the conceptus had been exposed to radiolabel. A linear relationship between the amount captured and duration of exposure would not be expected, owing to the rapid growth of the tissue during the incubation period (Figure 2.1). Figures 2.2 - 2.7 show that there was little difference in tissue radioactivity at harvesting when the radiolabel was added at 24 hours or 30 hours of culture. However, to infer that the yolk sac is pinocytically inactive during this early phase of exposure would be erroneous. Figure 2.1 indicates that the amount of protein contained in the yolk sac between 24 and about 30 hours of culture was relatively small compared to later stages of culture. As the protein content of the yolk sac increased, apparently linearly, from about 30 hours of culture to 48 hours of culture, so the rate of pinocytic capture of substrates by yolk sac likewise increases. It is noteworthy that the pattern of glucose utilisation by rat conceptuses cultured by the New method (Sanyal, 1980) varied in a similar fashion to the pinocytosis

of non-digestible radiolabelled substrates. Culturing 10.5-day rat

conceptuses, it was observed that little depletion of medium glucose

occurred between 0 and about 9 hours of culture (equivalent to 24

and 33 hours in the present study). After this time glucose was

rapidly utilised by the conceptuses up to 24 hours of culture

(48 hours here). This pattern of glucose utilisation coincides with

the increase in protein content of yolk sac and embryos shown in

Figure 2.1, and the increase in pinocytic rates of yolk sacs shown

in Figures 2.2 - 2.7.

In the 17.5-day yolk sac uptake of ^{125}I-PVP is by fluid-phase non-

adsorptive pinocytosis (Williams et al., 1975a; Roberts et al., 1977).

Since the rates of uptake of $\left[^{3}H\right]$ dextran and ^{125}I-PVP in the present

experiments are quantitatively similar, when expressed as clearances,

it is likely that adsorption to plasma membrane does not occur with

either substrate in the 9.5-11.5 day yolk sac. This conclusion is

strengthened by the observation that uptake of both substrates

appeared to be independent of substrate concentration.

The linear uptake of both ^{125}I-PVP and $\left[^{3}H\right]$ dextran by the yolk sac

over the last 12-15 hours of culture enables a reliable rate of

uptake of substrate to be calculated. Such a rate, termed the Endocytic

Index by Williams et al. (1975a, b) allows direct numerical comparisons

to be drawn between experiments performed with the same substrate at

the same or different concentrations in the culture medium, and

between experiments employing different pinocytic substrates.

The mean Endocytic Index of all experiments using ^{125}I-PVP as pinocytic

substrate was 1.387 μl/mg protein/hour and compares well with the mean

Endocytic Index of $\left[^{3}H\right]$ dextran of 1.272 μl/mg/hour. These values,

though slightly lower, also compare well with the mean endocytic

indices of ^{125}I-PVP by 17.5-day yolk sacs of 1.74 μl/mg/hour reported by Williams et al., (1975a) and 1.57 μl/mg/hour reported by Roberts et al. (1977), suggesting that the pinocytic capacity of yolk sac is maintained at a similar level until late in gestation, and that in common with the 17.5-day yolk sac, these substrates are taken into the early organogenesis-stage yolk sac in the fluid-phase. The similarity between the computed Endocytic Indices for the experiments reported here indicates further that the culture technique is capable of providing reliable and reproducible quantitative biochemical data on the pinocytic mechanisms of the early organogenesis-stage conceptus.

CHAPTER THREE

THE DIGESTIVE CAPACITY OF THE YOLK SAC OF THE
EARLY ORGANOGENESIS-STAGE RAT CONCEPTUS

3.1   Introduction

The data presented in the last chapter demonstrated the pinocytic properties of the visceral yolk sac during early organogenesis. Pinocytosis by the yolk sac must function either to provide a barrier to potentially harmful macromolecular substances reaching the embryo, and/or as a mechanism by which macromolecules are gathered as a nutritional source for the embryo. In a nutritional process, degradation of the pinocytosed material within the yolk sac would be necessary since it has already been shown that exogenous macromolecules do not penetrate into the tissues of the embryo.

Digestion by the yolk sac during this early period of gestation has never been adequately demonstrated. Beck et al. (1967a) suggested that the disappearance of horseradish peroxidase from yolk sac cells during early organogenesis was due to digestion, and hence loss of enzymic activity, of the protein, although other explanations (see Section 1.1) account equally well for this observation. The presence of only two acid hydrolases, acid phosphatase and β-glucuronidase, has been demonstrated histochemically in the 9.5-11.5-day yolk sac (Bulmer, 1963; Beck et al., 1967a).

In contrast to the paucity of data for early organogenesis yolk sac, the 17.5-day rat yolk sac in culture has been shown to degrade pinocytosed protein to the level of amino acids (Williams et al., 1971; Williams et al., 1975b; Livesey and Williams, 1979). In addition to these studies which used ^{125}I-labelled bovine serum albumin as pinocytic and proteolytic substrate, the near-term yolk sac has been observed to degrade in vitro ^{125}I-human serum albumin (Goetze et al., 1975), ^{125}I-rat gamma globulins (Ibbotson, 1977), deglycosolated yeast invertase (Brown et al., 1979) and [14C]haemoglobin (Fridhandler

and Zipper, 1964). Also the activities of several lysosomal hydro-
lases have been demonstrated in homogenates of near-term rat
(Beck et al., 1967b; Lloyd et al., 1968) and rabbit (Jones and Hemmings,
1971) yolk sac.

The aim of the experiments described in this Chapter was to establish
the digestive capacity of the yolk sac from the early organogenesis
conceptus. A dual experimental approach was adopted. In the first
series of experiments a protein, formaldehyde-denatured ^{125}I-bovine
serum albumin (^{125}I-dBSA) was added to the culture medium in which
conceptuses were being cultured. The distribution of both tri-
chloroacetic acid-insoluble radioactivity (indicating intact radio-
labelled protein and -soluble radioactivity (indicating radiolabelled
breakdown products of protein) were measured in yolk sac and embryo
tissues, and in the culture medium. This particular protein was
chosen for two reasons. Firstly, it has been found that formaldehyde-
denatured BSA, when compared with native BSA, is rapidly ingested,
by adsorptive pinocytosis in the 17.5-day yolk sac in culture
(Moore et al., 1977). In the present study, the culture medium
comprised 100% homologous serum, and it was therefore desirable to use
a substrate which would be pinocytosed in measurable quantities in
the presence of a very high concentration of competing protein, as
was present in the culture medium. Secondly, since radioiodination
of BSA preferentially labels tyrosine residues and $\left[^{125}\text{I}\right]$ iodotyrosine
released upon hydrolysis of the protein is not accepted by any tRNA
(Alexander, 1964; Cartouzou et al., 1964), radiolabelled digestion
products are not re-utilised in de novo protein biosynthesis, thus
simplifying the quantitation of proteolysis.

The second approach used to assess the digestive capacity of yolk sac was by direct assay of several lysosomal hydrolases in yolk sac homogenates. Yolk sacs were obtained from 9.5-day conceptuses that had been cultured for 48 hours, and hydrolase activities were compared to those of 11.5-day yolk sacs removed directly from the mother.

3.2   Materials

Radiochemicals

Na $\begin{bmatrix} ^{125}I \end{bmatrix}$ iodide (preparation IMS.[30])   The Radiochemical Centre, Amersham,

Bucks,

Chemicals

Bovine serum albumin (-042t)   Koch-Light Laboratories Ltd, Coln-
                                                brook, Bucks

4-methylumbelliferyl-2-acetamido-   Koch-Light Laboratories Ltd, Coln-
  2-deoxy-B-D-glucopyranoside   brook, Bucks

4-methylumbelliferyl-D-glucoside   Koch-Light Laboratories Ltd, Coln-
                                                brook, Bucks

Chloramine-T   Sigma Chemical Co., Poole, Dorset

Benzoyl-D, L-Arginine-Napthylamide   Sigma Chemical Co., Poole, Dorset

Fast Garnet (4-amino-2;3-dimethyl-   Sigma Chemical Co., Poole, Dorset
  azobenzene)

Mersalyl Acid (2-$\begin{bmatrix} 3-(\text{hydroxymercuri}) \end{bmatrix}$ Sigma Chemical Co., Poole, Dorset
  -2-methoxypropyl$]$ carbamoyl phenoxy-
  acetic acid)

Brij 35 (polyoxyethylene lauryl ether) Sigma Chemical Co., Poole, Dorset

p-nitrophenyl phosphate   Boehringer-Mannheim Ltd, West

Germany

Equipment

Vertis homogeniser "45"   Vertis Research Equipment, New York,

                                                                    USA

3.3   Methods

3.3.1   Culture of Conceptuses in the Presence of ^{125}I-dBSA

3.3.1.1 Preparation of ^{125}I-dBSA

Radiolabelling of BSA was performed according to the method of Williams

et al. (1971). Bovine serum albumin (20 mg) was dissolved in 9.0 ml

of phosphate buffer (0.05 M Na_2HOP_4-KH_2PO_4, pH 8.0) and cooled in an

ice-bath. Sodium $\left[^{125}I\right]$ iodide (1 mCi) was added using a 1 ml disposable

syringe and the ampoule rinsed with phosphate buffer (1.5 ml) and the

washings added to the reaction vessel. After stirring for 2 minutes,

chloramine-T solution (4 ml of a 1 mg/ml solution) was added and the

reaction allowed to proceed for 8 minutes before being stopped by the

addition of sodium metabisulphite solution (3 ml of a 2 mg/ml solution).

After stirring for a further 2 minutes, solid potassium iodide (100 mg)

was added to the reaction mixture to aid displacement of unreacted

$\left[^{125}I\right]$ iodide during the subsequent dialysis. An equal volume of

formalin (10% formaldehyde in 0.5 M Na_2CO_3, pH 10) was added and the

resulting solution allowed to stand for 72h at 4°C (Moore et al., 1977).

The solution was then freed of inorganic iodide and formaldehyde by

dialysis (72h at 4°C) in Visking tubing against at least four changes

of aqueous sodium chloride (1%, w/v); the initial dialysate contained

0.05% sodium iodide to ensure complete removal of unreacted $\left[^{125}I\right]$

iodide from the reaction mixture. The protein concentration of the

final preparation was determined by absorbance at 280nm using a

standard curve prepared in saline (1%, w/v).

3.3.1.2 Culture Experiments

Cultures were established as already outlined (Section 2.3.1). ^{125}I-dBSA at a final concentration of 5.5 or 11.0 μg/ml was added to the culture medium at some time-point between 12 and 0.5 hours before harvesting. Uptake of substrate was measured during the last 12 hours of culture because only during this time is uptake demonstrably linear (see Chapter 1). At harvesting, conceptuses were solubilised, neutralised and diluted as described.

Following assay of 1.0 ml samples of tissue solutions for total radioactivity, 0.5 ml of 20% TCA and 0.1 ml of carrier protein (calf serum) was added. The solution was then centrifuged for 20 minutes at 2,000 g, the supernatant (1.5 ml) decanted into a fresh tube and its radioactivity re-measured to give the TCA-soluble count. Duplicate 1.0 ml fractions of culture medium were assayed for total and TCA-soluble radioactivity in the same way, but without carrier protein. TCA-insoluble radioactivity was determined by substracting the TCA-soluble counts from the total counts. Total radioactivity in each yolk sac and embryo was calculated by multiplying the observed count for that tissue by 1.1.

Owing to the increase in volume of the TCA-soluble count and the occlusion of some TCA-soluble material within the precipitate, the observed TCA-soluble count was multiplied by a correction factor to give a more accurate value. The correction factor was determined empirically as follows. To sextuplicate samples of either yolk sac solution, embryo solution or culture medium was added a small quantity of $\left[^{125}I \right]$ iodotyrosine (10^5 cpm) to give a final volume of 1.0 ml. The total and TCA-soluble radioactivity of each sample was measured as described above. Theoretically, these counts should be equal.

However, for the reasons stated, the TCA-soluble counts were seen to be lower than the total counts. The ratio of total radioactivity to TCA-soluble radioactivity, a value greater than unity, was the correction factor employed.

3.3.1.3 Quantitation of Uptake

When ^{125}I-dBSA is used as substrate, the yolk sac is found to degrade the pinocytosed protein to the level of its constituent amino acids. One of these, $\left[^{125}I\right]$ iodotyrosine, is largely released back into the culture medium. This digestion product appears as the TCA-soluble radioactivity which was estimated as described above. The equation expressing uptake, U, the quantity of radioactivity that would have accumulated in the yolk sac in the absence of any proteolysis, becomes:

$$U = \frac{Y + (S-F)}{M \times Py}$$

As with non-digestible substrates, the numerator represents the total radioactivity taken up by the yolk sac. In the case of a digestible substrate such as a protein this is the sum of two terms; Y, the total radioactivity taken up and retained in the whole yolk sac (c.p.m., corrected for background) and (S-F), the TCA-soluble radioactivity generated by the yolk sac and released into either the culture medium or the embryo. S is the sum of the TCA-soluble radioactivity found in the culture medium and the embryo at harvesting (c.p.m., corrected for background). F is the amount of TCA-soluble radioactivity present that has been generated elsewhere than in the yolk sac, *i.e.* TCA-soluble radioactivity present initially in the substrate preparation and any TCA-soluble radioactivity generated by proteolysis within the

culture serum. This latter component was shown to be essentially
zero (Figure 3.1) and F was therefore determined by measuring the
TCA-soluble radioactivity in the substrate preparation before
culture.

Since the only radioactivity found in the embryo was TCA-soluble,
the yolk sac was considered to be the only site of pinocytosis (and
degradation) of the radiolabelled protein. The expression of uptake
of radioactivity into the embryo was, therefore,

$$U = \frac{E}{M \times Pe}$$

3.2.2   Enzyme Analyses of 11.5-day Yolk Sac
3.2.2.1 Preparation of Yolk Sacs for Assay

Yolk sacs were obtained either from 9.5-day conceptuses cultured for
48 hours (gestational age at harvesting = 11.5 days) or direct from
the mother at 11.5 days of gestation. All yolk sacs from a single
mother were explanted into an ice-cold 3 ml disposable tube and
washed by centrifugation in 1 ml of distilled water at 2,000 g at
4°C for 10 minutes. The washings were discarded and yolk sacs were
homogenised in 1 ml of distilled water in a Virtis homogeniser. The
resulting yolk sac homogenate was used immediately in enzyme assays.
Duplicate 0.05 ml portions of homogenate were assayed for total protein
as described in Section 2.3.4.

3.2.2.2 Measurement of Acid Phosphatase

Acid phosphatase was assayed by the method of Torriani (1960) using
20mM p-nitrophenyl phosphate as substrate in 0.2 ml of citrate buffer,
pH 5.0 (0.1M citric acid, pH adjusted with 1 M NaOH). To this was
added 0.05 ml of yolk sac homogenate and the mixture was incubated

at 37°C for 4 hours. The reaction was terminated by the addition

of 2.75 ml of Tris-phosphate buffer, pH 8.5 (0.4M Tris and 0.4 M

sodium dihydrogen phosphate, pH adjusted with 1 M NaOH). Liberated

p-nitrophenol was measured at 400nm. A standard curve was drawn

of absorbances of different concentrations of p-nitrophenol in

Tris-phosphate buffer, and was used to calculate the extent of

substrate hydrolysis by the yolk sac homogenate. Blanks were performed

by incubation of substrate and homogenate separately, mixing immediately

prior to addition of Tris-phophate buffer. Specific enzyme activity

was expressed as nmoles of p-nitrophenol released per hour per mg

of homogenate protein.

3.2.2.3 Measurement of N-acetyl-β-glucosaminidase

N-Acetyl-β-glucosaminidase was assayed using the method described by

Griffiths et al. (1978) using 2.5 mM 4-methylumbelliferyl-2-acetamido-

2-deoxy-β-D-glucopyranoside as substrate in 0.1 ml of citrate-phosphate

buffer, pH 4.3 (0.1 M sodium citrate adjusted to the appropriate pH

with 0.2 M sodium phosphate). To this was added 0.05 ml of a 1 in 10

dilution of yolk sac homogenate and the mixture was incubated for

10 minutes at 37°C. The reaction was terminated by the addition

of 2.85 ml of 0.17 M glycine-sodium carbonate buffer, pH 10.5. The

fluorescence of liberated 4-methylumbelliferone was determined

using an excitation wavelength of 365 nm and an emission wavelength

of 450 nm. A standard curve was drawn of fluorescence of different

concentrations of 4-methylumbelliferone and was used to calculate the

extent of substrate hydrolysis by the homogenate. Blanks were

performed as described for acid phosphatase. Specific enzyme activity

was expressed as nmoles of 4-methylumbelliferone released per hour

per mg homogenate protein.

3.2.2.4 Measurement of ⍺-glucosidase

⍺-Glucosidase was assayed using the method described by Griffiths et
al. (1978) using 5 mM 4-methylumbelliferyl-⍺-D-glucoside as substrate
in 0.1 ml of citrate-phosphate buffer, pH 5.2. To this was added
0.05 ml of homogenate and the mixture was incubated at 37°C for 30
minutes. Other procedures were as for N-acetyl-β-glucosaminidase.
Specific enzyme activity was expressed as nmoles of 4-methylumbelliferone
released per hour per mg homogenate protein.

3.2.2.5 Measure of Cathepsin D

Cathepsin D was assayed by the method described by Barrett and Heath
(1977) using bovine haemoglobin as substrate. Haemoglobin powder
was mixed to a paste with water, dialysed for 2 days against distilled
water at 4°C, centrifuged and diluted to 8.0% (w/v) solids for storage
at -20°C. To 0.05 ml of haemoglobin substrate was added 0.1 ml of
homogenate and 0.05 ml of assay buffer, prepared by mixing 1.0 M sodium
formate with 1.0 M formic acid as required to give a pH of 3.0 (rising
to 3.2 in the incubation mixture), and the mixture was incubated
at 45°C for 60 minutes. The reaction was terminated by adding 1.0 ml
of 3% (w/v) trichloroacetic acid. After 5 minutes the mixture was
centrifuged at 2,000 g for 20 minutes and 1.0 ml of the clear super-
natant was transferred to a clean tube. To it was added 2.5 ml of
modified alkaline copper reagent which was prepared as follows.
Stock (a) contained 1.0 g of trisodium citrate dihydrate with 0.50 g
of $CuSO_4.5H_2$) in 100 ml of water. Stock (b) contained 16.0 g of NaOH
with 50 g of Na_2CO_3 in 500 ml of water. Working solution was prepared
by adding 1.0 ml of stock (a) to a 100 ml measuring cylinder and
diluting to 80 ml with water and making up to 100 ml with the addition of
20 ml of stock (b). Following addition of the modified alkaline copper

reagent, the mixture was allowed to stand for 10 minutes at room temperature. To this was added 0.25 ml of diluted Folin-Ciocalteau reagent (1:2 with distilled water) with immediate mixing. After 30 minutes the absorbance of the blue solution was measured at 700 nm. Standards were made containing different dilutions of tyrosine in 2.5% (w/v) TCA and a standard curve was drawn to calculate the extent of substrate hydrolysis by homogenate. Blanks were performed by delaying the addition of the homogenate until the end of the incubation, mixing it in just before the addition of TCA. Enzyme activity was expressed as nmole of tyrosine released per hour per mg homogenate protein.

3.2.2.6 Measurement of Cathepsin B

Cathepsin B was assayed by the method of Barrett (1972) using benzoyl-D, L-arginine-2-naphthylamide hydrochloride as substrate in dimethylsulphoxide (40 mg/ml). To 0.1 ml of homogenate was added 0.3 ml of buffered activator. The latter was prepared by dissolving 32 mg of cysteine in 100 ml of EDTA-phosphate buffer (KH_2PO_4, 12.0 g; $Na_2HPO_4.12H_2O$, 4.3 g; disodium $EDTA.2H_2O$, 0.495 g), and making up to 1 litre with distilled water to a pH of 6.0. The mixture was incubated for 5 minutes at 40°C before adding 0.01 of substrate stock with mixing and incubating for 10 minutes at 40°C. Substrate hydrolysis yielded 2-naphthylamine which was assayed colorimetrically by coupling with a diazonium salt, Fast garnet. The coupling reagent was added to the incubation mixture to terminate the reaction. (Termination was effected by a mercurial compound included in the coupling reagent). The coupling reagent was made up of two solutions, a base stock solution and Mersalyl-Brij reagent. Base stock solution was prepared by dissolving 225 mg of Fast garnet (4-amino -2', 3-dimethylazobenezene) in 50 ml of

ethanol. To this was added 30 ml of M HCI with stirring, and the solution diluted to 100 ml with distilled water. Mersalyl-Brij reagent was prepared by dissolving 2.43 g of Mersalyl acid (2-[3-(hydroxymercuri) -2-methoxypropyl] carbamoyl phenoxyacetic acid) in 60 ml of 0.5 M NaOH. To this was added 0.3 g of disodium EDTA and the solution diluted to 450 ml with distilled water, adjusting the pH to 4.0 by slow addition of M HCI before making up to a final volume of 500 ml with water. To this solution was added 500 ml of 4% (w/v) Brij 35 (polyoxyethylene lauryl ether) in water. The coupling reagent was prepared as follows: 1.0 ml of base stock solution was pipetted into a test-tube standing in a beaker of ice and water. To it was added 0.1 ml of 0.2 M $NaNO_2$ and the mixture was left to stand for 5 minutes before diluting with Mersalyl-Brij reagent to a final volume of 100 ml.

After adding the coupling reagent to the incubation mixture colour was allowed to develop for 10 minutes before measuring the absorbance of the final solution at 520nm. Standards were prepared by replacing the homogenate of the original incubation mixture with 0.1 ml of a solution of 2-naphthylamine. Blanks were performed by adding the homogenate, which had been incubated separately, after the coupling reagent. Specific enzyme activity was expressed as nmoles of 2-naphthylamine released per hour per mg of homogenate protein.

3.4   Results

3.4.1   Culture Experiments

The amount of radioactivity present in yolk sacs and embryos at harvesting following culture in the presence of 5.5 or 11.0 $\mu g/ml$ ^{125}I-dBSA is shown in Figures 3.2 and 3.3 respectively. The results are essentially identical at both concentrations used. In the yolk sac the level of radioactivity was independent of duration of
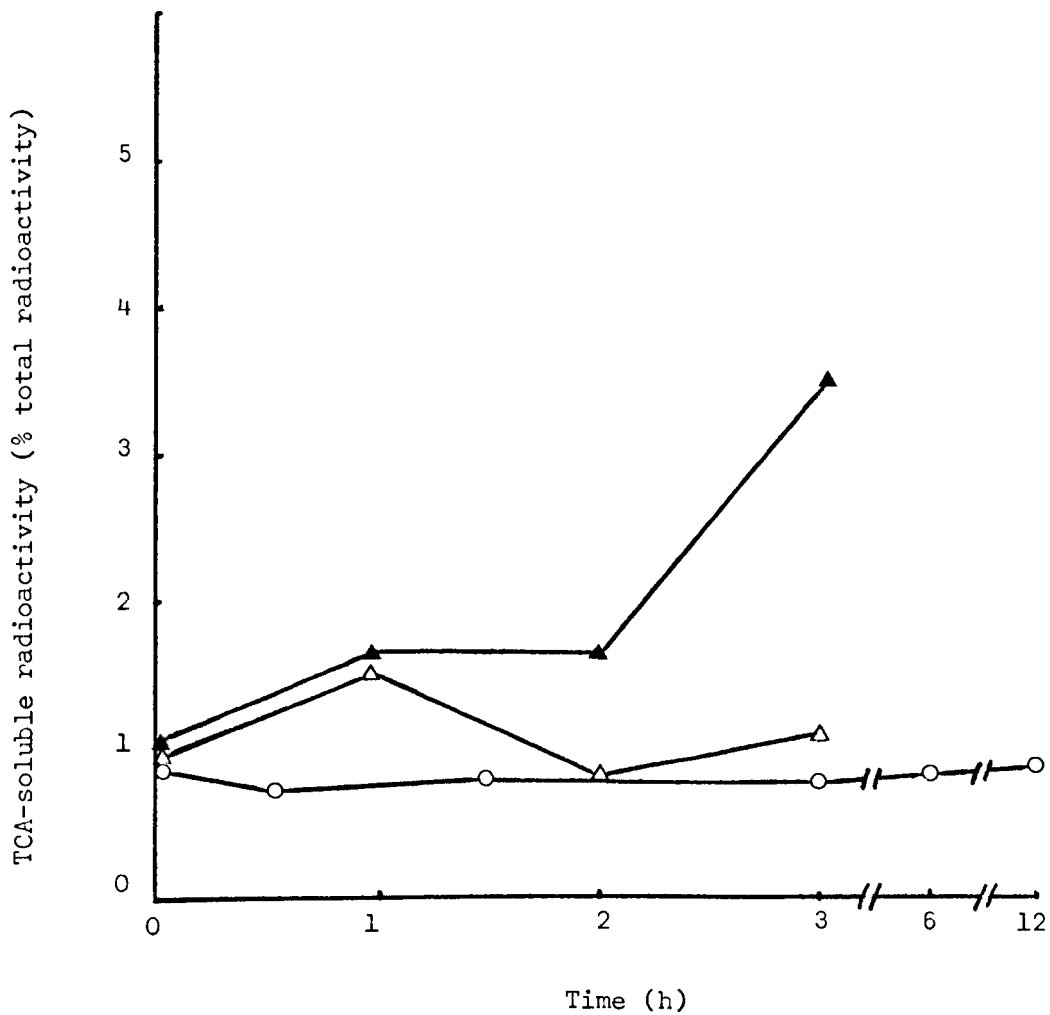
Figure 3.1 Increase in TCA-soluble radioactivity after incubation at 37°C in roller culture apparatus of ^{125}I-dBSA in 0.25 M NaOH (▲), water (△), and heat-denatured whole rat serum (O). Each point represents mean of at least 4 separate determinations.
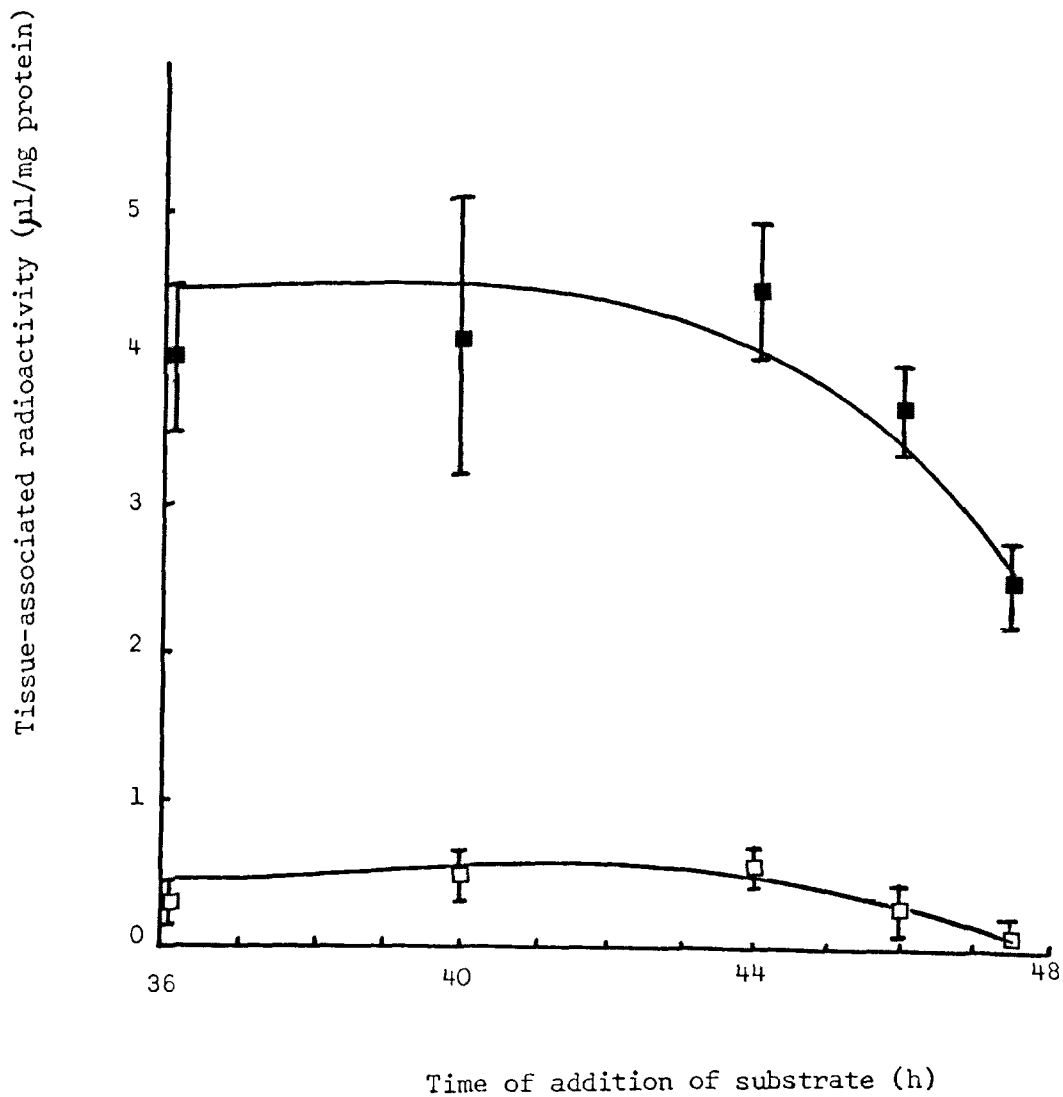
Figure 3.2 Radioactivity associated with yolk sac (■), and embryo (□) after incubation of conceptuses in the presence of ^{125}I-dBSA (5.5 μg/ml). Each point represents mean ($\pm$ S.D.) of at least 6 determinations.
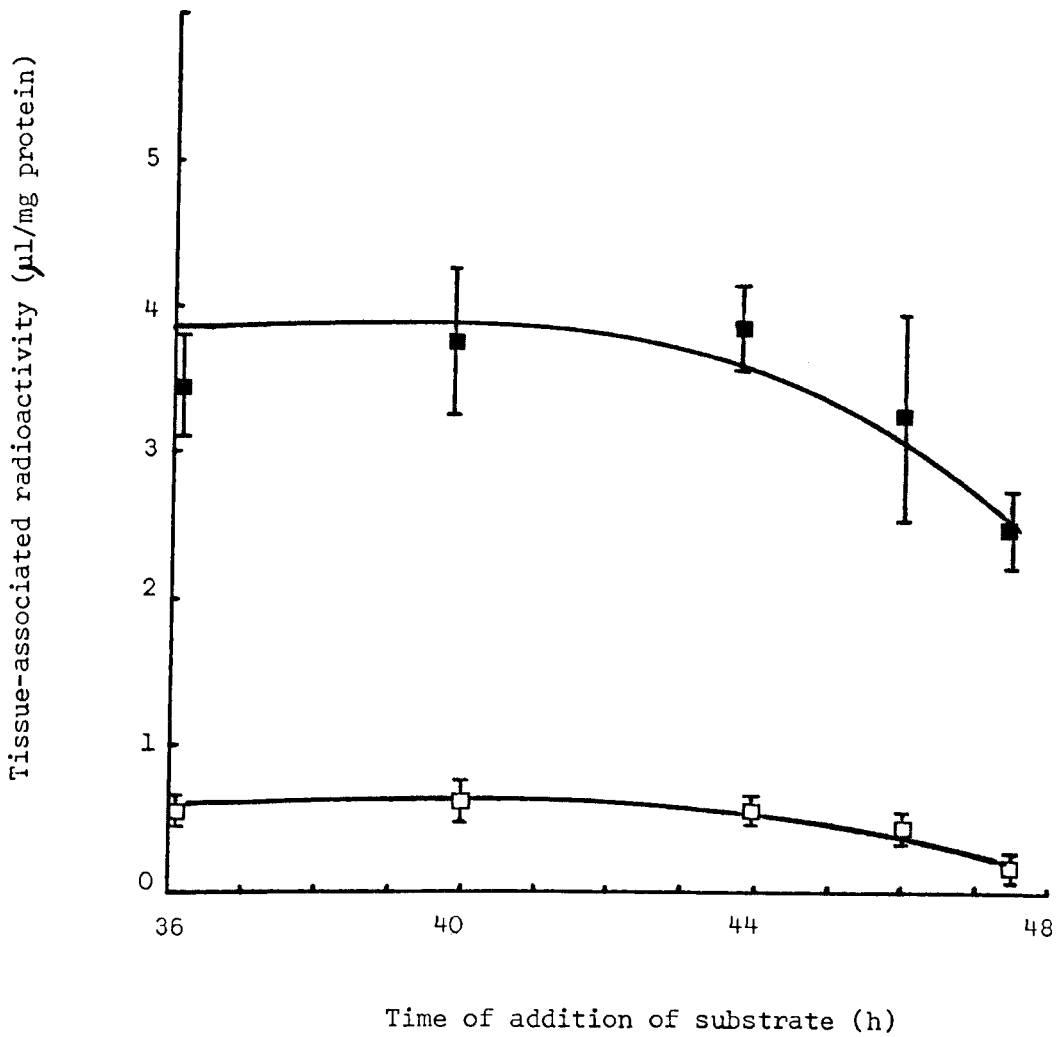
Figure 3.3 Radioactivity associated with yolk sac (■), and embryo(□) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml). Each point represents mean (± S.D.) of at least 6 determinations.

exposure to ^{125}I-dBSA, except for durations of exposure of less than 2 hours. A qualitatively similar pattern was observed for radioactivity in the embryo, though at a much lower level. The percentage of yolk sac and embryo radioactivity that was TCA-soluble is shown in Figures 3.4 and 3.5. The small quantity of radioactivity reaching the embryo is all in TCA-soluble form. The percentage TCA-soluble radioactivity in the yolk sac was, like total yolk sac radioactivity, independent of duration of exposure to radiolabelled protein, except where this duration was less than 2 hours, and levelled off at about 50%.

Analysis of the medium at harvesting revealed that the amount of TCA-soluble radioactivity had progressively increased with time of exposure of conceptuses to substrate. This TCA-soluble radioactivity must have been released from the tissues following hydrolysis of radiolabelled protein, and must properly be included in any calculation of uptake. The pattern of total uptake of radioactivity by yolk sacs, including that which is retained within the yolk sac tissue and that released back into the medium and the embryo, is shown in Figures 3.6 and 3.7. Uptake was linear over the 12 hours. That this TCA-soluble radioactivity was generated by conceptuses is clearly shown in Figure 3.1. In the absence of conceptuses, there was essentially no increase in TCA-soluble radioactivity in the culture medium with time.

The solubilisation of tissues after harvesting involves digestion of the tissues in 0.25 M NaOH. Since peptide bonds are alkali-labile, it was important to measure the effect of NaOH on the stability of ^{125}I-dBSA. Hydrolysis of tissue-associated radiolabelled protein during tissue solubilisation would lead to a falsely high percentage of TCA-soluble radioactivity. Figure 3.1 shows that only after 1 hour of incubation of 0.1 ml ^{125}I-dBSA with 0.5 ml 0.25 M NaOH at 37°C does
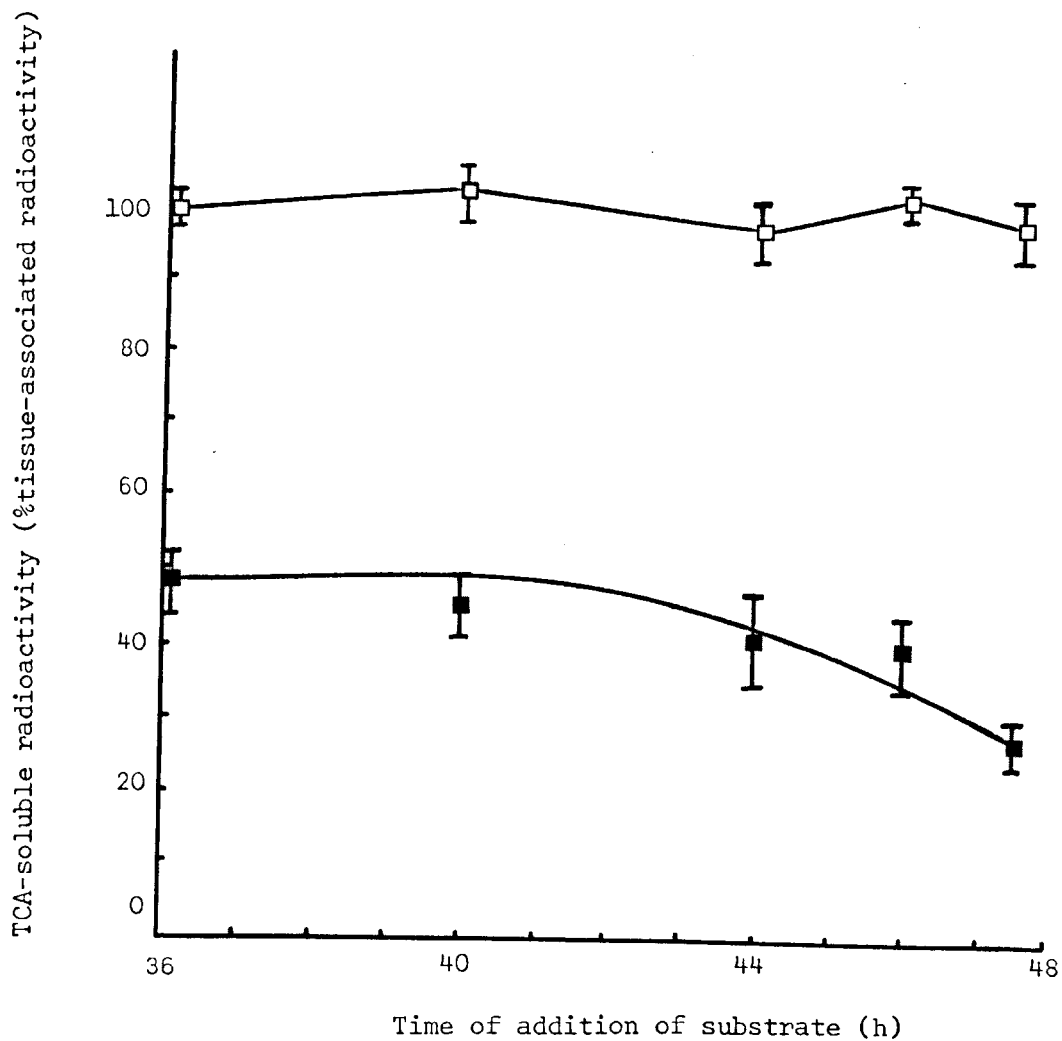
Figure 3.4 Percentage of tissue radioactivity soluble in TCA
after incubation of conceptuses in the presence of ^{125}I-dBSA (5.5 µg/ml)
Each point represents mean ($\pm$ S.D.) of at least 6 determinations.
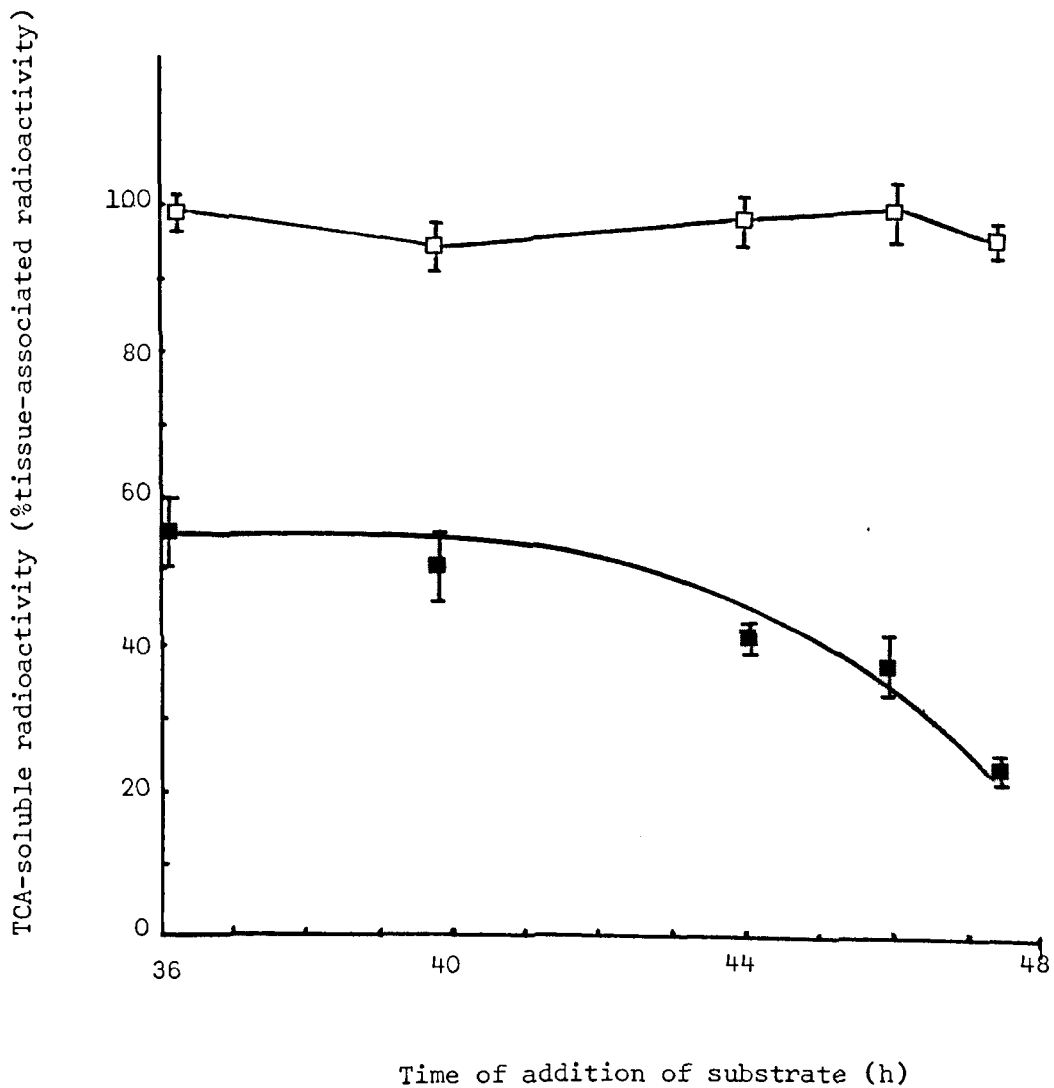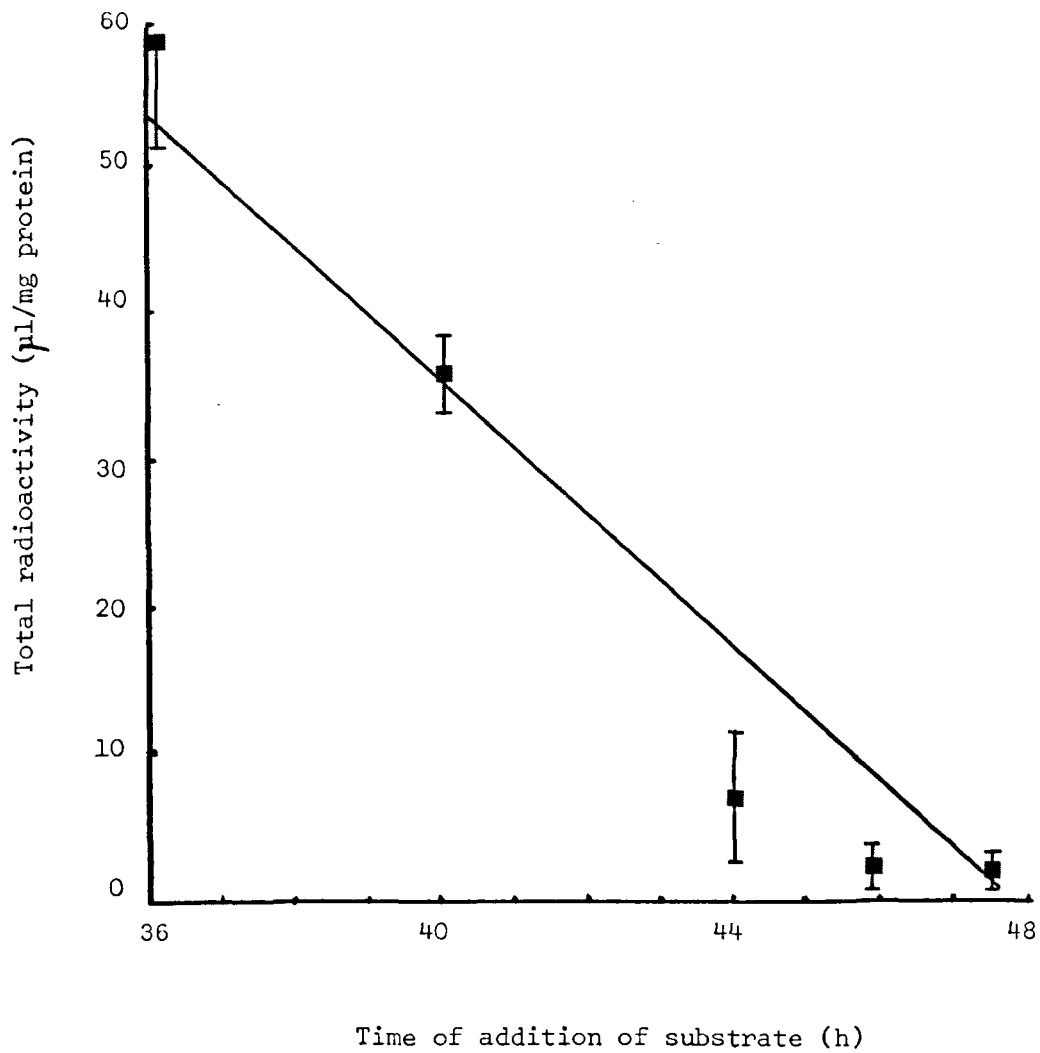(■) yolk sac, (□) embryo

Figure 3.5 Percentage of tissue radioactivity soluble in TCA after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml). Each point represents mean ($\pm$ S.D.) of at least 6 determinations. (■) Yolk sac, (□) embryo.
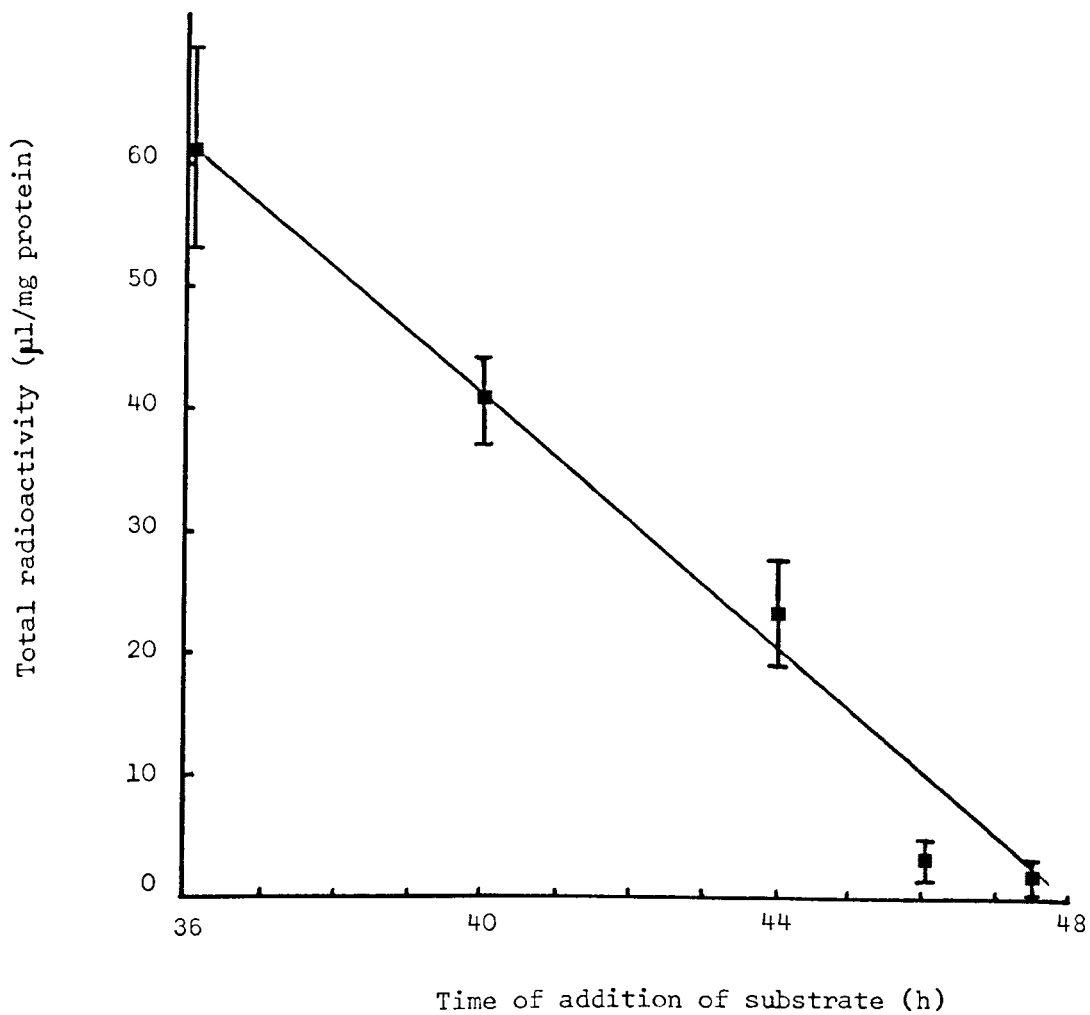
Figure 3.6 Total radioactivity taken up by yolk sac after incubation of conceptuses in the presence of ^{125}I-dBSA (5.5 µg/ml). Each point represents mean ($\pm$ S.D.) of at least 6 determinations.

Figure 3.7 Total radioactivity taken up by yolk sac after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml). Each point represents mean ($\pm$ S.D.) of at least 6 determinations.

Enzyme	CULTURED YOLK SAC		NON-CULTURED YOLK SAC	
	Specific Activity ± SE	Range of Activity	Specific Activity ± SE	Range of Activity
α-glucosidase	37.00 ± 9.09	23.33 – 46.67	49.35 ± 11.93	35.17 – 62.50
N-Acetyl-β-glucosaminadase	2372.31 ± 302.99	2025.5 – 2852.0	2818.71 ± 772.88	1948.17 – 3574.2
Acid phosphatase	1771.20 ± 240.19	1425 – 2067	2005.60 ± 148.57	1831.0 – 2237.0
Cathepsin D	604.4 ± 81.10	543 – 738	449.0 ± 55.1	408 – 543.0
Cathepsin B	989.00 ± 204.00	798 – 1129	856.0 ± 26.0	818.0 – 885.0

Table 3.1   Comparison of lysosomal hydrolase activities in cultured and non-cultured 11.5-day yolk sac. Each specific activity is the mean of groups of yolk sac from 5 animals. Units of activity are described in the text.

the increase in percentage TCA-soluble radioactivity in the mixture begin to depart from control values, obtained by incubating ^{125}I-dBSA in water.

3.4.2. Enzyme Analyses

Table 3.1 shows the specific activities of five lysosomal enzymes measured in homogenates of 11.5-day yolk sacs, explanted from the mother at 11.5 days, or at 9.5 days and cultured for 48 hours. In all assays, depletion of the substrate was less than 1%. For all enzymes assayed considerable activity was measured in both groups of yolk sacs, under the assay conditions described. The acid phosphatase activity recorded here compares well with the only other reported value (Beck et al., 1967a) for 11.5-day yolk sac of 2,310 nmoles/hour/mg protein, obtained from a single animal. The 11.5-day yolk sac possesses considerable proteolytic activity as judged by the specific activities of cathepsins D and B. Cathepsin D activity in cultured yolk sacs was about 1½ times that in the non-cultured tissue, but the ranges of activity of the enzyme in each group overlapped.

It is notable that little difference existed in hydrolase activity between cultured yolk sac and non-cultured yolk sac. Evidently, the conditions under which conceptuses were cultured did not lead to an altered level of hydrolase activity in yolk sac such as might have occurred for example through exocytosis of lysosomal enzyme.

3.5   Discussion

The data presented in this chapter demonstrate the ability of the early organogenesis-stage yolk sac to digest pinocytosed protein. When conceptuses were cultured in the presence of ^{125}I-dBSA, there was little accumulation of radioactivity by the conceptus (Figures 3.2 and 3.3),

but a considerable release of TCA-soluble radioactivity into the
culture medium was observed, the extent of which depended on the
duration of exposure to radiolabelled protein. Except for very
short exposures, the amount of radioactivity in the tissues did not
depend on the duration of exposure to substrate. This pattern is
noticeably similar to results obtained using the 17.5-day yolk sac
in culture (Williams et al., 1975b; Moore et al., 1977) and indicates
that the substrate is being pinocytosed and hydrolysed within the
tissues.

The demonstration of both TCA-soluble and -insoluble radioactivity in
the yolk sac, but only TCA-soluble radioactivity in the embryo
(Figures 3.4 and 3.5), strongly suggests that ^{125}I-dBSA is being both
taken up by, and digested in the yolk sac, the TCA-soluble radioactivity
in the embryo deriving from the products of digestion within yolk sac
lysosomes. The known inability of $\left[^{125}I\right]$ iodotyrosine to participate
in protein biosynthesis (Alexander, 1964; Cartouzou et al., 1964)
renders it of no value to the tissues and it is released back into the
culture medium. In addition to demonstrating yolk sac proteolysis, the
data also indicate that the embryo is unable to capture directly, or
receive intact from the yolk sac, undigested radiolabelled protein,
thus confirming and extending those observations made using ^{125}I-PVP
and $\left[^{3}H\right]$ dextran as substrates.

If it is assumed that the yolk sac alone is responsible for the
hydrolysis of ^{125}I-dBSA, the total uptake of substrate by the yolk sac
can be calculated. Figures 3.6 and 3.7 show that total uptake of
radioactivity is linear over the time period studied. The calculated
Endocytic Indices are 5.43 and 5.73 μl/mg protein/hour at substrate
concentrations of 5.5 and 11.0 μg/ml respectively. These values of

Endocytic Index are very much lower than those obtained for 17.5-day

yolk sac of 65.0 ± 11.0 (Moore et al., 1977) and 68.1 ± 10.6 (Ibbotson

and Williams, 1979). The difference can probably be explained by the

use in the present study of 100% serum as the culture medium.

Ibbotson and Williams (1979) showed that when 10% calf serum, normally

present in the culture medium of the 17.5-day yolk sac, was removed,

the Endocytic Index of ^{125}I-dBSA rose to an average value of $253.0 \pm$

93.2. These workers concluded that the protein present in calf serum

competed with ^{125}I-dBSA for the adsorptive sites on the plasma membrane

of yolk cells that facilitate the relatively rapid pinocytosis of this

substrate. Removal of the competing serum proteins resulted in a

somewhat faster and more effective clearance of ^{125}I-dBSA from the

culture medium. That pinocytosis of ^{125}I-dBSA by the early organo-

genetic yolk sac is aided by adsorption to the plasma membrane seems

most probable. The Endocytic Index of ^{125}I-dBSA is about 4 times that

of both ^{125}I-PVP and $\left[^{3}H\right]$ dextran, both of which were considered to

be pinocytosed in the fluid-phase.

The uptake and digestion of protein by yolk sac, and the inability of

the embryo to acquire intact protein, either directly or by transport

through the yolk sac, are observations entirely consistent with, and

providing direct experimental support for, the postulated mechanism

of histiotrophic nutrition. However, it is acknowledged that the data

presented here for a single heterologous protein need not necessarily

extent to other, particularly homologous, proteins. Indeed several

reports have presented evidence that both homologous and heterologous

proteins are transported across the yolk sac into the embryo without

undergoing digestion within yolk sac lysosomes. This evidence is discussed

later in this Thesis (Chapter 10).

Additional evidence that the yolk sac possesses considerable digestive capacity comes from the assay of lysosomal acid hydrolases in tissue homogenates (Table 3.1). All of the enzymes assayed were found to be active in the 11.5-day yolk sac.

The characteristics of these lysosomal enzymes have been extensively reviewed by Barrett and Heath (1977) and it would be inappropriate to discuss these characteristics here. However, it is worth recording that one of the five enzymic activities assayed here is also present in non-lysosomal sites in the cell. Acid phosphatase has been demonstrated in other membrane systems of cells (Neil and Horner, 1964a, b), and furthermore Beaufay (1972) has pointed out that para-nitrophenyl phosphate, which was used as substrate in the present study, is hydrolysed by non-lysosomal acid phosphatase. However, non-lysosomal acid phosphatase is relatively unstable at $37^{\circ}C$ and the long incubation period of 4 hours used in this study would minimise the contribution of this enzyme to the extent of substrate hydrolysis measured.

The data presented in Table 3.1 further demonstrate that lysosomal enzyme activities measured in 11.5-day yolk sac taken directly from the mother, are not noticeably different from those measured in 11.5-day yolk sacs of conceptuses which had been cultured for 48 hours. This most likely indicates that cultured yolk sac develops a normal complement of lysosomal enzymes and also that these lysosomal enzymes are not secreted into the culture medium. This latter point is important when one considers that the hydrolysis of ^{125}I-dBSA in culture described in this study, might have occurred as a result of the release of proteolytic enzyme(s) by tissues into the culture medium. That this is clearly not the case reinforces the conclusion that the early organogenesis yolk sac is capable of digesting macromolecules.

CHAPTER FOUR

UTILISATION OF EXOGENOUS PROTEIN BY THE
EARLY ORGANOGENESIS-STAGE RAT CONCEPTUS

4.1   Introduction

It has never been demonstrated that the products of yolk sac lysosomal protein digestion, amino acids, are utilised in the biosynthesis of embryonic protein. That such a process operates is central to the model of histiotrophic nutrition proposed by Beck et al. (1967a), which is, in view of this lack of definitive data, seriously weakened.

The culture technique affords an excellent opportunity to investigate this problem, and the experiments of this Chapter have attempted to provide a solution.

Conceptuses were cultured in the presence of $\left[^3\text{H}\right]$ leucine-labelled proteins which, unlike ^{125}I-dBSA, would yield radiolabelled digestion products capable of being incorporated into newly-synthesised protein. The distribution of TCA-insoluble and -soluble radioactivity in the conceptus was monitored, and an attempt was made to separate the tissue proteins to examine the distribution of radiolabel among the protein fractions.

The demonstration that embryonic proteins contain amino acids supplied by yolk sac proteolysis does not, in itself, indicate whether the process is of quantitative significance in embryogenesis. A preliminary investigation was therefore performed in which an excess of non-radioactive amino acid was included in a culture medium already containing radiolabelled protein. The effect of the excess amino acid upon the incorporation of radioactivity into the conceptus was measured.

4.2   Materials

Radiochemicals

L-$[4,5-^3H]$ leucine (preparation TRK.510)   The Radiochemical Centre,
Amersham, Bucks.

Chemicals

Heparin (5,000 i.u./ml)   Boots Co. Ltd, Nottingham

2-mercaptoethanol   Calbiochem, USA

TEMED (NNN'N'-Tetramethylethylene   Hopkins and Williams, Chadwell
                        diamine)   Heath, Essex

Sodium dodecyl sulphate   BDH Chemicals Ltd, Poole, Dorset

Ammonium persulphate   BDH Chemicals Ltd, Poole, Dorset

Acrylamide   BDH Chemicals Ltd, Poole, Dorset

Methylenebisacrylamide   BDH Chemicals Ltd, Poole, Dorset

Bromophenol blue   E. Merck AG, Darmstadt, W. Germany

Other chemicals were of analytical grade.

Equipment

LKB 2117-301 Multiphor basic unit   LKB Ltd, Bromma, Sweden

LKB 2117-201 Multiphor Electrophoresis   LKB Ltd, Bromma, Sweden
                        Kit

LKB 2117-601 Multiphor Electrophoresis   LKB Ltd, Bromma, Sweden
    Kit for SDS polyacrylamide gels

LKB 2209 Multitemp Cooling Unit   LKB Ltd, Bromma, Sweden

LKB 2197 Power Supply   LKB Ltd, Bromma, Sweden

4.3   Methods

4.3.1 Preparation of Rat Serum in which Proteins have been labelled
with $[^3H]$ leucine

Male rats weighing approximately 250 g received an intraperitoneal

injection of $\left[^3H\right]$ leucine contained within 1.0 ml of 0.9% saline, at
a dosage of 40 μCi/100 g body weight. After 2 hours, at which time
incorporation of radioactivity into serum proteins is maximal[*]
(Schreiber et al., 1966), serum was collected as described in 2.3.1.
To remove free $\left[^3H\right]$ leucine the serum was dialysed at 4°C for 4 days
against four changes of a balanced salt solution (Cockroft, 1979).
The balanced salt solution contained, per litre of water, 6.9 g NaCl,
0.3 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.05 g $MgCl_2.6H_2O$, 0.1 g $NaH_2PO_4.2H_2O$,
2.0 g $NaHCO_3$ and 0.2 g $CaCl_2$. The dialysed serum contained approximately
1% of its total radioactivity in a form soluble in 6.7% (w/v) TCA.
The dialysed serum was stored at -20°C until use, whereupon it was
heat-denatured and centrifuged as previously described. In order to
support normal embryonic development in culture, 1.5 mg/ml of D-glucose
and 10 μl/ml of vitamin concentrate were added to the serum
(Cockroft, 1979).

The effect of incubation in the presence and absence of 0.25 M NaOH
at 37°C on the stability of protein-bound radioactivity was studied
as described for ^{125}I-dBSA. In order to assess whether the $\left[^3H\right]$ leucine
was incorporated into the primary structure of the radiolabelled protein
and not non-covalently bound, $\left[^3H\right]$ leucine-labelled serum from the same
batch was divided into several portions and dialysed in the presence
of different concentrations of non-radioactive L-leucine. The latter
would compete with any non-covalently bound $\left[^3H\right]$ leucine thereby
decreasing the specific radioactivity of the TCA-insoluble radioactivity
of the serum after dialysis.

[*] Specific radioactivity of serum was 3,600 c.p.m /mg serum protein.

4.3.2 Preparation of [³H] leucine-labelled Haemoglobin

[³H] Leucine-labelled haemoglobin was prepared from rat reticulocytes
according to the unpublished procedure of Ballard. Reticulocytosis
was produced in 2 male adult rats by daily injections over 5 days of
a 1% solution of phenylhydrazine. Phenylhydrazine hydrochloride was
dissolved in water and neutralised to pH 7.0 ± 0.5 with 10% Na_2CO_3.
To this solution was added 0.9% saline to give a final concentration
of phenylhydrazine of 10 mg/ml. The dosage employed was 0.2 ml of
phenylhydrazine solution per 200 g body weight per day. After the
last injection the animals were rested for two days. Blood was
then collected from aorta of the ether-anaesthetised animals using
a 10 ml syringe and a serum needle that had been filled with heparin
solution to prevent clotting of blood. Approximately 7 ml of blood
were withdrawn followed by 2 ml of air and the barrel of the syringe
was gently rolled to allow mixing of blood with the heparin. All the
blood collected from both animals was pooled. Aliquots of 2 ml of
pooled blood were placed in 50 ml glass centrifuge tubes and to each was
added 30 ml of 'reticulocyte washing solution' prepared as follows by
a modification of the method of Burka and Marks (1964). To 500 ml of
distilled water was added 4.24 g NaCl, 0.187 g KCl, 0.153 g $MgCl_2.6H_2O$
and 0.229 g of TES. The pH of the solution was adjusted to 7.5 with
M NaOH. Each tube was centrifuged at 2000 g for 3 minutes at 4°C.
Following centrifugation the washing solution was decanted and the
procedure repeated twice more. To the pelleted material (reticulocytes)
remaining after three washes was added 10 ml of 'reticulocyte incubation
medium' containing 100 μCi of [³H] leucine. The incubation medium
was made up by adding to 50 ml of distilled water 349 mg NaCl, 18.7 g KCl,
15.3 mg $MgCl_2.6H_2$), 229 mg TES, 49.5 mg glucose and 3.9 mg $Fe(NH_4)_2SO_4.6H_2O$

and adjusting the pH to 7.5. The ungassed, uncapped tubes were incubated in a water bath at 37°C for 1 hour, shaking at 5 minute intervals throughout this period. At the end of the incubation period, 30 ml of 'reticulocyte washing solution' was added to each tube and the mixture was centrifuged at 2,500 g for 5 minutes at 4°C. The washing procedure was repeated twice more. The remaining pelleted reticulocytes were haemolysed by adding 2 ml of distilled water to each tube and freezing and thawing through 4 cycles using liquid nitrogen and a 37°C water bath. The free haemoglobin was separated by centrifuging this solution at 3,000 g for 45 minutes at 4°C. The clear solution was decanted from the pellet and dialysed overnight against distilled water at 4°C, after which less than 1% of the total radioactivity in the solution was TCA-soluble. In order to check radiolabelling of haemoglobin, the dialysed solution was subjected to chromatography on a Sephadex G-75 column (2.2 cm x 45 cm, 100 ml bed volume) and eluted with 0.9% saline. Blue dextran was added to the radiolabelled haemoglobin solution to indicate the elution position of the void volume. Successive 2 ml fractions of the eluant were assayed for haem absorbance at 570nm and for total radioactivity.

4.3.3   Culture Experiments

4.3.3.1 Experiments using [^{3}H] leucine-labelled serum proteins as

substrates

Conceptuses were transferred from a culture medium of homologous serum to one of dialysed [^{3}H] leucine-labelled serum, at different intervals between 12 and 0.5 hours before harvesting. At harvesting, conceptuses were washed as before, and tissues solubilised, neutralised and diluted as before. The 1.0 ml of tissue solution for radioactivity counting was divided into two 0.5 ml fractions, one of

which was added to 4.5 ml of Lumagel scintillation fluid and assayed for total radioactivity. To the remaining 0.5 ml of tissue solution was added 0.25 ml of 20% TCA and 0.05 ml of carrier protein. After centrifugation at 2,000 g for 20 minutes, the supernatant (0.75 ml) was added to 4.5 ml Lumagel scintillation fluid and counted to give the TCA-soluble radioactivity. This figure was adjusted as for ^{125}I-dBSA by multiplication by an empirically determined correction factor. Appropriate adjustments were also made to allow for background and quenching. A quench curve equation was calculated as described in 2.3.5 only using $\left[^{3}H\right]$ leucine rather than $\left[^{3}H\right]$ dextran.

Duplicate 0.5 ml samples of culture medium were similarly assayed for total and TCA-soluble radioactivity, with the omission of carrier protein.

In order to assess any effect that the dialysed serum might have upon pinocytosis by the yolk sac, a culture was performed in which the culture medium comprised non-radioactive dialysed serum supplemented with the necessary glucose and vitamins, and ^{125}I-PVP was used as the pinocytic substrate following the procedure described in 2.3.2.

4.3.3.2 Experiments using $\left[^{3}H\right]$ Leucine-labelled Haemoglobin as Substrate

The experimental protocol was identical to that described for ^{125}I-dBSA (3.3.2), with the exception that the culture medium comprised 50% homologous serum, 50% Hanks Balanced Salt Solution. Conceptuses develop equally well in this medium with respect to protein content and the chosen developmental criteria (2.3.1) as in 100% homologous serum. Haemoglobin solution was added at a ratio of 0.05 ml per ml of culture medium. At harvesting, conceptuses were treated as previously described and total and TCA-soluble radioactivity of tissues and culture medium was assayed as described in 4.3.3.1.

4.3.4 Quantitation of Uptake

In experiments in which both $[^3H]$ leucine-labelled serum proteins and
$[^3H]$ leucine-labelled haemoglobin were used as substrates, no significant
depletion of radioactivity in the culture medium following culture
was observed. Furthermore there was no significant alteration in the
percentage of TCA-soluble radioactivity in the culture medium during
culture. Uptake was therefore calculated in the same way as
described in 2.3.6.

4.3.5 SDS-polyacrylamide gel electrophoresis of tissues following
culture in the presence of $[^3H]$ leucine-labelled haemoglobin

SDS-polyacrylamide gel electrophoresis was performed according to the
method of Fehrnstrom and Moberg (1977), using a phosphate buffer
system. Phosphate buffer stock solution (0.2 M, pH 7.1) containing
SDS was prepared by dissolving in 5 litres of distilled water 39.0 g
$NaH_2PO_4.H_2O$, 258.0 g $Na_2HPO_4.12H_2O$ and 10.0 g SDS. The gel was
prepared by adding 33.0 ml of phosphate buffer stock solution to
22.2 ml of acrylamide solution and 7.5 ml of distilled water.
Acrylamide solution was prepared by dissolving 22.2 g acrylamide and
0.6 g methylenebisacrylamide in 100 ml of distilled water. The gel
mixture was then deaerated for about 2 hours by connecting to a
vacuum pump and periodically shaking. Following deaeration, 3.2 ml
of a 1.5 mg/ml solution of ammonium persulphate and 0.1 ml of TEMED
were added to the gel mixture to effect polymerisation. The gel
mixture was poured into the gel moulding set and allowed to polymerise
for 45 minutes. The moulded 7.5% gel was then placed in a fridge for
5 minutes to facilitate removal of the mould and the gel was stored
on a glass plate overnight at room temperature. For electrophoresis
the gel was placed on a cooling plate in the centre of the apparatus

to which had been added a few drops of 0.1% Triton X-100 to improve thermal conductivity. Each of the buffer reservoirs were filled with 1 litre of electrode buffer, a 1:1 dilution with distilled water of phosphate buffer stock solution, and electrode wicks, seven strips of Whatman No. 1 filter paper on each side, were dipped into the electrode buffer and positioned along each side of the gel, overlapping it by about 15 mm. Before applying samples to the gel pre-electrophoresis of the gel was performed at a constant current of 150mA for 30 minutes. Samples (0.01 ml) were then applied to the pre-formed slots at the cathodic pole of the gel using a Hamilton syringe. Samples were concentrated by running at a low current of 20mA for 10 minutes. Electrophoresis was then started by adjusting the potential difference to give a current of 195mA, and the system was run at this current for about 4 hours.

The samples were prepared as follows. A culture was performed using $[^3H]$ leucine-labelled haemoglobin as substrate. The substrate was present in the culture medium for either the last 12 hours of culture or the last hour of culture. At harvesting conceptuses were washed as usual and the yolk sacs dissected from the embryos. All yolk sacs and embryos from a single experiment were pooled separately. Pooled tissues were homogenised in 0.25 M NaOH and once completely solubilised were neutralised with an equal volume of 0.25 M HNO_3. Samples were then dialysed overnight to remove non-protein-bound radioactivity. To 0.2 ml of dialysed sample solution was added 0.1 ml of sample buffer, a solution comprising 6% SDS and 30% 2-mercaptoethanol in 0.01M phosphate buffer stock solution, and the mixture was heated at 100°C for 5 minutes in a water bath. This latter step promoted dissociation of proteins into sub-units with a rod-like shape of constant diameter, the long axis of the rod varying in proportion to the molecular weight.

The binding of the SDS to the protein sub-unit introduced one negative charge per bound molecule of SDS. Thus proteins carried a net negative charge and migrated in one direction only, towards the anode, migrating a distance which was related to their molecular weight. Following heat treatment of the sample 0.005 ml of 0.25% (w/v) bromophenol blue in sample buffer was added. The bromophenol blue acted as front marker and indicated when the run was complete. In addition to tissue samples a sample of dialysed $[^{3}H]$ leucine-labelled haemoglobin was treated with SDS/2-mercaptoethanol, as described, and electrophoresed.

Following electrophoresis, the gel was immediately sliced. Each lane was first excised from the gel slab using a razor blade and the strip was then sliced with a purpose-built gel-slicer. The thickness of each gel slice was 1.8 mm. Each slice was individually homogenised in distilled water (0.5 ml) and the homogenate was added to 4.5 ml of Lumagel scintillant and counted for total radioactivity.

4.3.6 The effect of L-leucine on the incorporation of radioactivity by conceptuses following culture in the presence of ^{3}H-labelled serum proteins

Conceptuses were cultured for the last six hours of a 48 hour culture period in dialysed $[^{3}H]$ leucine-labelled serum to which had been added L-leucine at a concentration of 50, 100, 500 or 1,000 µg/ml. A control group of conceptuses received no L-leucine in their culture medium. At harvesting, conceptuses were washed, dissected and solubilised as described previously and assayed for total and TCA-soluble radioactivity as described in 4.3.3.1.

Results were expressed graphically as the uptake of radioactivity (µl/mg protein) in 6 hours as a function of L-leucine concentration in the culture medium.

4.4   Results

4.4.1 Stability and authenticity of radiolabelled substrates

Figure 4.1 shows that the increase in TCA-soluble radioactivity when

both $\begin{bmatrix} 3H \end{bmatrix}$ leucine-labelled serum proteins and $\begin{bmatrix} 3H \end{bmatrix}$ leucine-labelled

haemoglobin were incubated separately under standard culture conditions

in the absence of conceptuses was negligible. In contrast, the

percentage of TCA-soluble radioactivity released when 0.1 ml of

radiolabelled serum or 0.1 ml of radiolabelled haemoglobin were

incubated with 0.5 ml of 0.25M NaOH at $37^{o}C$ rose appreciably after 1

hour of incubation. It was for this reason that tissues were

solubilised in 0.25 M NaOH for no longer than 1 hour.

The incorporation of $\begin{bmatrix} 3H \end{bmatrix}$ leucine into the primary structure of serum

proteins following its injection into a rat is demonstrated by the data

of Table 4.1. The addition of a different concentration of L-leucine

to each of 3.0 ml aliquots of radiolabelled serum led to there being

no significant difference in the total radioactivity of the serum

following dialysis for 4 days at $4^{o}C$. Evidently the protein-bound

$\begin{bmatrix} 3H \end{bmatrix}$ leucine in the radiolabelled serum cannot be displaced by the

addition of an excess of non-radioactive L-leucine.

The authenticity of $\begin{bmatrix} 3H \end{bmatrix}$ leucine-labelled haemoglobin is demonstrated

in Figure 2. Haemoglobin and radioactivity were eluted simultaneously

from a Sephadex G-75 chromatography column.

4.4.2 Culture experiments

When conceptuses were cultured in vitamin- and glucose-supplemented

dialysed serum containing radiolabelled proteins, the level of

radioactivity in both the yolk sac and the embryo increased linearly

with time of exposure to substrate (Figure 4.3). Following short periods
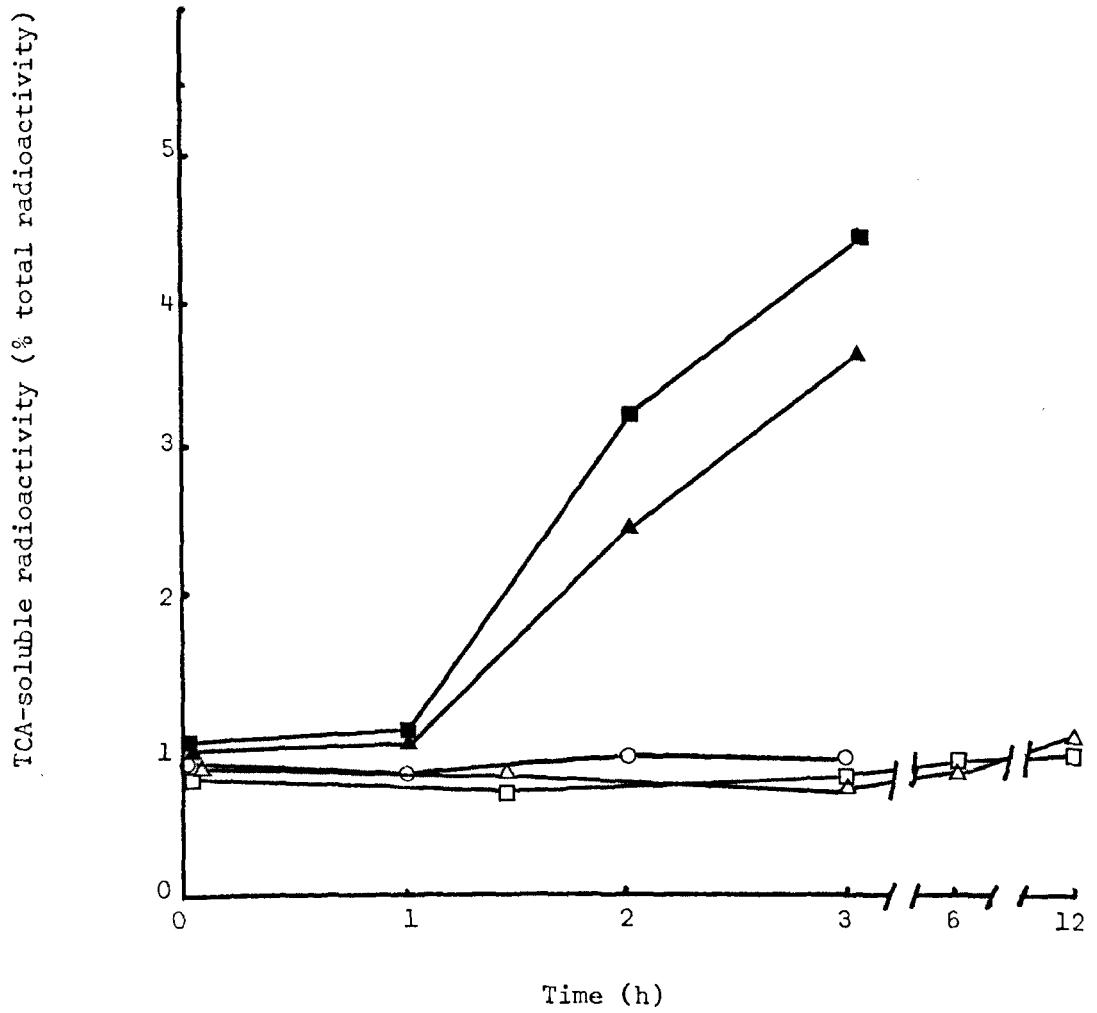
Figure 4.1 Increase in TCA-soluble radioactivity after
incubation at 37°C in roller culture apparatus of vitamin-
and glucose-supplemented dialysed [³H] leucine-labelled serum
in 0.25 M NaOH (■), water (O) or alone (□), and of [³H] leucine-
labelled haemoglobin in 0.25 M NaOH (▲) and rat serum (Δ).
Each point represents mean of 4 separate determinations.
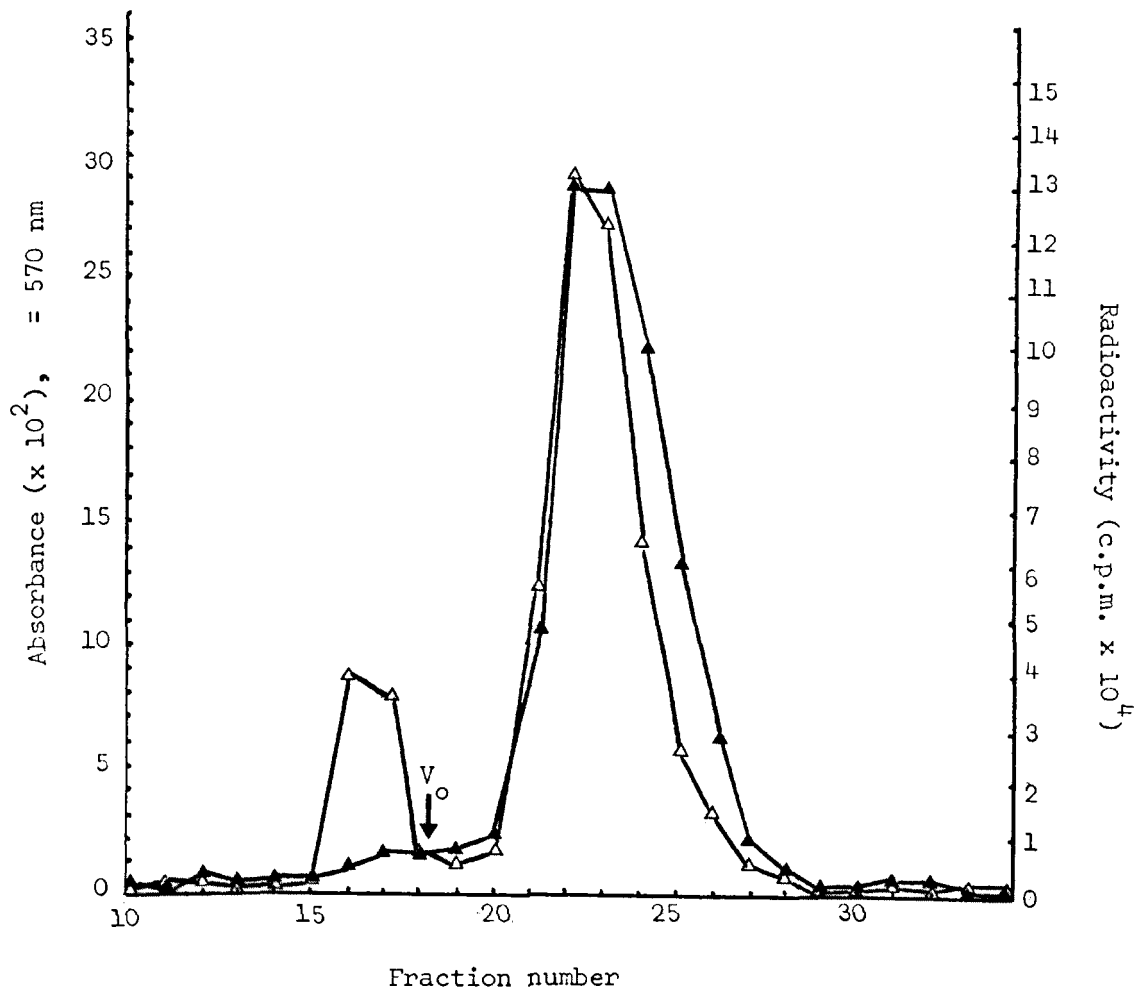
Figure 4.2 Co-elution from Sephadex G-75 column of haem,
estimated by absorbance (△) at 570 nm, and radioactivity (▲)
after application to column of $[^3H]$leucine-labelled
haemoglobin. V_o indicates void volume.
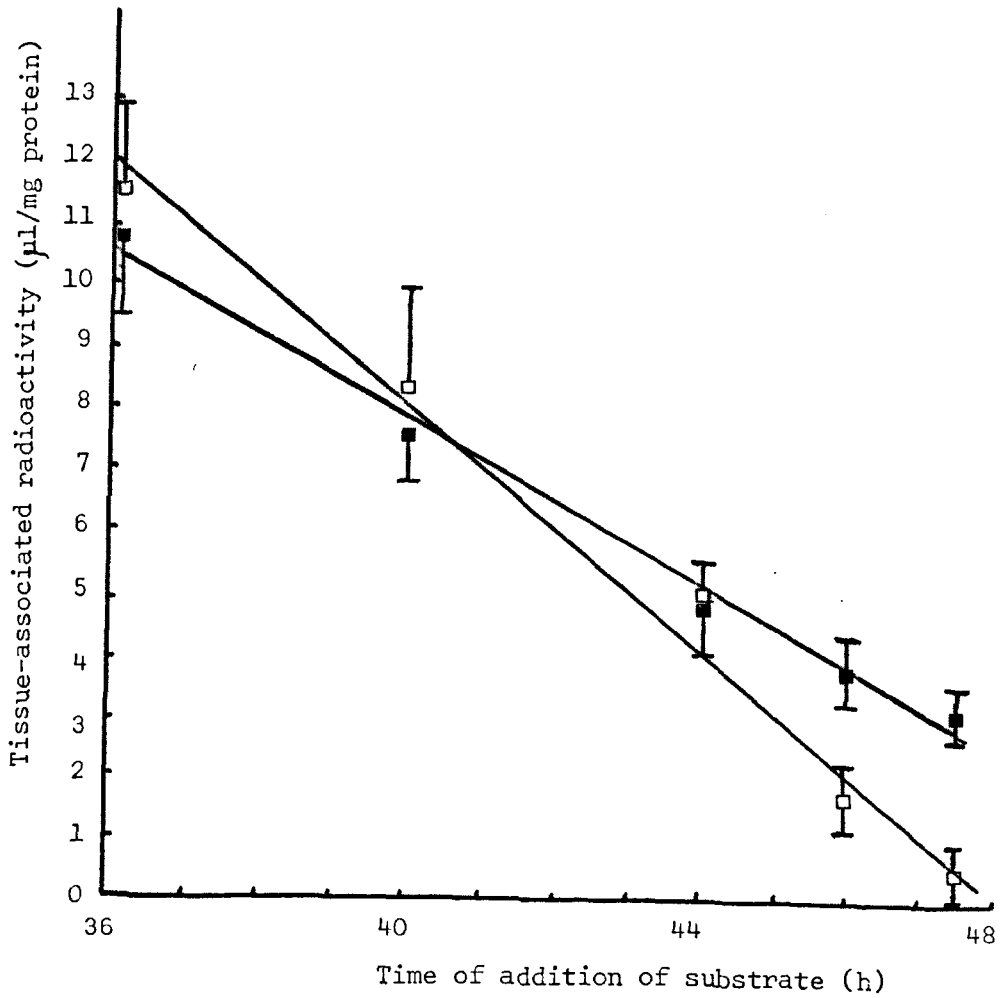
Figure 4.3 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of [3H] leucine-labelled serum proteins. Each point represents mean (± S.D.) of at least 6 determinations.

of exposure to radiolabelled substrate, uptake of radioactivity into the yolk sac exceeded that into the embryo; this trend was reversed after longer exposures to substrate. The percentage of tissue radioactivity that was TCA-soluble is shown in Figure 4.4. After short periods of exposure to substrate, a high percentage of yolk sac radioactivity was TCA-soluble. On longer exposures, this percentage steadily declined, apparently tending towards a minimum value of 30%. A qualitatively similar pattern was observed in the embryo, where the percentage of TCA-soluble radioactivity was higher than in the yolk sac at short exposures and declined more rapidly with longer exposures to a steady level of about 20%, lower than that seen in the yolk sac. Assuming that the radioactivity in the entire conceptus is derived from initial uptake by the yolk sac, the clearance of radiolabelled proteins from the culture medium can be calculated. Figure 4.5 shows that this clearance is linear with time over the 12 hours incubation period studied, and has an Endocytic Index of 2.19.

The necessary use of dialysed serum as a culture medium in these experiments raises the question of whether dialysis of the serum affects the conceptuses' ability to capture macromoleculre substrates. Therefore the uptake of ^{125}I-PVP was studied following exactly the same procedure described in 2.3.2.1, but using glucose- and vitamin-supplemented dialysed serum as a culture medium. At a concentration of 2.8 μg ^{125}I-PVP/ml, the pattern of uptake of radioactivity (Figure 4.6) did not differ from that observed when whole serum was used as culture medium (Figure 2.3).

The pattern of uptake of total radioactivity by yolk sac and embryo (Figure 4.7), and the percentage of this radioactivity that was TCA-soluble (Figure 4.8) when $[^{3}H]$ leucine-labelled haemoglobin was used
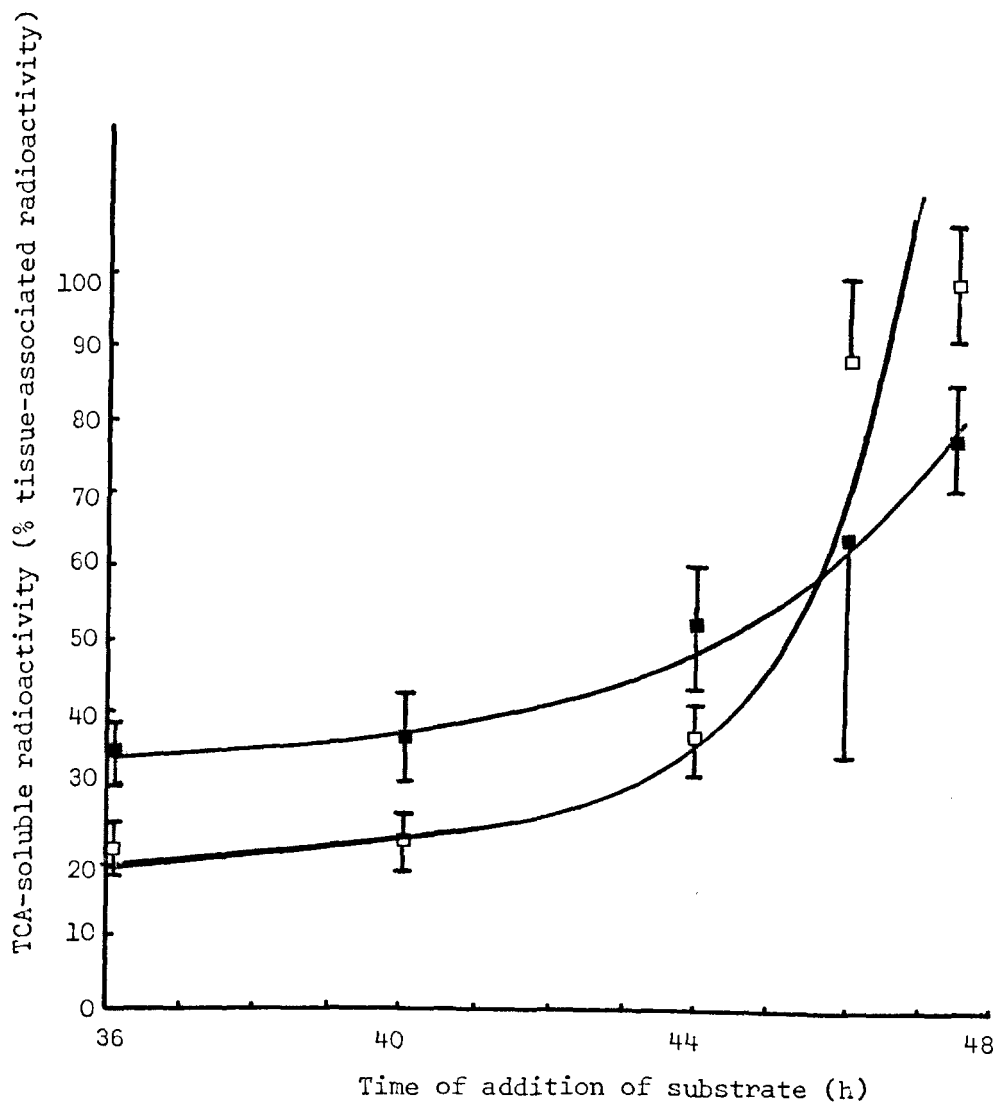
Figure 4.4 Percentage of tissue radioactivity soluble in TCA after incubation of conceptuses in the presence of $\left[^3H\right]$ leucine-labelled serum proteins. Each point represents the mean (± S.D.) of at least 6 determinations. (■) yolk sac, (□) embryo.
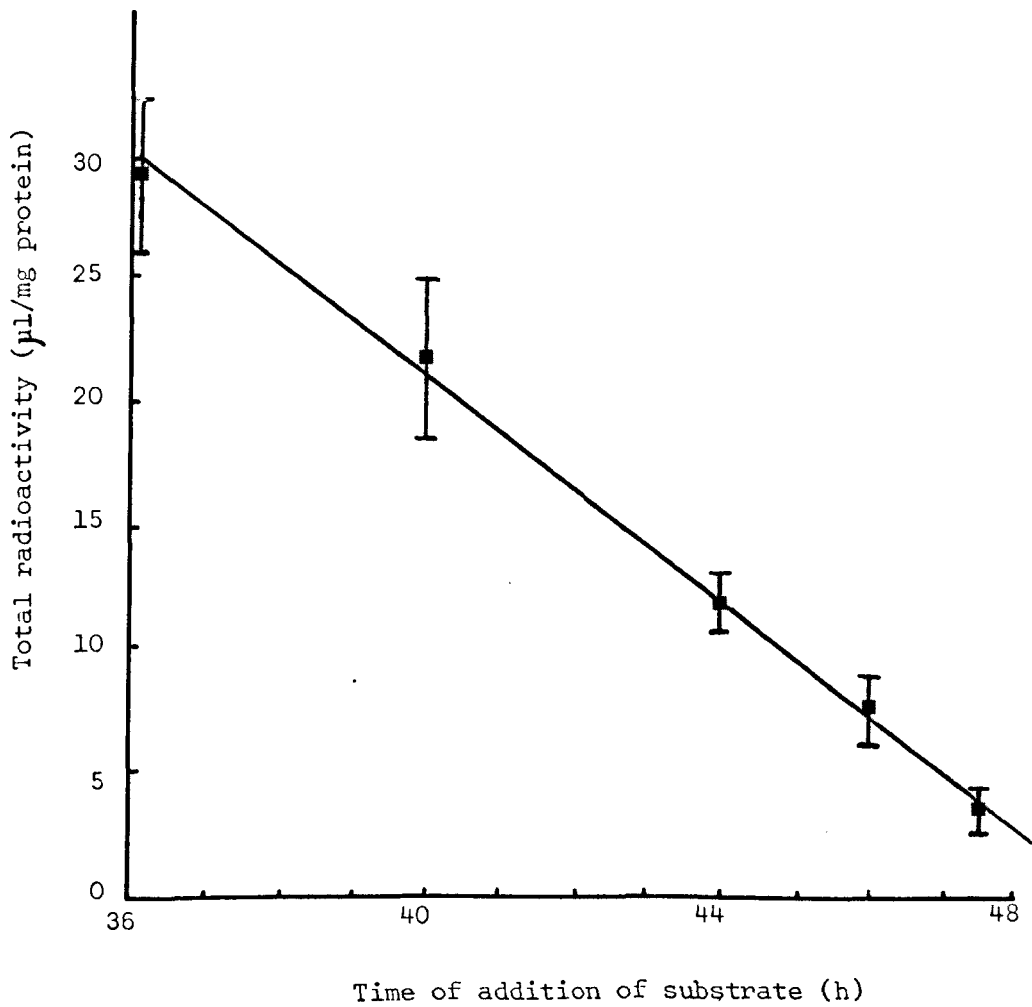
Figure 4.5 Total radioactivity taken up by yolk sacs after incubation
in the presence of [3H] leucine-labelled serum proteins. Each point
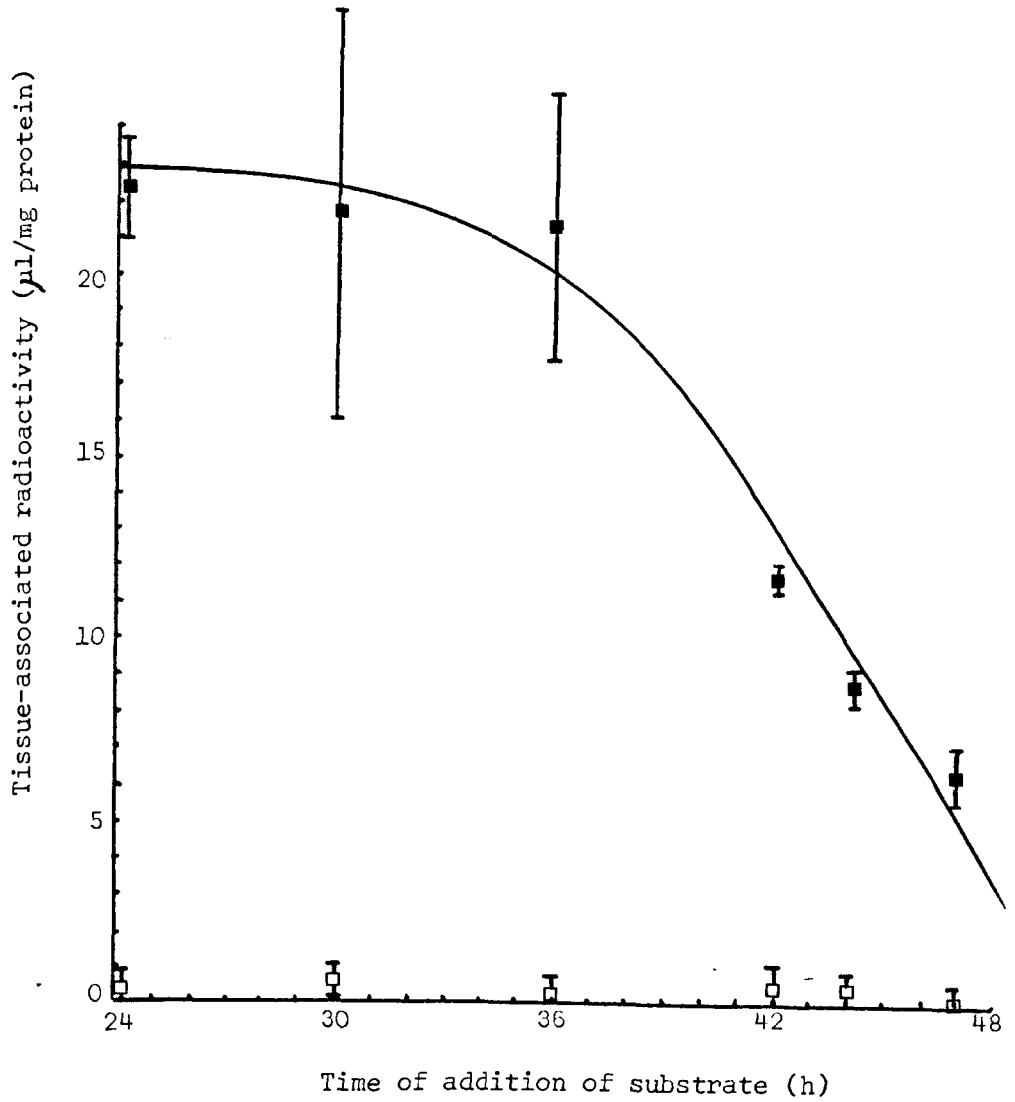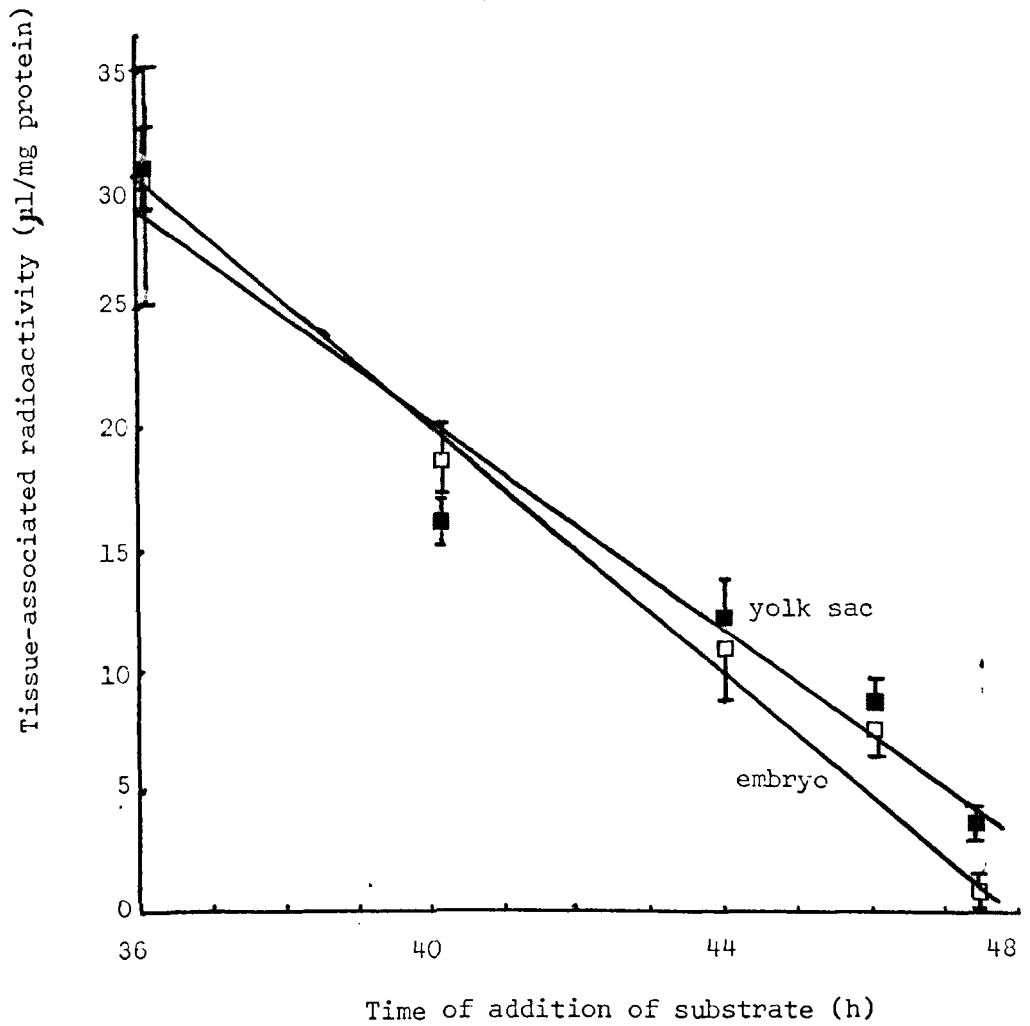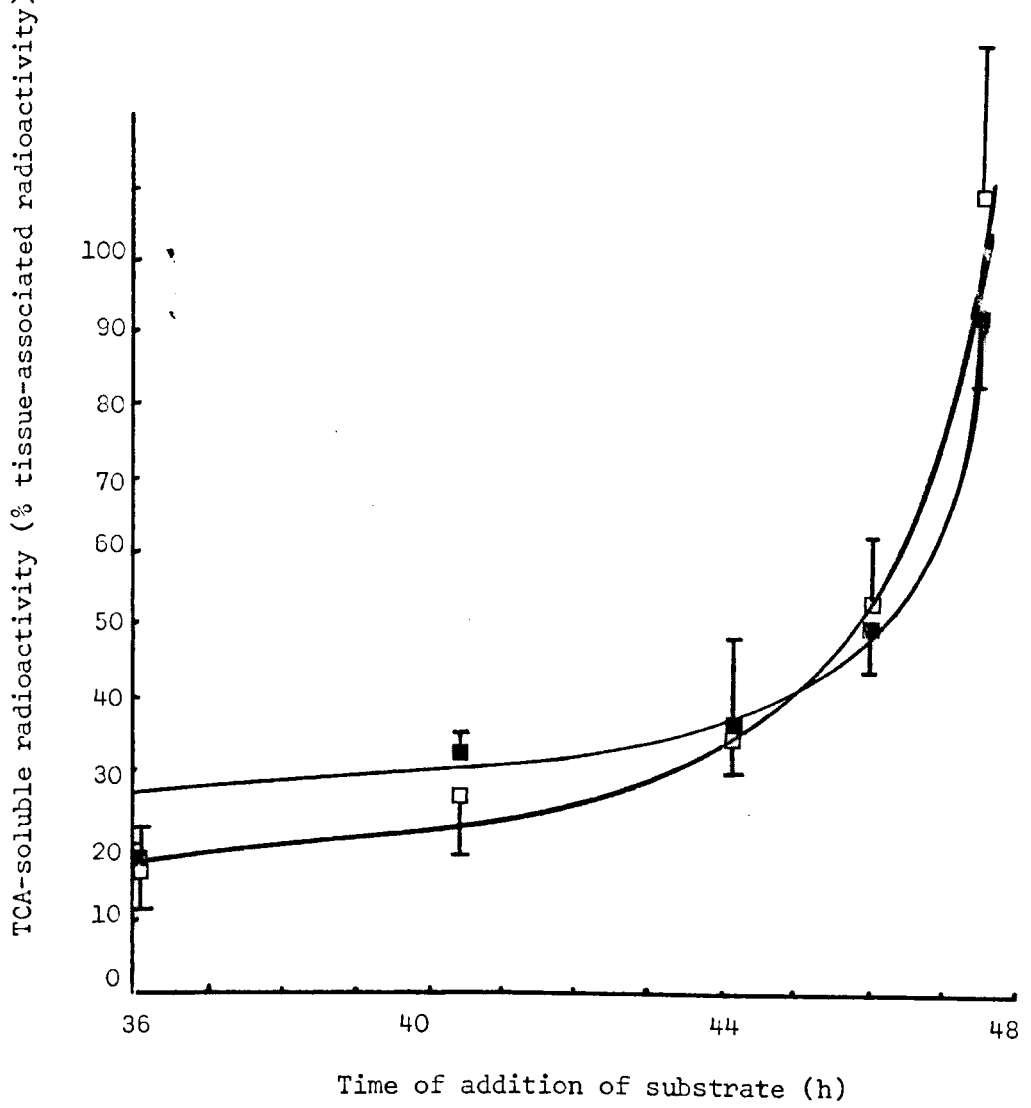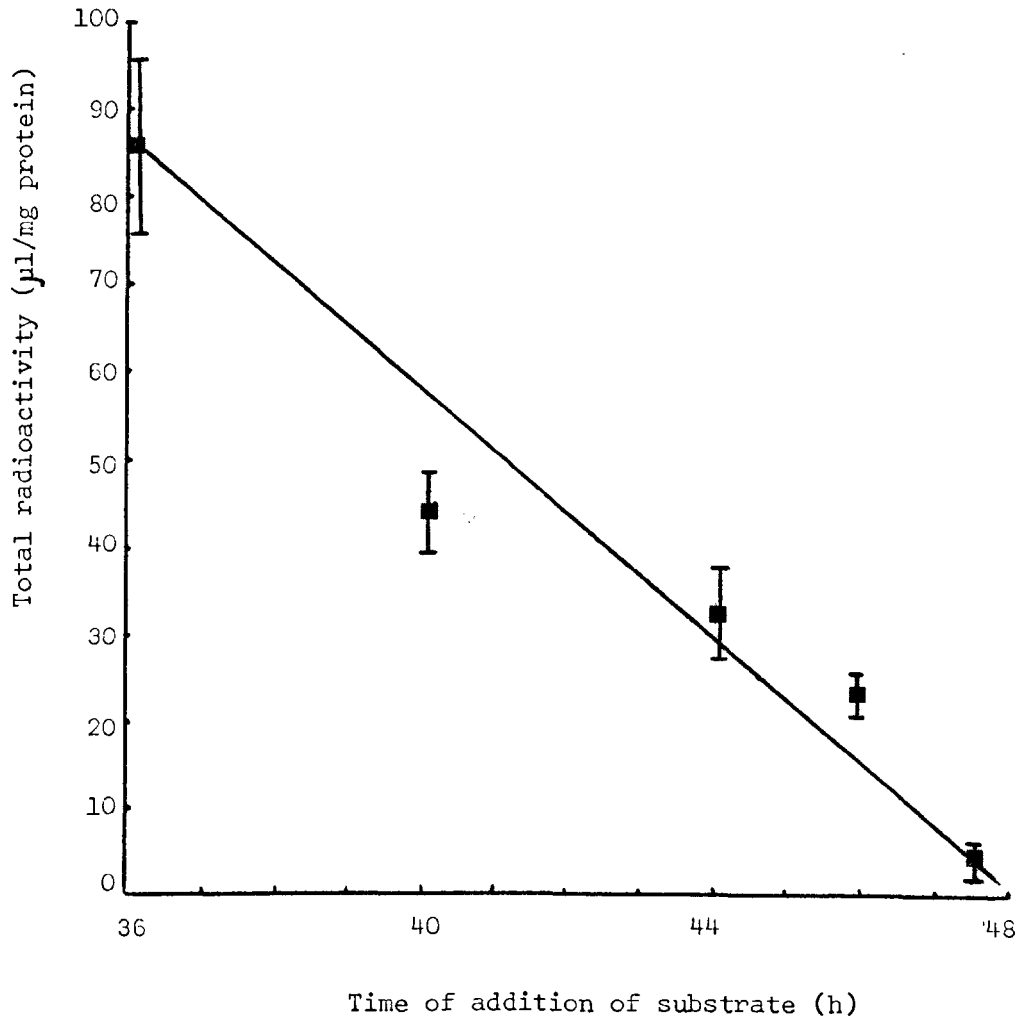represents the mean (+ S.D.) of at least 6 determinations.

Figure 4.6 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 μg/ml). Conceptuses were cultured in vitamin- and glucose-supplemented dialysed rat serum. Each point represents the mean (± S.D.) of at least 6 determinations.

Figure 4.7 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of [³H] leucine-labelled haemoglobin. Each point represents the mean (± S.D.) of at least 6 determinations.

Figure 4.8 Percentage of tissue radioactivity soluble in TCA after incubation of conceptuses in the presence of $\left[^3_H\right]$ leucine-labelled haemoglobin. Each point represents the mean ($\pm$ S.D.) of at least 6 determinations. (■) Yolk sac, (□) embryo.

Figure 4.9 Total radioactivity taken up by yolk sacs after
incubation of conceptuses in the presence of [³H] leucine-
labelled haemoglobin. Each point represents the mean (± S.D.)
of at least 6 determinations.

as substrate, was qualitatively similar to that described for ^{3}H-labelled serum proteins. Quantitatively however, the uptake of $\left[^3H\right]$ haemoglobin by both yolk sac and embryo, expressed as a function of yolk sac and embryo protein respectively, was about $2\frac{1}{2}$ times greater than was ^{3}H-labelled serum protein uptake. Clearance of radiolabelled haemoglobin by conceptuses is shown in Figure 4.9. On exposure to substrate for 12 hours, clearance, expressed as a function of yolk sac protein, was about 3 times that observed with ^{3}H-labelled serum proteins.

4.4.3 Electrophoretic Separation of Conceptus Tissue Proteins following Culture in the Presence of $\left[^3H\right]$ leucine-labelled haemoglobin

In order to gain further information on the mechanism of accumulation of radioactivity by yolk sac and embryo following culture in the presence of $\left[^3H\right]$ haemoglobin, tissues were solubilised and subjected to SDS-polyacrylamide gel electrophoresis. Figure 4.10 shows the electrophoretic profile of the sample of $\left[^3H\right]$ haemoglobin used in this study. Radioactivity was concentrated in a single peak. The profile of radioactivity associated with yolk sac proteins after 1 hour of exposure to substrate (47 to 48 hours of culture; Figure 4.11) shows a single peak coincident with that of the substrate sample. Embryonic proteins under the same incubation conditions demonstrated no radio-activity. These observations indicate that the yolk sac pinocytoses radiolabelled haemoglobin and that this process precedes the uptake of radioactivity by the embryo, confirming the data of Figure 4.7. After exposure to $\left[^3H\right]$ haemoglobin for the last 12 hours of culture, both yolk sac and embryo display a whole range of radiolabelled proteins (Figures 4.12 and 4.13). These spectra of radiolabelled proteins clearly demonstrate that radioactivity from a single source, $\left[^3H\right]$ leucine-labelled

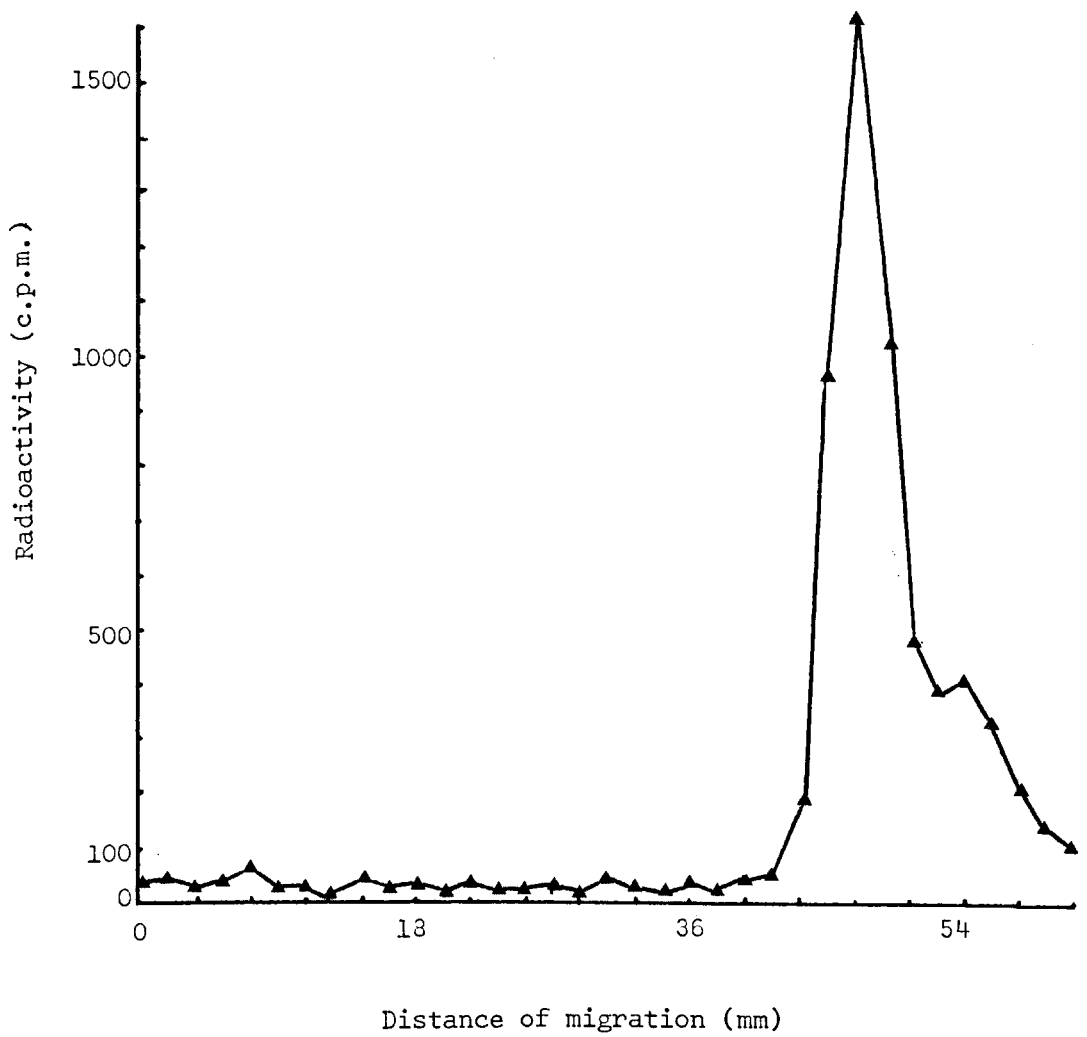Figure 4.10 Profile of protein-associated radioactivity after SDS polyacrylamide gel electrophoresis of $\left[^3\text{H}\right]$ leucine-labelled haemoglobin. Details of method are given in the text.
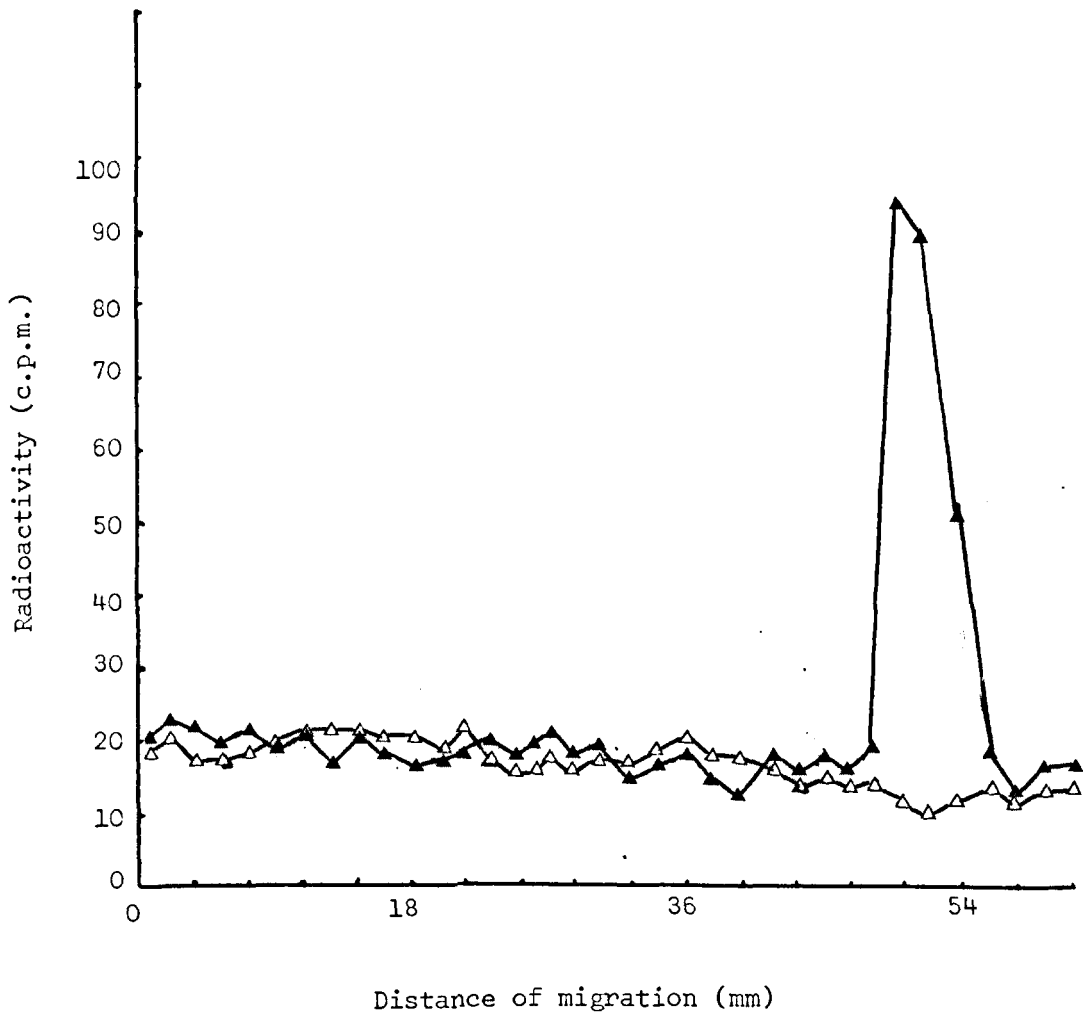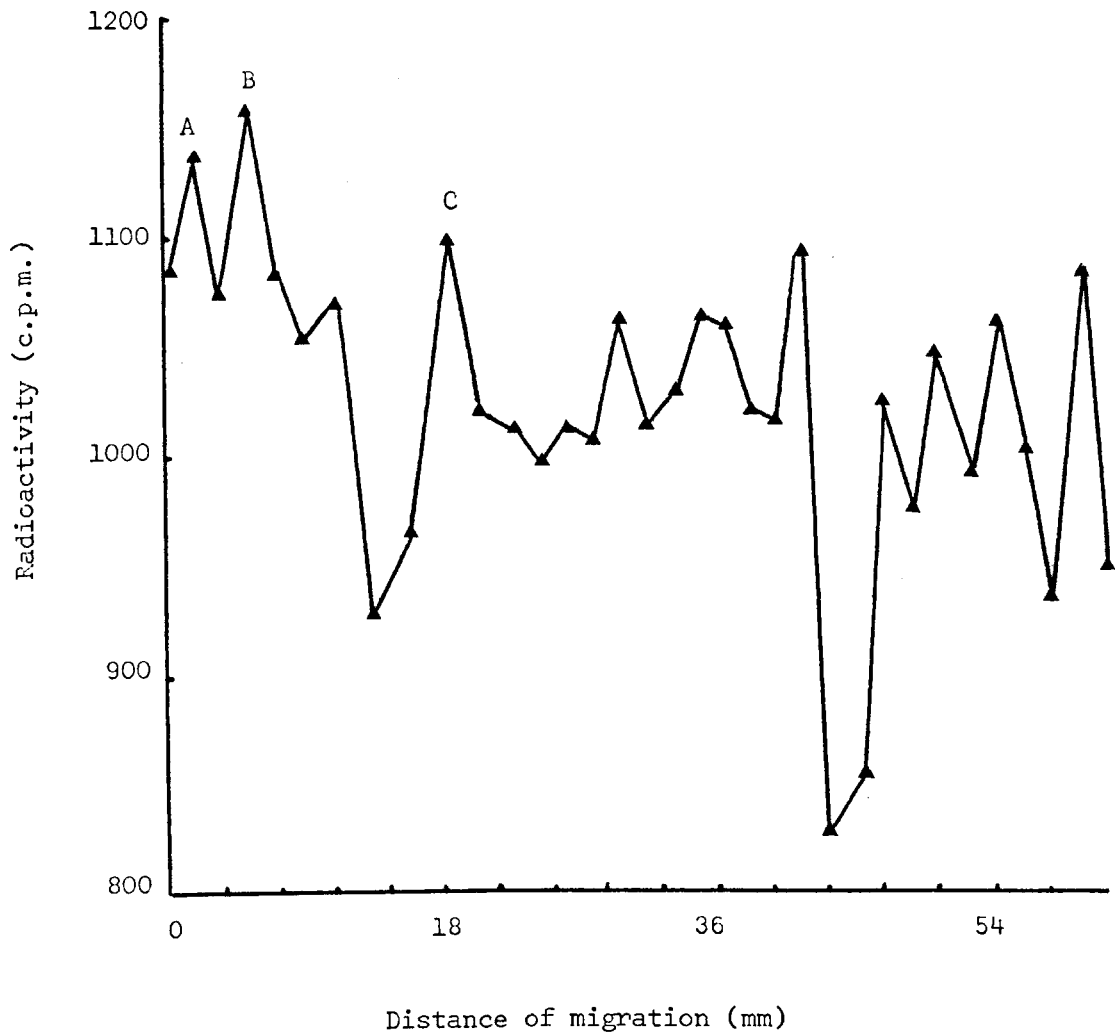
Figure 4.11 Profile of protein-associated radioactivity after
SDS polyacrylamide gel electrophoresis of yolk sac proteins(▲)
and embryo proteins (△) from conceptuses that were incubated
in the presence of [^{3}H]leucine-labelled haemoglobin for the last
hour of a 48-hour culture period. Details of method are given
in the text.

Figure 4.12 Profile of protein-associated radioactivity after
SDS polyacrylamide gel electrophoresis of yolk sac proteins from
conceptuses that were cultured in the presence of [3H]leucine-
labelled haemoglobin for the last 12 hours of a 48-hour culture
period. Details of method are given in the text.
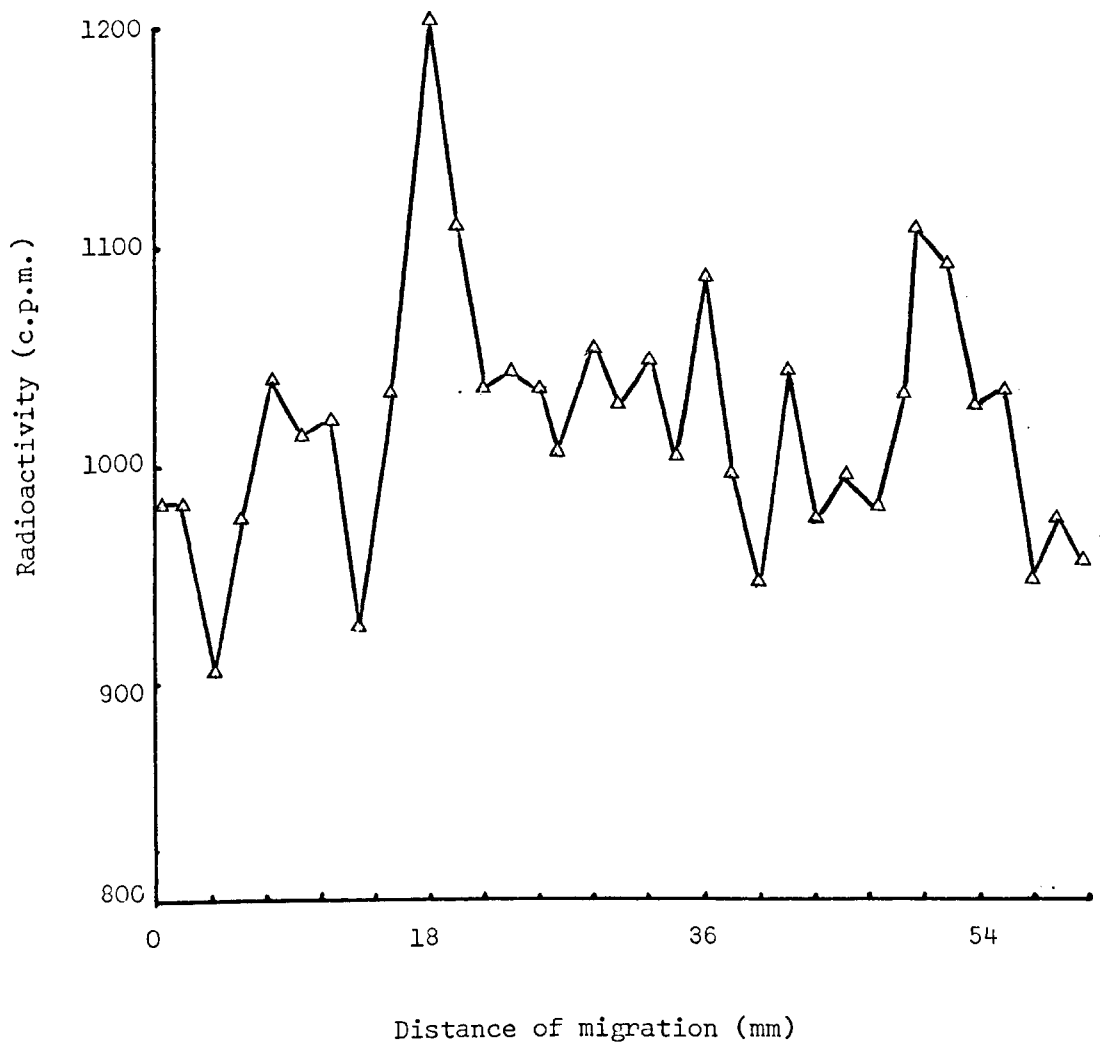
Figure 4.13 Profile of protein-associated radioactivity after

SDS polyacrylamide gel electrophoresis of embryo proteins from

conceptuses that were cultured in the presence of $\left[^3\text{H}\right]$ leucine-

labelled haemoglobin for the last 12 hours of a 48-hour culture

period. Details of method are given in the text.

haemoglobin, has become incorporated into a large array of tissue proteins in both the yolk sac and the embryo. Among the radiolabelled yolk sac proteins three major peaks predominate (labelled A, B and C in Figure 4.12), while a single major peak is observed among embryonic proteins, corresponding to peak C of the yolk sac. All of these major bands of radioactivity are quite distinct from haemoglobin.

4.4.4 The Effect of L-leucine on the Incorporation of Radioactivity by Conceptuses following Culture in the Presence of ^{3}H-labelled Serum Proteins

Figure 4.14 shows that L-leucine reduced the extent of incorporation of radioactivity into both yolk sac and embryo when conceptuses were cultured in the presence of ^{3}H-labelled serum proteins for the last 6 hours of culture. This reduction was proportional to the concentration of L-leucine in the culture medium. At all concentrations of L-leucine used, there was no observed increase in TCA-soluble radioactivity in the medium during culture, and the protein content of conceptuses at harvesting were not noticeably different from that of control conceptuses incubated in the absence of L-leucine (Table 4.2).

The percentage of yolk sac- and embryo-associated radioactivity that was TCA-soluble is shown in Figure 4.15. It may be seen that the proportion of TCA-soluble radioactivity increases as the concentration of L-leucine in the culture medium increases. At low L-leucine concentrations the percentage TCA-soluble radioactivity rises rapidly in both yolk sac and embryo. At L-leucine concentrations of more than 100 µg/ml, percentage TCA-soluble radioactivity increases more slowly, the yolk sac TCA-soluble radioactivity increasing rather more slowly than that in the embryo. At L-leucine concentrations of 1,000 µg/ml, all of the embryo radioactivity is TCA-soluble while more than 80% of yolk sac radioactivity is TCA-soluble.
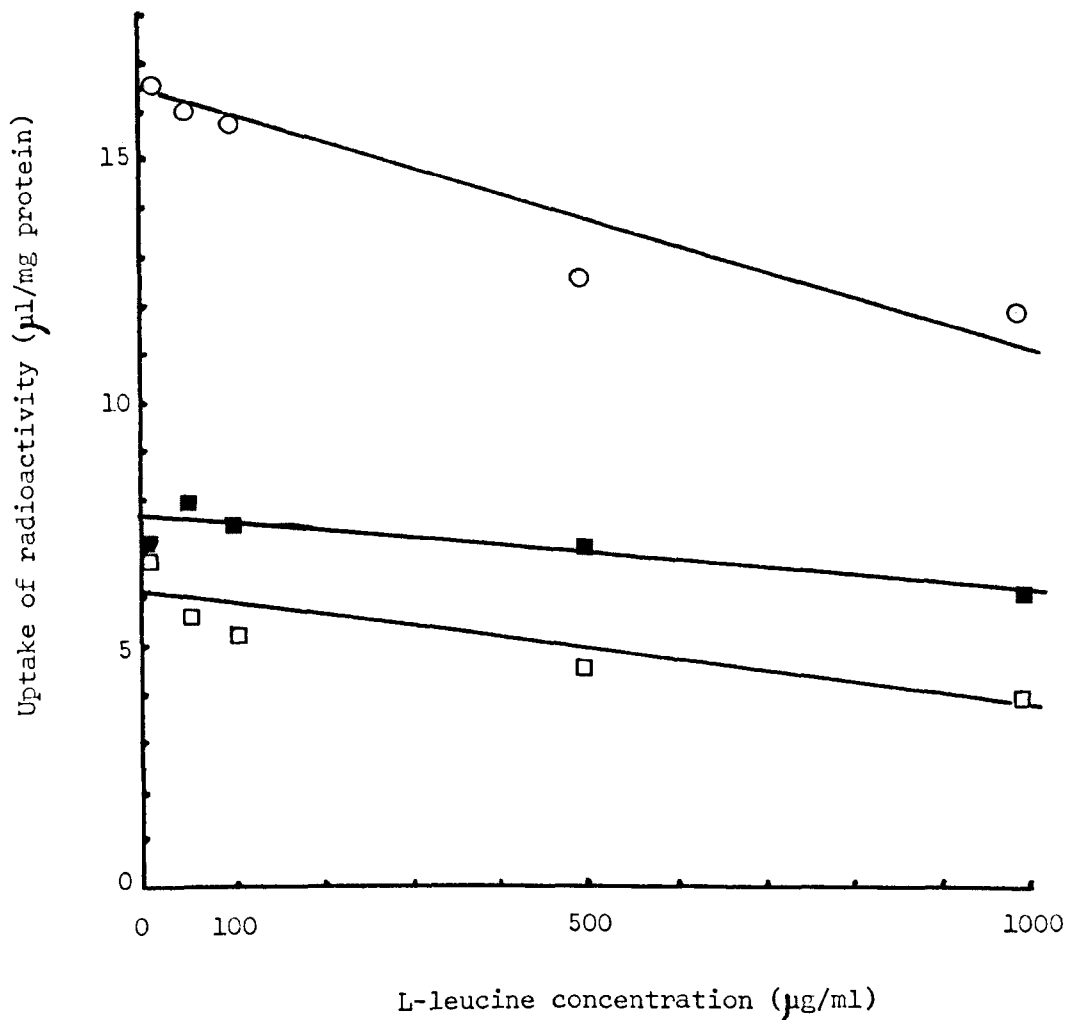
Figure 4.14 Radioactivity associated with yolk sac (■)and
embryo (□), and total radioactivity taken up by yolk sac (O)
after incubation of conceptuses in the presence of $[^{3}H]$ leucine-
labelled serum proteins and L-leucine for the final 6 hours
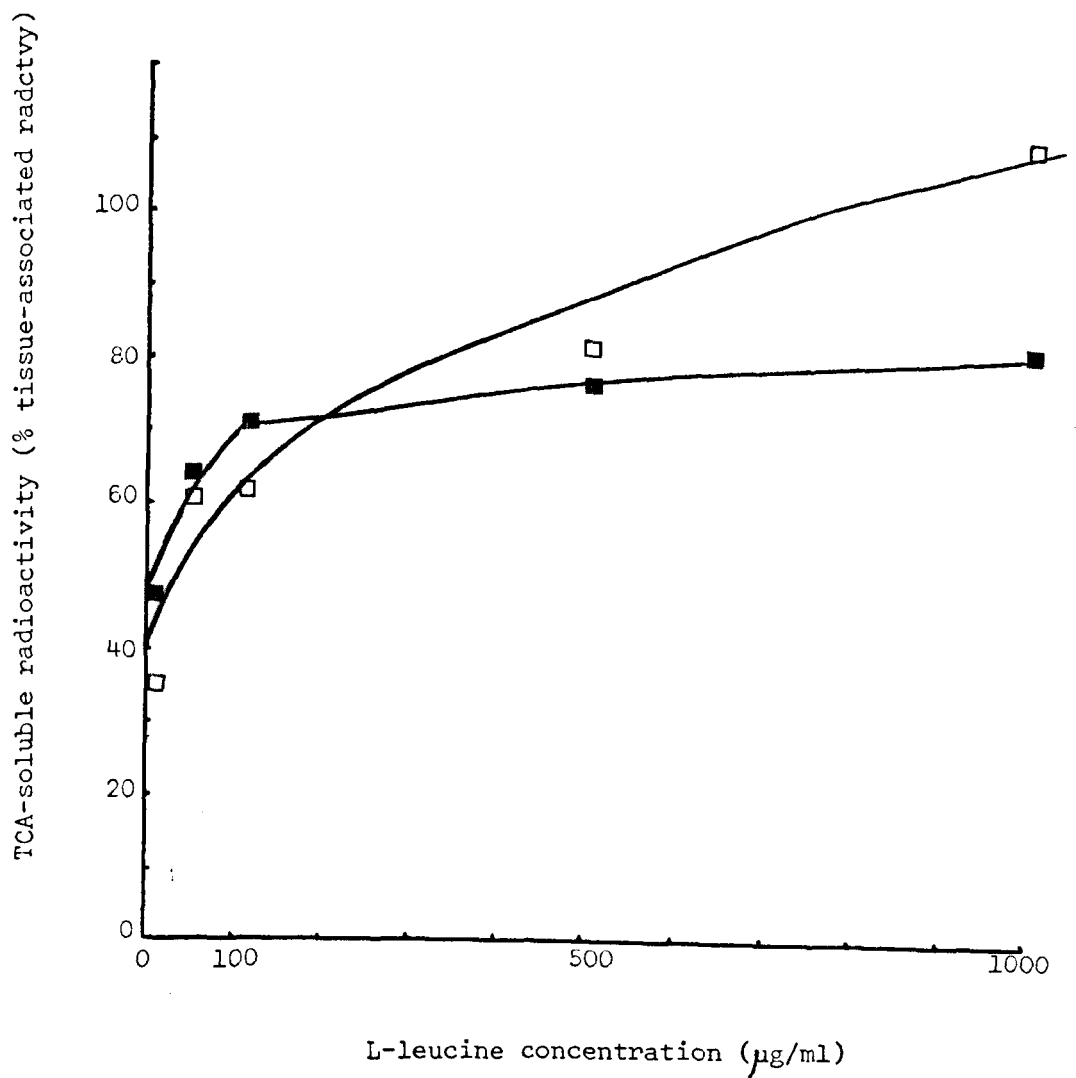of culture. Each point represents the mean of 4 separate
determinations.

Figure 4.15 Percentage of tissue radioactivity that was soluble in TCA after incubation of conceptuses in the presence of $\begin{bmatrix}3\\H\end{bmatrix}$ leucine-labelled serum proteins and L-leucine for the final 6 hours of culture. Each point represents the mean of 4 separate determinations. (■) Yolk sac, (□) embryo.

Concentration of added L-leucine (μg/ml)	Mean radioactivity of dialysed serum (c.p.m. per 0.5 ml)
0	6488 $\pm$ 187 (6)
1	6389 $\pm$ 122 (6)
2	6317 $\pm$ 158 (6)
5	6367 $\pm$ 149 (6)

Table 4.1 The effect of adding L-leucine to [^{3}H]leucine-labelled rat serum before dialysis on the level of radioactivity associated with serum after dialysis. Details of procedure for dialysis are given in the text. Figures in brackets indicate the number of determinations performed. No significant difference (p > 0.1) exists between values as judged by students t-test.

Concentration of L-leucine in culture medium (μg/ml)	TCA-soluble radioactivity in culture medium after culture (% total radio-activity)	Protein contents of embryos/ yolk sacs at harvest-ing (μg)
0	0.65	192.4/117.3
50	0.73	174.6/113.0
100	0.74	217.0/121.8
500	0.73	187.0/125.2
1000	0.60	189.2/120.2

Table 4.2 Lack of effect of L-leucine present in culture for the final 6 hours on the percentage TCA-soluble radioactivity in the culture medium and the protein contents of embryos and yolk sacs at harvesting. Values are the means of 4 determinations.

4.5   Discussion

$\left[^3H\right]$ Leucine-labelled proteins yield a radiolabelled digestion product, $\left[^3H\right]$ leucine, which cells are able to utilise in the de novo biosynthesis of proteins. It is interesting to note therefore that when such proteins were presented to conceptuses in culture, radioactivity was accumulated in the yolk sac and the embryo, the extent of the accumulation being directly proportional to the duration of exposure of the conceptus to the radiolabelled proteins.

The percentage of this radioactivity that was TCA-soluble decreased with increasing duration of exposure to substrate, to steady levels. These data are consistent with the embryo drawing for its amino acid require- ments on the products of yolk sac lysosomal proteolysis, although other explanations are possible. For example, proteins may be taken up by the embryo either directly, or by a route through the yolk sac that avoids lysosomal action. In any such event it must be further postulated that proteins are rapidly degraded by the embryo, and the amino acids generated progressively re-utilised in the synthesis of new protein. Alternatively, radioactivity accumulated by the embryo might derive from a combination of the latter pathway and a mechanism involving yolk sac proteolysis. However, in the light of observations made using the non- digestible substrates ^{125}I-PVP and $\left[^3H\right]$ dextran, and the digestible protein ^{125}I-dBSA, together with the observations of Sharma and Peel (1979) that FITC-serum proteins could only be detected by fluorescence microscopy, in the endoderm of the visceral yolk sac and not in the subjacent mesoderm, any mechanism postulating uptake of intact protein by the embryo is much less plausible than one in which proteolysis by the yolk sac is necessary for biosynthesis of embryonic protein. Taken together, these data provide the first direct experimental

evidence for the mechanism of histiotrophic nutrition postulated by Beck et al. (1967a), described earlier.

Certain quantitative differences are apparent between the uptake of ^{3}H-labelled serum proteins and $\left[^3\text{H}\right]$ haemoglobin by conceptuses. The total quantity of radioactivity pinocytosed by yolk sac when $\left[^3\text{H}\right]$ haemoglobin was present in the culture medium for 12 hours (Figure 4.9) was about 3 times that when conceptuses were exposed to ^{3}H-labelled serum proteins for the same period (Figure 4.5), giving an Endocytic Index of 7.25 for $\left[^3\text{H}\right]$ haemoglobin and 2.19 for ^{3}H-labelled serum proteins. However, direct comparison of these figures is misleading, owing to the differences in the culture medium used in the two experiments. The discussion to the last chapter drew attention to the importance that the protein concentration of the culture medium has upon the extent of pinocytosis of a single protein. The relatively high Endocytic Index reported here with $\left[^3\text{H}\right]$ haemoglobin is probably due, at least in part, to the reduced protein content (and hence competition) in the culture medium. This explanation assumes that haemoglobin uptake is to some extent an adsorptive process. Fridhandler and Zipper (1964) measured $\left[^{14}\text{C}\right]$ haemoglobin uptake by 17.5-day yolk sac cultured in serum-free medium. From their data an Endocytic Index of 4.06 can be calculated, which is approximately twice the fluid-phase rate of capture of ^{125}I-PVP by the 17.5-day yolk sac in serum-free culture medium reported by Ibbotson and Williams (1979). This suggests that haemoglobin uptake by the near-term yolk sac is assisted by some adsorption to the plasma membrane.

The separation of yolk sac and embryo proteins by gel electrophoresis following exposure of conceptuses to $\left[^3\text{H}\right]$ haemoglobin for the last 12

hours or the last hour of culture, has been described. Although the data arising from this study contribute little to the understanding of the mechanism of protein assimilation by yolk sac and embryo, certain qualitative conclusions can be drawn. Firstly, if the embryo does receive intact [^{3}H] haemoglobin from the culture medium, then either the process is slow or the protein is rapidly degraded and re-utilisation of the generated amino acids is slow. Either way, no radioactivity would be demonstrated in the proteins of the embryo after 1 hour of exposure to radiolabel, confirming the kinetic data of Figure 4.7. The yolk sac on the other hand, after a 1 hour exposure to radiolabel, contained a single peak of radioactivity with a slightly higher electrophoretic mobility than the radiolabelled substrate. It is quite probable that this yolk sac radioactivity is derived from pinocytosed [^{3}H] haemoglobin that has been partially hydrolysed. Secondly, both yolk sac and embryo display a whole range of radiolabelled proteins, or protein sub-units, after a 12 hour exposure to [^{3}H] haemoglobin. This conclusively demonstrates that a single protein captured by the conceptus can be utilised by the tissue to form many and varied cellular proteins.

The evidence presented so far all indicates that the yolk sac, through pinocytosis and catabolism of protein, is able to supply the conceptus with amino acids during early organogenesis. Whether or not this process contributes significantly to histiotrophic nutrition in vivo is less certain. It may well be that the conceptuses' requirements for amino acids are met largely by diffusion of exogenous amino acids into embryonic and yolk sac cells, the yolk sac -mediated pathway of protein utilisation being of only supplementary value. Certainly, exogenous amino acids are incorporated into yolk sac and embryonic

protein during early organogenesis (Kernis, 1971; Payne and Deuchar, 1972). On the other hand, the very fact that conceptuses develop normally in vitro in a culture medium of dialysed serum, which contains minute quantities of amino acids, between 9.5 and 11.5 days gestation (Gunberg, 1976; Cockroft, 1979) indicates that protein alone can provide the amino acids necessary for development and growth.

In the present study, the effect on protein utilisation by conceptuses of an excess of L-leucine in a dialysed culture medium containing ^{3}H-labelled serum proteins, was investigated. Over the 6 hour incubation period studied, L-leucine reduced the quantity of radiolabel measured in the yolk sac and embryo tissues by an amount proportional to the concentration of L-leucine in the culture medium. Furthermore, the percentage of tissue-associated radioactivity at harvesting that was TCA-soluble, increased with increased concentration of L-leucine. Indeed, at the highest concentration of L-leucine employed, 1,000 μg/ml, all of the embryo radioactivity and more than 80% of the yolk sac radioactivity was TCA-soluble. At all concentrations of L-leucine used there were no noticeable differences in the protein contents of yolk sacs and embryos at harvesting, compared with controls cultured in the absence of L-leucine. These data are interpreted as follows: pinocytosis of serum proteins and their intralysosomal catabolism in yolk sac, proceed at a normal rate. The generation of $\left[^3H\right]$ leucine by yolk sac proteolysis provided a source of radiolabelled amino acid for protein synthesis in the cells of the conceptus. However, this pool of $\left[^3H\right]$ leucine is diluted by L-leucine which has diffused into the cells from the culture medium, and which competes with $\left[^3H\right]$ leucine for the appropriate tRNA. The greater the concentration of L-leucine, the greater the dilution of $\left[^3H\right]$ leucine in the intracellular leucine pool, and the less the

radiolabelled amino acid will become incorporated into synthesised protein. Evidently, not all the free $\left[^{3}H\right]$ leucine has remained within the tissues of the conceptus and must have been lost, although no increase in the TCA-soluble radioactivity of the medium was observed at the end of culture. Either the small amount of radioactivity lost was excreted into the culture medium and was not detected, or more likely, $\left[^{3}H\right]$ leucine diffused out of the tissues during washing (see Wheatley, 1980). Since all tissues were washed in an identical manner, the extent of the diffusion would depend entirely on the concentration of free $\left[^{3}H\right]$ leucine within the tissues which, as already explained, increases with increased concentration of L-leucine. This effect could be eliminated by the addition of an excess of L-leucine to the washing medium.

The reduced re-incorporation of proteolytically-derived $\left[^{3}H\right]$ leucine into new protein as a result of the presence of an excess of unlabelled L-leucine in cells, has been observed in isolated rat hepatocytes (Hopgood et al., 1977), BHK21 fibroblasts (Hendil, 1980) and mouse peritoneal macrophages (Ehrenreich and Cohn, 1968) and is commonly used in studies of cellular protein degradation of $\left[^{3}H\right]$ leucine-labelled proteins to specifically prevent underestimation of proteolysis due to re-utilisation of $\left[^{3}H\right]$ leucine.

In the present study, the data demonstrate that free amino acid in the culture medium successfully competes with that amino acid derived from proteolysis in the yolk sac for incorporation into yolk sac and embryonic proteins. Therefore, the source of amino acids for protein synthesis in the conceptus is most probably determined by the composition of the histiotroph that bathes the early organogenesis conceptus in utero. This topic is further discussed in Chapter 10.

CHAPTER FIVE

THE EFFECT OF TRYPAN BLUE ON YOLK SAC-MEDIATED NUTRITION

5.1 Introduction

Throughout the preceding chapters reference has been made to the mechanism of histiotrophic nutrition, postulated by Beck et al. (1967a) to function during early organogenesis in the rat. In a companion paper the same authors (Beck et al., 1967b) suggested that the bisazo dye trypan blue might elicit congenital malformations in the offspring of rats treated with the dye by disturbing this nutritional process, a hypothesis that was subsequently modified in detail (Williams et al., 1976). This evidence, along with the corroborative observations of other workers, was described in Chapter 1. However, although the balance of evidence favours yolk sac function as the primary site of teratogenic action of trypan blue, other (less well-formulated) theories have proposed that some aspect of maternal metabolism or the embryo itself might be the principal target of trypan blue's action.

Gilbert and Gillman (1954) suggested that trypan blue-induced teratogenesis in rats was due to erythrophagocytosis in the mother, leading to an acute anaemia and consequent hypoxia. Hommes (1959), citing the observation of Gillman et al. (1948) that trypan blue injection brought about adrenal hypertrophy in the pregnant rat, proposed that embryonic development might in some way be impaired by a disturbance of the maternal hypophyseo-adrenal system. The well-documented alterations in maternal serum proteins occasioned by trypan blue treatment (reviewed by Beck and Lloyd, 1966) led Beaudoin and Kahkonen (1963) to suggest that in rats these changes, generally a fall in the absolute levels of serum albumin and a rise in α- and β-globulins, might in themselves be the principal cause of congenitally malformed offspring, particularly since the composition of yolk sac fluid (Beaudoin and Ferm, 1961) and fetal serum (Beaudoin

and Kahkonen, 1963) are also altered. In a subsequent study however, Beaudoin and Roberts (1965) observed that injection of α- and β-globulins from trypan blue-treated pregnant rats into 8-day pregnant rats did not cause fetal malformations. These workers concluded that the altered α- and β-globulins were not, in themselves, responsible for teratogenesis. Christie (1964) postulated that maternal thyroid dysfunction following trypan blue administration may cause a decrease in oxidative metabolism and a consequently reduced supply of oxygen and essential metabolites to the embryo. But in a later publication the same author (Christie, 1965) observed that, although thyroidectomy potentiated the teratogenicity of trypan blue in rat, thyroidectomy alone failed to produce malformations. Furthermore, Beck and Lloyd (1966) administered excess thyroxine between 8.5 and 10.5 days of gestation to trypan blue-injected rats and failed to reduce the dye's teratogenicity. Therefore the teratogenic effect would seem not to be mediated, directly at least, through the thyroid gland.

Wilson et al. (1959) correlated the end of the teratogenic period of trypan blue in rats with the closure of the yolk stalk and hence the complete surrounding of the embryo by the yolk sac. It was suggested by these authors that trypan blue might act directly on the embryo in some way, and that sensititivy to trypan blue abruptly ceased once this route of access to the embryo was closed, a suggestion also advanced by Greenhouse et al.(1969). However, in a subsequent study using ^{14}C-labelled trypan blue (Wilson et al., 1963), contrary to expectation, no radioactivity above background levels could be detected in the embryo following maternal injection, though the yolk sac contained high levels. Even so there are reports in the literature (Barber and Geer, 1964; Davis and Gunberg, 1968; Greenhouse et al, 1969; Davis and Sauter, 1977; Dencker, 1977) that demonstrate trace quantities

of the dye in the embryonic endoderm. Furthermore Turbow (1966) was able to show that rat conceptuses explanted at 10-11 days of gestation with the yolk sac intact, and cultured for periods up to 48 hours on plasma clots, were more susceptible to the toxic effects of trypan blue when the dye was injected, at low concentrations, into either the amniotic cavity or the extra-embryonic coelom, than when it was present solely in the culture medium and thus in contact with the yolk sac only. Interestingly, at higher concentrations of trypan blue abnormal development was more common when the dye was present in the culture medium only. (The in vitro toxicity of trypan blue, present in the culture medium, to the day 10 rat conceptus has recently also been observed by Beaudoin and Fisher, 1981). All of these observations lend support to the theory of direct action of the dye on the embryo and, as Hamburgh et al. (1975) have pointed out, "the cells of the head process, early notochord and possibly the primitive streak are exposed to fluids in the yolk sac cavity prior to the complete envelopment of the embryo by the yolk sac", suggesting that a surface action of the dye on embryonic cells might well be a causative factor in teratogenesis. Hamburgh et al. (1975) were also perplexed by the fact that only a fraction of those conceptuses exhibiting impaired yolk sac lysosomal enzyme function (indicated as a reduced acid phosphatase activity) as a result of exposure to trypan blue, actually went on to develop abnormally, and they suggested that the effect of trypan blue on the yolk sac might not be the primary cause of teratogenesis.

Another reported effect of trypan blue on the developing rat conceptus was the increase in absorption of certain inorganic ions by the yolk sac, and in some cases by the embryo as well (Kernis and Johnson, 1969). This effect may be of importance in the production of the changes of

volume and pressure of the circulating fluids of rat and chick embryos which Grabowski and his co-workers (Kaplan and Grabowski, 1967; Grabowski et al., 1971; Grabowski, 1977) have suggested as a mechanism of teratogenic action of trypan blue. Grabowski (1977) has observed similar effects in chick embryos resulting from hypoxia. This latter observation is of particular interest since Kaplan and Johnson (1968) suggested that trypan blue might produce hypoxia in chicks by uncoupling oxidative phosphorylation, a mechanism also proposed in rats by Christie (vide supra). Rogers (1978) observed that a teratogenic dose of trypan blue injected into 8.5-day pregnant rats, resulted, by 11.5 days in a substantial increase in the osmolality of embryonic amniotic fluid which correlated closely with the abnormal development of the embryo.

That trypan blue can induce so many and profound effects in biological systems, together with its wide-ranging teratogenicity in many species, including amphibia and reptilia, makes it unlikely that a single mechanism of action of the dye is responsible for the production of malformations.

In the present study, the effect of teratogenic trypan blue on the pinocytic and proteolytic capacity of the yolk sac, and the ability of the conceptus to utilise $\left[^{3}H\right]$ leucine-labelled serum proteins, properties of the early organogenesis conceptus that were established in the preceding chapters, was investigated.

According to Williams et al. (1976), it is the pinocytic ingestion of nutrients by the yolk sac that is inhibited by trypan blue and probably constitutes the primary defect in trypan blue-induced teratogenesis. However, the evidence for this mechanism was obtained using the 17.5-day yolk sac in culture, and, as the authors recognise,

the dye has long ceased to be teratogenic _in vivo_ by this stage.
Furthermore, the composition of the culture medium used in this study
probably differs greatly from the plasma transudate that bathes the
early conceptus _in vivo_. The importance of the composition of the
culture medium in _in vitro_ studies upon the degree of uptake of
macromolecules, has already been stressed. The New culture technique
is therefore ideal to investigate the claim of Williams et al. (1976)
since it uses entire conceptuses from early organogenesis, a stage of
gestation which approximates to the period _in vivo_ when trypan blue
is teratogenic (Wilson et al., 1959), and also since it uses as a
culture medium 100% homologous serum, which probably resembles closely
the environment of the conceptus _in vivo_ during the period of
teratogenic susceptibility to trypan blue.

Several factors are known to influence the teratogenicity of trypan
blue in rodents. Wilson et al. (1959) demonstrated that the time
during pregnancy at which trypan blue was administered to pregnant
rats determined the frequency of malformations. These workers found
that a maximum teratogenicity occurred following dye injection at
8.5 days of gestation, and no teratogenic activity was apparent when
trypan blue given after day 10 of gestation. Several reports
(Gunberg, 1958; Tuchmann-Duplessis and Mercier-Parrot, 1959; Beck
et al., 1960; Hamburgh and Callahan, 1967) have indicated that the
teratogenicity of trypan blue varies with the strain of species used,
while Beck (1967) showed that the position in the uterus occupied
by embryos influenced their susceptibility to trypan blue. Beck (1961)
made the interesting observation that different commercial samples of
trypan blue produced varied response when equivalent doses (in terms
of titratable azo linkage) were administered at the same stage of
pregnancy to the same strain of rat. It was concluded that commercial

preparations of trypan blue were mixtures of substances the proportions of which varied between commercial samples, and consequently doubts were raised as to whether trypan blue was the teratogenically active component of such mixtures.

Trypan blue, whose structure is shown in Figure 5.1, is synthesised by coupling tetrazotized o-tolidine to H-acid (8-amino-1-naphthol-3, 6-disulphonic acid) in alkaline solution (Hartwell and Fieser, 1936). During preparation of the dye, precipitation with NaCl removes trypan blue from solution, thus allowing it to be easily separated from the reaction mixture. However, this treatment can lead to considerable contamination of the dye with NaCl, which in the context of teratogenic testing is undesirable, since it can lead to a grossly inaccurate estimation of the teratogenic dose. In a survey of randomly selected commercial samples of trypan blue, Lloyd and Beck (1963) showed that the NaCl content varied from less than 10% in one sample to more than 70% in another. Clearly, on a weight to weight basis, one preparation contains considerably more trypan blue than the other. In addition to contamination with NaCl, moisture can account for up to 10% of the dye's weight, and a number of coloured impurities, generated through side reactions during synthesis, are also often observed in the dye preparation. Beck and Lloyd (1963) isolated blue, red and purple fractions from one commercial sample of trypan blue by paper chromatography and found that only the blue fraction was teratogenic in rats. Barber and Geer (1964) confirmed that the blue fraction of "trypan blue" was responsible for the production of deformed mouse fetuses, the red and purple fractions being at most only very slightly teratogenic. Only Bertini and Sacerdote (1970), using mice, have reported a teratogenic activity of the red fraction; Field et al. (1977)
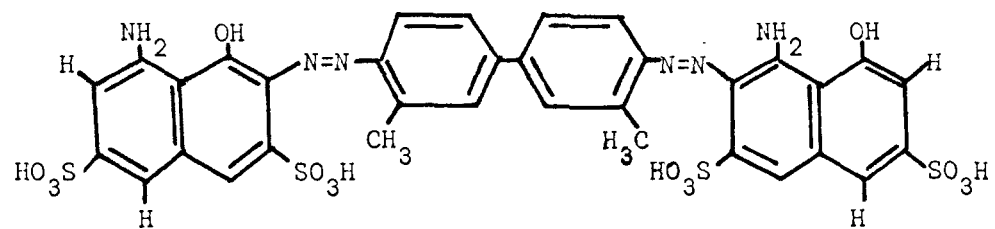
Figure 5.1   Structural formula of trypan blue

also using mice, were however unable to support this finding. The latter authors did however demonstrate a potent teratogenicity of the blue fraction in mice. Thus it seems reasonable to conclude that trypan blue itself, and not a coloured contaminant, is the active teratogenic component of commercial preparations of the dye, and that furthermore, the intact dye molecule and not a reduction product is necessary for this activity (Wilson, 1955; Turbow, 1966; Field et al., 1977).

In view of the above discussion, it is essential that the purity of the dye be evaluated before an accurate estimation of the dosage can be obtained.

5.2   Materials

Chemicals

Trypan blue was a generous gift of Professor F. Beck, University of
<div align="right">Leicester</div>

Alizarin Red S   Koch-Light Laboratories Ltd,

<div align="center">Colnbrook, Berks.</div>

Dowex-1 (H^+ form)(x4, 20-50 mesh)   BDH Ltd, Poole, Dorset

Other chemicals were of analytical grade.

Equipment

Lab. pH meter Model 7020   Electronic Instruments Ltd,

<div align="center">Chertsey, Surrey</div>

Shandon Electrophoresis Apparatus   Shandon Ltd, Cheshire

 Model 477

Chromatography Tank   Shandon Ltd, Cheshire

Chandos power pack Model No. E 26   Chandos, Stockport, Cheshire

Edwards Modulyo Freeze-Drier   Edwards High Vacuum,Crawley, Sussex

Cecil Scanning Spectrophotometer   Cecil Instruments Ltd, Cambridge

5.3   Methods

5.3.1   Estimation of purity of trypan blue preparation

5.3.1.1 Estimation of salt content of trypan blue preparation

Sodium chloride content of the dye sample was estimated using the

method of Lloyd and Beck (1963). Dye sample (10 mg) was accurately

weighed into a 25 ml beaker and dissolved in 5 ml of distilled water.

After 5 minutes of constant stirring, 5 ml of glacial acetic acid was

added, and the mixture, stirred throughout, was titrated with

0.1 M $AgNO_3$ solution, using a 5 ml microburette and the electrode

assembly described by Sanderson (1952). A pH meter was used as the

millivoltmeter, and the end-point, always sharp, taken as 200mV.

A schematic diagram of the apparatus is shown in Figure 5.2.

5.3.1.2 Evaluation of coloured contaminants of trypan blue preparation

(a) Paper Chromatography

Dye sample was prepared as a 2% solution for ascending paper

chromatography, according to the procedure of Lloyd and Beck (1963).

Chromatograms were prepared in cylindrical glass tanks by running

the solvent for 16 hours after an equilibration period of 7 hours.

Spots were applied to 28 x 43cm sheets of Whatman No. 1 chromatography

paper using a glass capillary tube. The solvent system used was

n-butanol-pyridine-water, 1:5:4 by volume. A sample of trypan blue

of known purity was included in the chromatogram for comparison.

(b) Paper Electrophoresis of Reduction Products of Dye

Dye sample (20 mg) was dissolved in 2 ml of water with stirring, and the

solution heated to about 80°C. With stirring and temperature maintained

throughout a freshly prepared 10% (w/v) aqueous solution of sodium

dithionite ($Na_2S_2O_4$) was added in drops until the dye solution had

0.1 M AgNO$_3$

mV meter

platinum electrode (+)

silver electrode (-)

trypan blue/acetic acid mixture
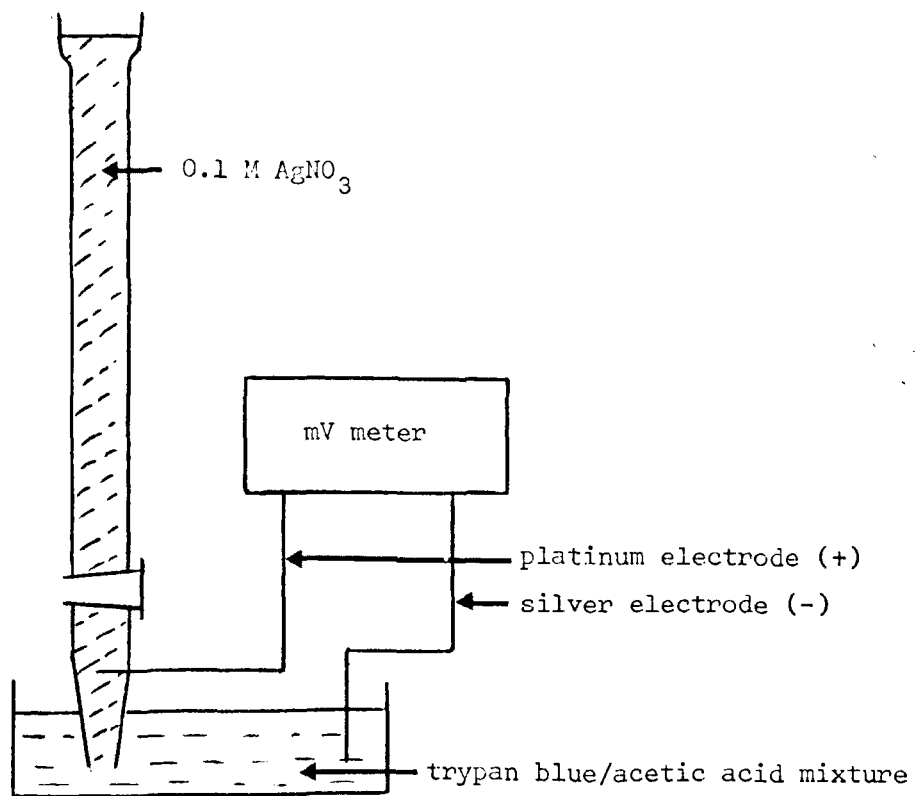
Figure 5.2 Apparatus of Sanderson (1952) for determination
of chloride ions by potentiometric titration

lost its blue colour. Electrophoresis was immediately performed
using the method of Lloyd and Beck (1964). Strips (16 x 4 inches)
of Whatman No. 1 paper were dipped in 0.6 M sodium acetate–acetic
acid buffer, pH 4.0, and excess solvent removed by dabbing with tissue.
Spots of the reduced dye solution were applied to the paper with a
glass capillary and electrophoresis carried out for 50 minutes with
a field strength of 10 V/cm. The papers were dried, sprayed with
Ehrlich's reagent (100 mg of p-dimethylaminobenzaldehyde in 50 ml of
ethanol to which had been added 1 ml of concentrated HCl) and dried
in an oven.

(c) Ion-exchange Chromatography of Reduction Products of Dye

Dye sample (5 mg) was dissolved in 2 ml of water and heated to 80°C before
reduction with 2% (w/v) sodium dithionite. To remove anions the
resulting colourless solution was acidified by the addition of 2 drops
of concentrated HCl. Ion-exchange chromatography was then performed
using the method of Field et al. (1977), using a resin of Dowex-1
(x4, 20–50 mesh) in a column 7 cm long x 1.5 cm diameter. The reduced
and acidified solution was applied to the column that had been
thoroughly washed with 0.01 M HCl, and eluted with 0.01 M HCl. Fractions
(2 ml) were collected and the presence of biphenyl determined by spotting
onto chromatography paper and spraying with Ehrlich's reagent. Those
fractions with demonstrable biphenyl content were diluted with 0.1 M HCl
and their ultraviolet absorption spectra obtained with a scanning
spectrophotometer.

5.3.2 Purification of Trypan Blue for Teratology and Culture Experiments

Crude trypan blue was made into a 1% solution and extensively dialysed
against running tap water. After 3 days of dialysis, the solution was

concentrated by rotary evaporation before being evaporated to dryness using a freeze-dryer. The resulting powder was then heated in an oven at 110°C for 16 hours (Lloyd and Beck, 1963) to remove moisture. The NaCl content of the dialysed sample was then estimated as described, and found to be less than 1% (w/v).

5.3.3   Teratogenic Testing of Trypan Blue

Pregnant rats received a subcutaneous injection of purified trypan blue at a dosage of 100 mg/kg body weight contained within 1.0 ml of 0.9% saline, at either 8.5 or 9.5 days of gestation. Animals were sacrificed at 20.5 days of gestation and the uterus examined for the number of implantations, viable young and resorption sites. All fetuses were weighed, examined for external malformations and were either fixed in Bouin's fluid (saturated picric acid solution-40% formaldehyde-acetic acid, 15:5:1) or preserved in 70% alcohol. Of every three fetuses, two were fixed in Bouin's fluid and one in alcohol. Following exposure to Bouin's fluid for 7 - 10 days, fetuses were sectioned free-hand with a razor blade to examine for visceral anomalies, according to the method outlined by Wilson (1965), and depicted in Figure 5.3.

Those fetuses preserved in alcohol were subsequently transferred to acetone to defat for 24 hours, before further treatment with alcohol for 24 hours. Fetuses were then transferred to 2% KOH for clearing of soft tissues. The bones were usually visible after 4 days in KOH. Fetuses were then stained overnight in 0.01% alizarin red S in 2% KOH, and subsequently destained in a solution of glycerol and 2% KOH (at a ratio of 1:3). Fetuses were examined for skeletal defects. Pregnant control rats received an injection of 1.0 ml of 0.9% saline.
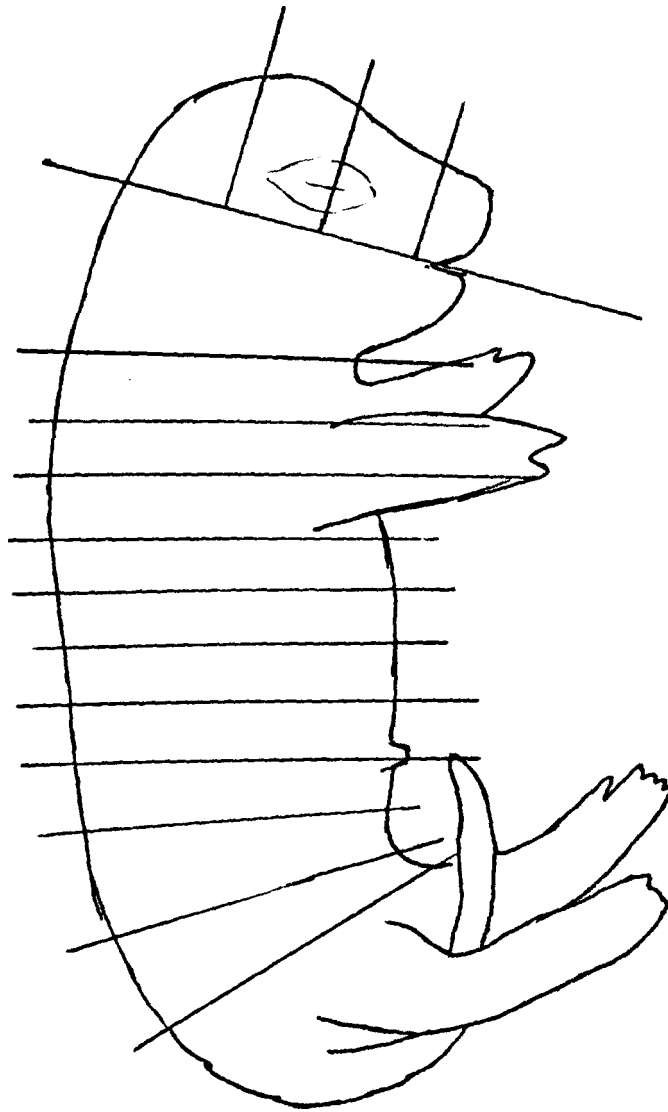
Figure 5.3   20.5-Day rat fetus marked to show approximate levels of freehand razor sections for preparing slices to examine for internal malformations

5.3.4   Culture Experiments

All cultures were of 48 hour duration. For the last 6 hours of all cultures, either ^{125}I-PVP (2.3 μg/ml) or ^{125}I-dBSA (11 μg/ml) were added to the culture medium, or conceptuses were transferred to a culture medium of glucose- and vitamin-supplemented dialysed serum containing [3H] leucine-labelled proteins. In those experiments which used as radiolabelled substrate ^{125}I-PVP, trypan blue was added to the culture medium at the same time as the substrate at concentrations of 10, 20, 40, 60, 80, 100, 200 or 400 μg/ml. Control conceptuses were cultured in the presence of ^{125}I-PVP only. On the basis of results of culture experiments using ^{125}I-PVP as substrate, two concentrations of trypan blue (50 and 200 μg/ml) were chosen, to investigate the effect of the dye on the uptake and digestion of ^{125}I-dBSA. Trypan blue was added to the culture medium at either the same time, or 6 hours before, the addition of ^{125}I-dBSA. In those experiments in which the dye was added before the radiolabelled substrate, conceptuses were transferred into fresh medium containing no trypan blue, before the addition of ^{125}I-dBSA. Therefore, the only trypan blue present during the last 6 hours of such cultures was either adsorbed onto the external surfaces of, or contained within, the tissues of the conceptuses. In cultures in which the dye was added to the culture medium at the same time as ^{125}I-dBSA, a control group of conceptuses received only ^{125}I-dBSA, while in cultures in which trypan blue was administered to the culture medium prior to substrate, control conceptuses were not exposed to dye but were transferred into fresh medium and exposed to ^{125}I-dBSA.

Precisely the same experimental protocol as that described for ^{125}I-dBSA was followed in cultures which used as radiolabelled substrates [3H]-labelled serum proteins. Uptake was measured as described in previous chapters.

5.4   Results

5.4.1   Estimation of Purity of Trypan Blue Preparation

Table 5.1 shows that the crude sample of trypan blue that was to be used in this study, contained an exceedingly high percentage, 83.2%, of NaCl. Paper chromatography of the sample indicated the presence of two coloured components, a major blue fraction (R_f value = 0.53) and a faint purple fraction (R_f = 0.73). Since the purple fraction evidently constituted a very small proportion of the total weight of the crude sample, the dye was purified by dialysis only. Dialysis of a 1% solution of dye against running tap water for 3 days, reduced the NaCl content to approximately 1% (w/w). Reduction of the dye sample by $Na_2S_2O_4$ produced a biphenyl that had an electrophoretic mobility identical to that of o-tolidine and the biphenyl of reduced pure trypan blue, and stained orange following treatment with Ehrlich's reagent. Since the electrophoretic mobilities of a number of aminobiphenyls are not dissimilar, the ultraviolet absorption spectra of the biphenyls were determined. In reduced crude trypan blue, reduced pure trypan blue and o-tolidine solutions, only one peak of maximum absorption, which was common to all three solutions, was seen. The wavelength at which this maximum occurred was 248nm, the ultraviolet absorption maximum of o-tolidine in acid solution (Field et al., 1977).

These observations were taken to indicate that the major coloured component of the dye sample used in this study was trypan blue.

5.4.2   Teratogenic Testing of Trypan Blue

Injection of the dialysed trypan blue (100 mg/kg) into pregnant rats at either 8.5 or 9.5 days of gestation resulted in the production of

	Sodium chloride content (% of total weight)	Paper chromatography		Biphenyl	
		R_f value of main blue spot	R_f value of other spots	colour on spraying with Ehrlich's reagent	Absorption maximum in 0.1 M HCl
Crude trypan blue	83.2	0.53	purple, 0.73	orange	248 nm
Pure trypan blue	−	0.53	−	orange	248 nm
o-tolidine	−	−	−	orange	248 nm

Table 5.1 Estimation of the purity of a crude sample of trypan blue used in the present study. A pure sample of trypan blue and the biphenyl of trypan blue, o-tolidine, were included in some of the analyses for comparison. Experimental details are given in the text.

malformed fetuses (Table 5.2). Following injection at 8.5 days, the number of resorption sites visible at 20.5 days of gestation constituted 14% of the total number of implantation sites. Following injection at 9.5 days of gestation, the resorption rate had fallen to 6%, barely different from that observed in saline-injected controls. Of the surviving fetuses, some 42% of those from rats injected at 8.5 days, and 22% at 9.5 days, were malformed. The types of defects observed are listed in Table 5.3. In both the 8.5 day- and 8.5 day-injected groups, the most common defect noted was hydrocephalus. A high incidence of tail defects was also observed in both groups, while in the 8.5 day-injected group there was a high frequency of kidney malformation. The occurrence of spina bifida in both groups was noted, and in addition the 8.5 day group included one case of anophthalmia, and somewhat surprisingly, three fetuses with limb defects. The latter are very rarely described in mammals following trypan blue treatment. The average weight of surviving fetuses in both 8.5 day- and 9.5 day-injected groups was significantly different ($p < 0.001$) than controls injected with 0.9% saline at corresponding days of gestation.

5.4.3   Culture Experiments

The viability of conceptuses cultured in the presence of trypan blue for a period of 6 hours, either from 36-42 hours of culture or 42-48 hours, did not differ from that in cultures in which trypan blue was absent from the culture medium, and was at least 95%. Furthermore, no developmental deviations were noted in conceptuses exposed to trypan blue during culture.

Nature and time of injection	No. of ♀ treated	No. of implantations (% resorptns)	Av. weight surviving fetuses (g)	External Abnormalities		No. of fetuses sectioned	No.(%) sectioned fetuses with anomalies	No.of fetuses stained with alizarin	No.(%) stained fetuses with anomalies
				No. %surviving fetuses	% total implntns				
Trypan blue 8.5 days	8	93 (14)	$3.47 \pm .92$*	8 10.00	8.60	53	22 (42)	27	0
Trypan blue 9.5 days	8	86 (6)	$3.36 \pm .65$*	5 6.17	5.81	55	12 (22)	26	0
Saline 8.5 days	4	45 (4)	$4.19 \pm .42$	0 0	0	28	0	15	0
Saline 9.5 days	4	44 (5)	$4.40 \pm .60$	0 0	0	30	0	12	0

* significantly different from saline-injected controls ($p < 0.001$)

Table 5.2   Effects on offspring of s.c. injection of trypan blue (100 mg/kg) or saline (0.9%) into pregnant rats. Details of method are given in text.

Day of injection of trypan blue	8.5	9.5
Abnormality		
Anophthalmia	1 (80)	0 (81)
Absent or abnormal tail	5 (80)	5 (81)
Kidney (deformed)	8 (53)	1 (55)
Limb defects	3 (80)	0 (81)
Spins bifida	3 (80)	2 (81)
Hydrocephalus	13 (80)	10 (81)

Table 5.3 Abnormalities of fetuses after
trypan blue treatment of pregnant rats.
Figures in brackets indicate the size of
the population examined for that particular
defect.

When trypan blue, at concentrations of 100 µg/ml or more, was present in the culture medium for the last 6 hours of culture, the protein contents of yolk sacs and embryos did not differ from control levels (Figure 5.4). The same can be said to apply to the protein levels of conceptuses following exposure to trypan blue between 36 and 42 hours of culture (Figure 5.4).

The uptake of radioactivity by conceptuses, incubated for the last 6 hours of culture in the presence of ^{125}I-PVP (2.3 µg/ml) and different concentrations of trypan blue, is shown in Figure 5.5. As previously observed, essentially no radioactivity was present in the embryo at harvesting. In the yolk sac, the uptake of radioactivity increased with increased concentration of trypan blue up to dye concentrations of 70 µg/ml. At concentrations of trypan blue in excess of 200 µg/ml, the uptake of ^{125}I-PVP by yolk sac was approximately that of control levels.

When conceptuses were incubated for the last 6 hours of culture in the presence of ^{125}I-dBSA (11 µg/ml) and trypan blue (50 or 200 µg/ml) (Figure 5.6), little radioactivity was measured in the embryos at harvesting, and all of this was TCA-soluble. At a trypan blue concentration of 50 µg/ml the total uptake of radioactivity by conceptuses (tissue-associated radioactivity plus TCA-soluble radio-activity released back into the culture medium) was about 80% of the control value. Of this radioactivity, a larger proportion was associated with the yolk sac (12%, 39% TCA-soluble) in experimental conceptuses, compared with controls (6%, 43% TCA-soluble). A qualitatively and quantitatively similar effect was observed when trypan blue at a higher concentration, 200 µg/ml, was present in the culture medium. Total uptake of radioactivity by conceptuses was decreased to about 80% of
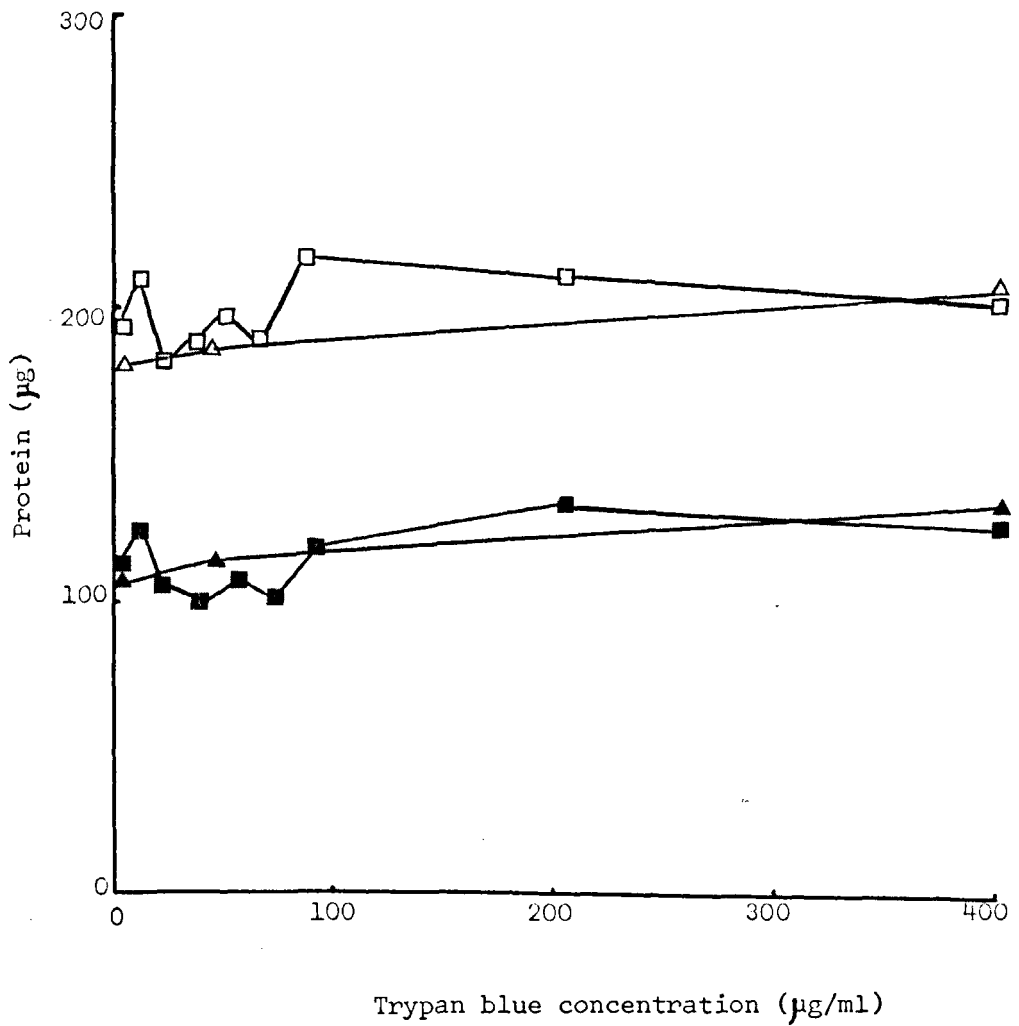
Figure 5.4 Protein contents of yolk sacs (filled symbols) and embryos (open symbols) after incubation of conceptuses in the presence of trypan blue for either the final 6 hours (squares) or the penultimate 6 hours (triangles) of culture. Values are means of at least 6 determinations.
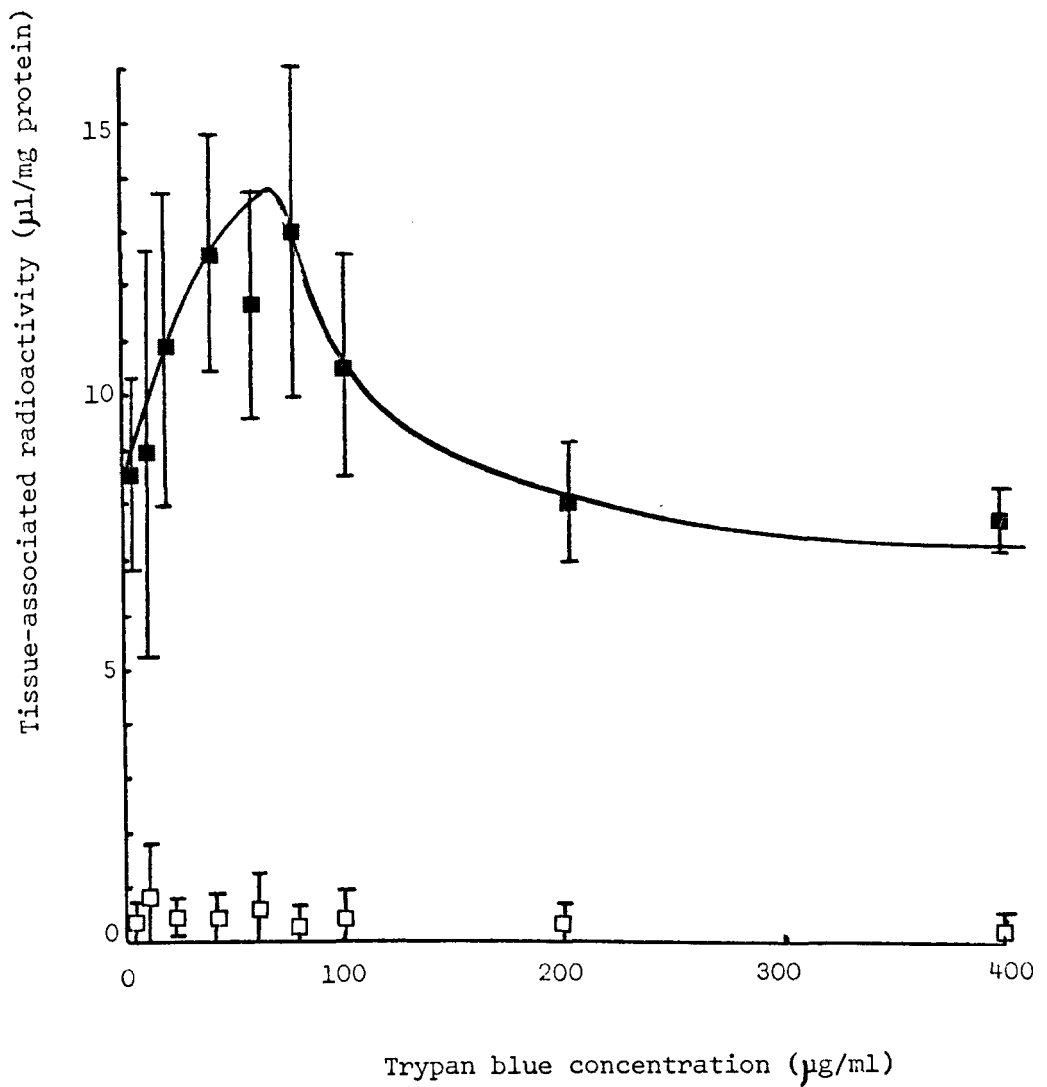
Figure 5.5 Radioactivity associated with the yolk sac (■) and embryo(□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 µg/ml) and trypan blue for the final 6 hours of culture. Values are means (+ S.D.) of at least 6 determinations
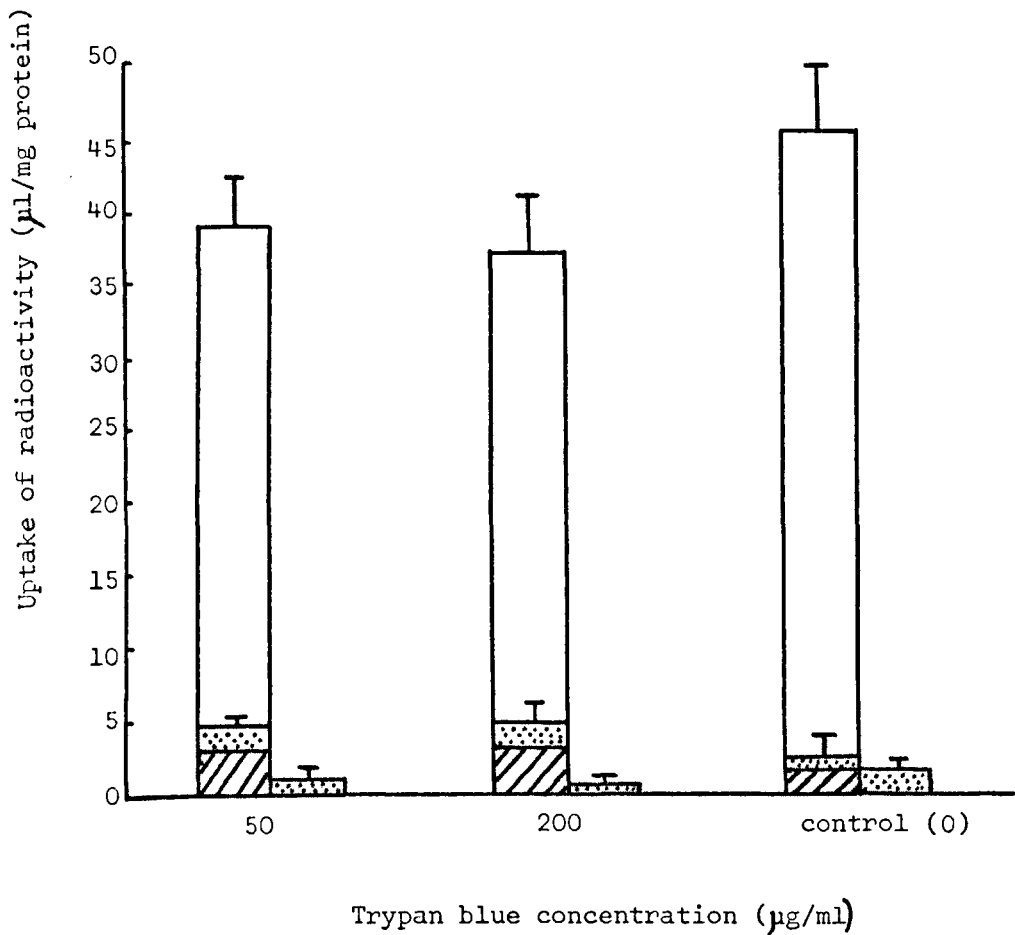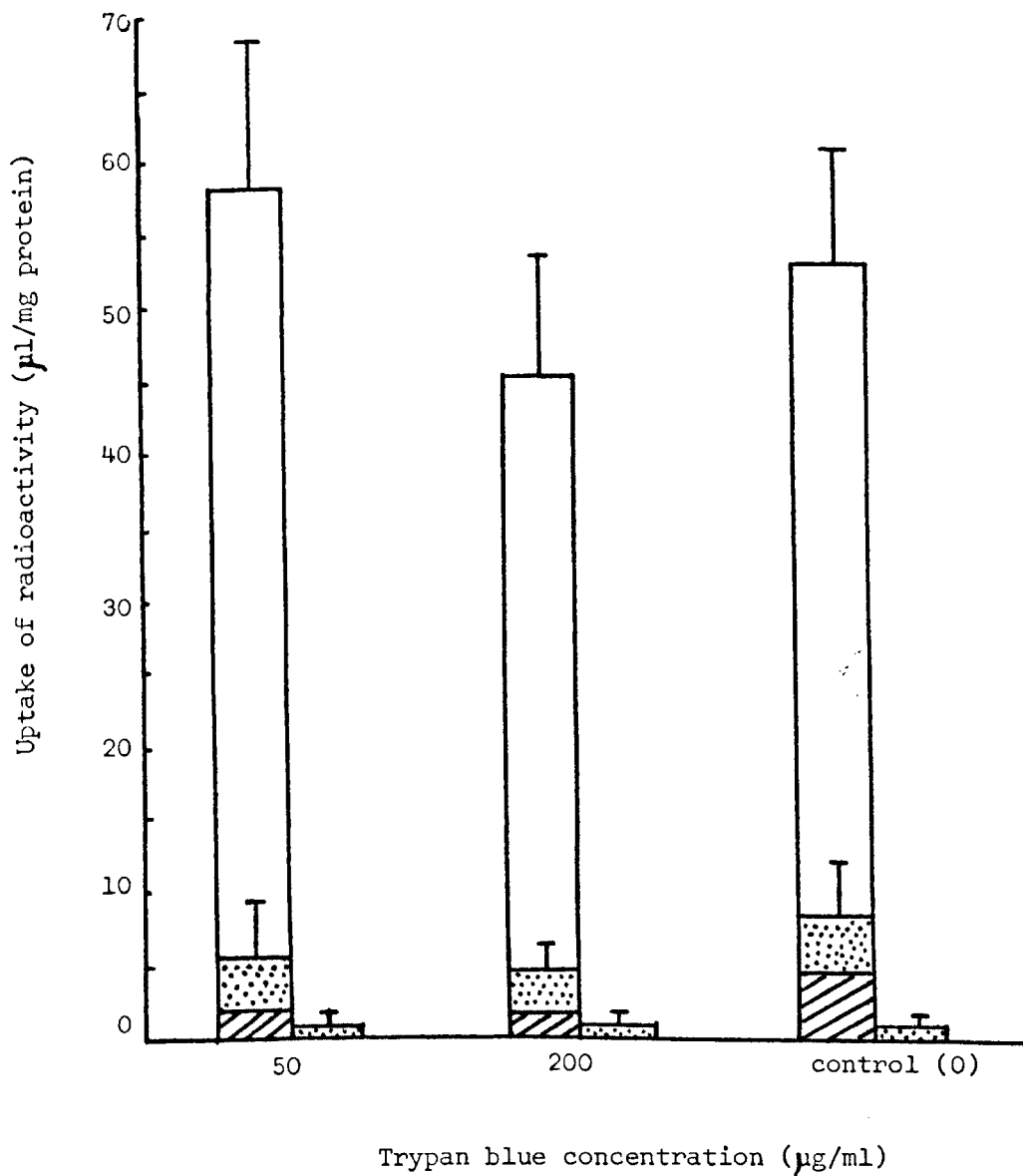
Figure 5.6 Total radioactivity taken up by the yolk sac
(each left hand column) and embryo (each right hand column),
and the radioactivity associated with the yolk sac (total
shaded area of each left hand column) after incubation of
conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) and trypan
blue for the final 6 hours of culture. The proportions of
yolk sac- and embryo-associated radioactivity that were TCA-
soluble ▒ and TCA-insoluble ▨ are indicated. Values are means
($\pm$ S.D.) of at least 6 determinations.

the control value. The proportion of this radioactivity that was yolk sac-associated was again higher than in controls, 13% compared to 6%. About 36% of yolk sac-associated radioactivity was TCA-soluble (controls 43%). The differences between the total uptake of radioactivity by conceptuses at both concentrations of trypan blue and the control value were significant ($p < 0.02$). The corresponding increases in yolk sac-associated radioactivity in these experiments were also significantly different from controls ($p < 0.02$).

The uptake of radioactivity by conceptuses following incubation with trypan blue (50 or 200 μg/ml) between 36 and 42 hours of culture, and with ^{125}I-dBSA but not trypan blue between 42 and 48 hours of culture, is shown in Figure 5.7. Little radioactivity, all TCA-soluble, was detected in embryos at harvesting. Total uptake of radioactivity by conceptuses exposed to trypan blue at a concentration of 50 μg/ml was slightly higher than the control value, while those conceptuses exposed to 200 μg/ml of trypan blue showed a slightly lower total uptake than controls. These differences were not statistically significant ($p > 0.1$). At both concentrations of trypan blue employed, the proportion of the total radioactivity processed by conceptuses that was associated with the yolk sac at harvesting was also insignificantly different from controls ($p > 0.05$).

The effect of trypan blue at a concentration of 50 or 200 μg/ml, present in the culture medium either between 36 and 42 hours of culture or 42 and 48 hours of culture, upon the uptake of radioactivity by conceptuses when ^{3}H-labelled serum proteins were present in the culture medium for the last 6 hours of culture, is shown in Figures 5.8 and 5.9. It may be seen that the exposure of conceptuses to trypan blue during the periods described did not noticeably affect ($p > 0.1$ for all comparisons
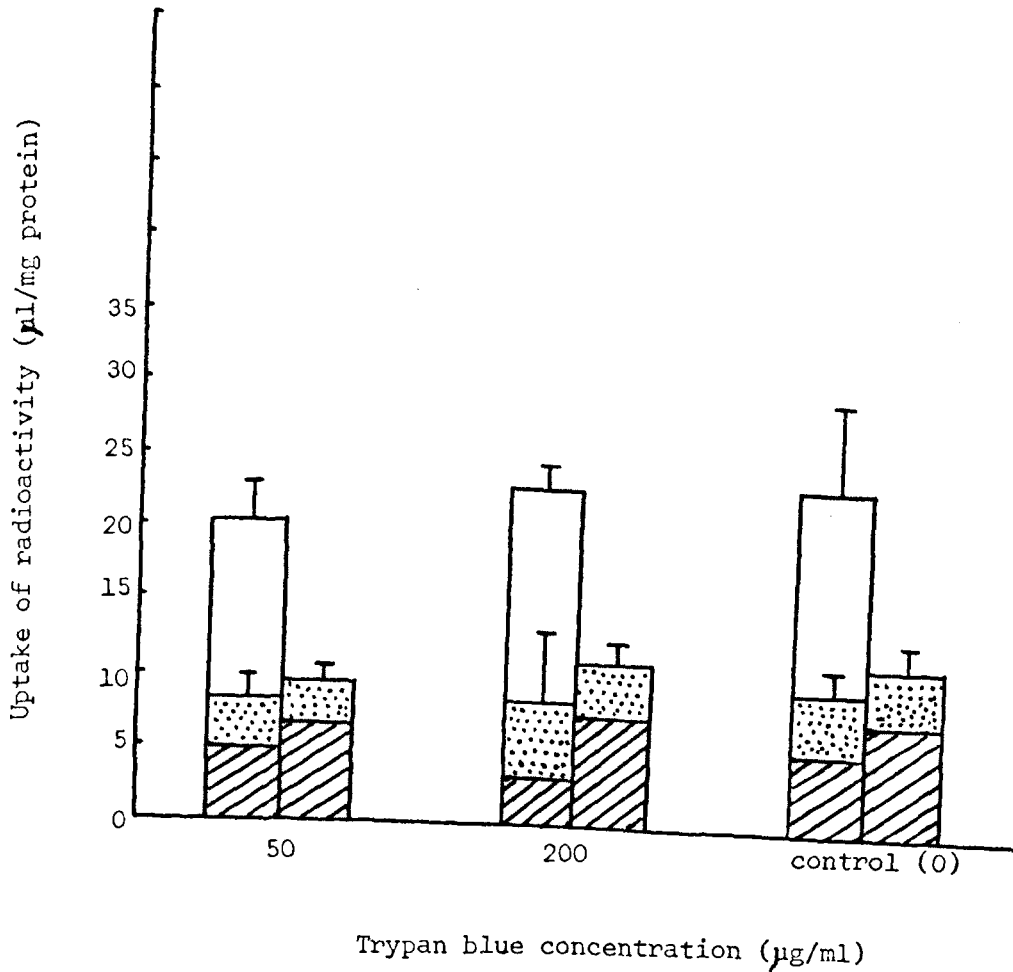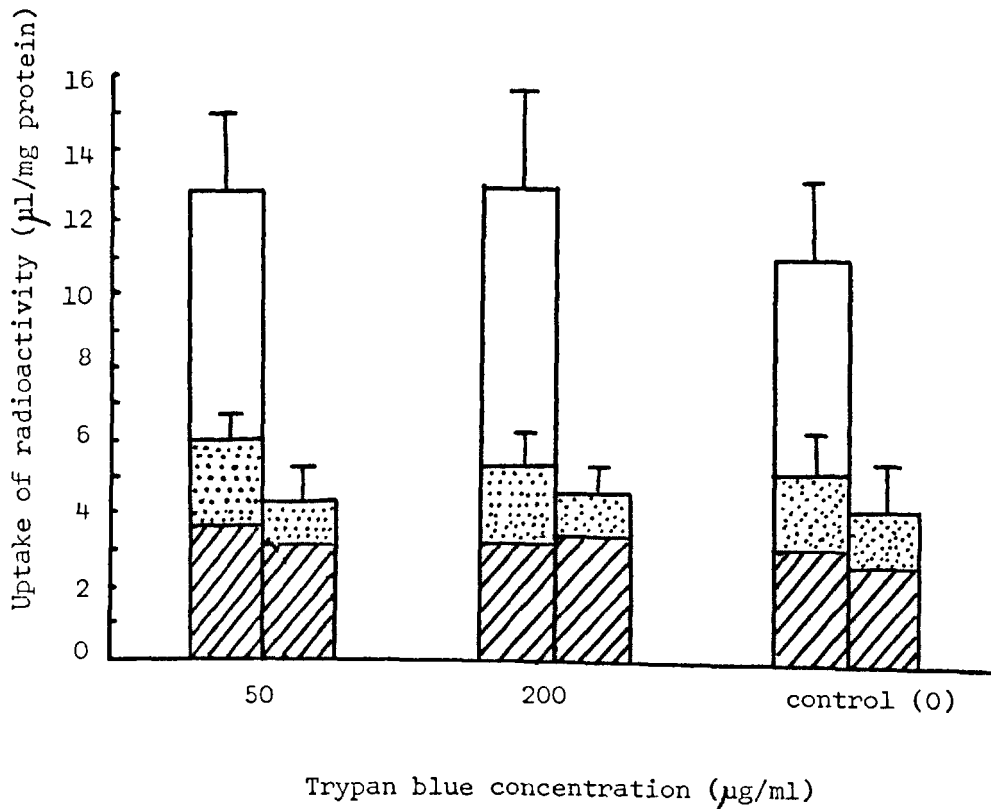
Figure 5.7 Total radioactivity taken up by the yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) for the final 6 hours, and trypan blue for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble and TCA-insoluble are indicated. Values are means ($\pm$ S.D.) of at least 6 determinations.

Figure 5.8 Total radioactivity taken up by the yolk sac
(each left hand column) and embryo (each right hand column),
and the radioactivity associated with the yolk sac (total
shaded area of each left hand column) after incubation of
conceptuses in the presence of $[^3H]$ leucine-labelled serum
proteins and trypan blue for the final 6 hours of culture.
The proportions of yolk sac- and embryo-associated radioactivity
that were TCA-soluble [▦] and TCA-insoluble [▨] are indicated.
Values are means (± S.D.) of at least 6 determinations.

Figure 5.9 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$ leucine-labelled serum proteins for the final 6 hours, and trypan blue for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▓ and TCA-insoluble ▨ are indicated. Values are means (± S.D.) of at least 6 determinations.

with control values) the uptake, digestion or distribution of radiolabel within the conceptus. In no instance was there an increase in the TCA-soluble radioactivity of the culture medium at harvesting over the initial level.

5.5   Discussion

The preparation of trypan blue used in the present study was shown by paper chromatography to be substantially free of coloured contaminants. It did however contain a high salt content which was effectively removed by dialysis, to give a highly purified sample of dye.

Before proceeding to quantitative studies of the effect of trypan blue upon histiotrophic nutrition in the cultured 9.5-11.5 day conceptus, it was essential to establish the dye's teratogenicity in the strain of rats used throughout this study. Maternal injection of a teratogenic dose of trypan blue on days 8.5 and 9.5 of pregnancy has been shown to produce the highest number of malformed offspring (Wilson et al., 1959), and therefore these times were selected. Although the resorption rate observed in the present study is lower than has previously been reported (Beck and Lloyd, 1963a,b; Beck et al., 1978), the percentage of survivors that were malformed (Table 5.2) is comparable to those figures in the reports cited. The types of malformation noted here have all previously been observed following trypan blue tratement. In contrast to the findings of Jensh and Brent (1967) and Berry (1970), but in agreement with Beaudoin and Kahkonen (1963), fetuses surviving trypan blue injection of the mother showed a significant decrease in weight at 20.5 days ($p < 0.001$).

The claims of Beck et al. (1967b) and Williams et al. (1976) that the mechanism of teratogenic action of trypan blue is an inhibition yolk sac-mediated histiotrophic nutrition were investigated using the New culture technique.

The protein contents of yolk sacs and embryos exposed to trypan blue for a period of 6 hours during culture are not different from those tissues cultured in the absence of any trypan blue. This is perhaps not surprising when it is considered that following trypan blue injection into day 7 pregnant rats, the embryonic protein content does not become significantly lower than that from untreated mothers until the 30-35 somite stage (approximately gestational day 11.5) (Berry, 1970). More recently Fisher (1981) observed that day-10 rat embryos, following a 4 hour in utero exposure to trypan blue, did not incur a protein deficit until after a further 24 hour period in culture. Therefore in the present study, it may well be that trypan blue was not present in culture for long enough, or that insufficient time was available after exposure to trypan blue for alterations in yolk sac and embryo protein levels to become apparent.

When trypan blue and ^{125}I-PVP were present together in the culture medium for the last 6 hours of culture, there was no uptake of radioactivity into the embryo. In the yolk sac, the uptake of radioactivity at harvesting varied biphasically as a function of the concentration of trypan blue. At concentrations of dye below 100 μg/ml, the pinocytosis of ^{125}I-PVP was stimulated, while concentrations of dye above 200 μg/ml had little effect on the uptake of radioactivity. A similar biphasic effect of trypan blue on ^{125}I-PVP uptake by the cultured 17.5-day yolk sac has been reported (Williams et al., 1973). These authors however observed an inhibition of pinocytosis at concentrations of 200 μg/ml and above. No such inhibition of pinocytosis was seen in the present study. Roberts et al. (1980) demonstrated that the stimulation of ^{125}I-PVP uptake into 17.5-day yolk sac by trypan blue was not due to an enhanced rate of pinosome formation. Trypan blue was shown to enter the yolk sac by adsorptive pinocytosis, and by interacting with

^{125}I-PVP enabled the latter substrate also to enter by an adsorptive mechanism; so-called piggy-back pinocytosis.

When trypan blue and ^{125}I-dBSA were present in the culture medium for the last 6 hours, little radioactivity, and all of it TCA-soluble, was detected in the embryos at harvesting. In the yolk sac, a trypan blue concentration in the culture medium of 50 μg/ml increased the tissue-associated radioactivity and reduced the total clearance of substrate by the yolk sac, relative to controls. A marginally higher tissue-associated radioactivity and lower total clearance of substrate by yolk sac was produced by 200 μg/ml of trypan blue. These data are consistent with both an inhibition of the yolk sac intralysosomal hydrolysis of ^{125}I-dBSA by trypan blue, and with an inhibition of pinocytosis of the substrate.

Pre-loading of yolk sac with trypan blue for 6 hours before incubation of conceptuses with ^{125}I-dBSA for the last 6 hours of culture resulted in there being little radioactivity in the embryo at harvesting. Again this was all in TCA-soluble form. At both concentrations of trypan blue used, the total clearance of radiolabelled protein by yolk sac was little affected. Though shown to be statistically insignificant, the yolk sac-associated radioactivity was reduced.

When trypan blue was presented to conceptuses in culture, either together with or before ^{3}H-labelled serum proteins, the dye had no measurable effect on the pinocytosis, digestion or distribution of radiolabel within the conceptus, over the time periods studied.

Taken together, these data lend no support to the hypothesis that trypan blue inhibits pinocytosis by the yolk sac, thereby depriving the developing embryo of essential nutrients during this critical period of embryogenesis. Only when trypan blue and ^{125}I-dBSA were added

simultaneously to the culture medium, was the uptake of radioactivity
by conceptuses decreased. However, concentrations of trypan blue that
decreased the uptake of ^{125}I-dBSA did not inhibit pinocytosis of
^{125}I-PVP or ^{3}H-labelled serum proteins. Two explanations are
consistent with these observations. Either trypan blue competes for
those sites on the plasma membrane to which ^{125}I-dBSA adsorbs and which
facilitate the more rapid entry of this substrate into yolk sac cells,
or trypan blue interacts with ^{125}I-dBSA to reduce the substrate's
affinity for these binding sites on the plasma membrane. This latter
explanation would seem the more plausible in view of trypan blue's
action in decreasing the hydrolysis of ^{125}I-dBSA in yolk sac (see
below). The stimulation of pinocytosis of ^{125}I-PVP by trypan blue
at concentrations of 100 μg/ml or less, appears to be specific for that
radiolabelled substrate since the uptake of ^{125}I-dBSA and ^{3}H-labelled
serum proteins was not similarly enhanced by trypan blue. This
conclusion agrees with that of Roberts et al. (1980) who found that in
the cultured 17.5-day yolk sac, trypan blue at concentrations that
stimulated the pinocytosis of ^{125}I-PVP had no effect upon the uptake
of colloidal $\left[^{198}\text{Au}\right]$ gold or ^{125}I-dBSA.

The hypothesis of Beck et al. (1967a) that trypan blue's teratogenicity
is due to an inhibition of proteolysis by yolk sac lysosomal enzymes,
receives only limited support from the data presented here. Only when
trypan blue was added to the culture medium at the same time as
^{125}I-dBSA was there observed an increase in the yolk sac-associated
radioactivity consistent with an inhibition of yolk sac proteolysis.
The simultaneous addition of the two substances makes it highly likely
that both are taken into the yolk sac within the same pinocytic vesicle.
Trypan blue is therefore on hand to inhibit the hydrolysis of ^{125}I-dBSA.

However, such an explanation cannot apply in the case of ^{3}H-labelled serum proteins. When the latter substrates were added to the culture medium at the same time as trypan blue there was no effect on their degradation. Trypan blue's inhibitory action would seem therefore to be confirmed to ^{125}I-dBSA. Two possible explanations could be advanced to account for this. Firstly, the proteinase responsible for the digestion of ^{125}I-dBSA is different from the proteinase(s) that digests the bulk of the ^{3}H-labelled serum proteins in being trypan blue-inhibitable. In view of the data presented in Chapter 9, this explanation is most unlikely. Secondly, trypan blue interacts with ^{125}I-dBSA in such a way as to reduce the protein's susceptibility to degradation, an explanation advanced also to account for the decreased uptake of ^{125}I-dBSA observed in these experiments (see above). The strongly anionic nature of trypan blue that promotes its binding to albumin (Rawson, 1943) makes this second explanation the more plausible. (Of course, ^{3}H-labelled serum proteins will contain a large proportion of native homologous albumin which will also bind trypan blue. The binding of the dye to the protein in this case though would appear not to affect its rate of degradation by the yolk sac). The observation that trypan blue, present in the culture medium between 36 and 42 hours of culture, has no effect on the uptake and digestion of ^{125}I-dBSA is also consistent with this explanation. Trypan blue, removed from the culture medium before the addition of ^{125}I-dBSA, would be unable to interact with the radiolabelled protein and thus reduce its rate of degradation, or even if the two substances became incorporated into the same heterolysosomes, there may be insufficient free trypan blue available at this site to bind, in significant quantities, to any undegraded ^{125}I-dBSA that may also be present.

The data of Williams et al. (1976), which show an inability of trypan blue to inhibit the intralysosomal digestion of ^{125}I-dBSA by the cultured 17.5-day yolk sac, though at first sight at variance with the current observations, may reflect the different methods of denaturation of the radiolabelled protein. In the present study, ^{125}I-dBSA was denatured by treatment with 10% formaldehyde, pH 10.0, and in the study of Williams et al., ^{125}I-dBSA was denatured by titrating with acetic acid to pH 3.5. These different denaturing protocols confer on the protein different properties evident in their rates of clearance by the cultured 17.5-day yolk sac and in their optical rotatory dispersion characteristics (Lloyd et al., 1976). It seems quite possible therefore that formaldehyde-denatured ^{125}I-BSA interacts with trypan blue in a way which the acid-denatured ^{125}I-BSA is unable to, and that this binding of the dye renders the protein less susceptible to proteolytic attack, and less able to adsorb to the plasma membrane. It must be emphasised here that such a specific action of trypan blue on ^{125}I-dBSA uptake and digestion by the early organogenesis-stage yolk sac, though interesting in itself, is likely to be of no significance to any teratogenic action of the dye on yolk sac-mediated histiotrophic nutrition.

Another anomaly between the present data, which show no evidence of any inhibition by trypan blue of pinocytosis by the yolk sac, and those of Williams et al. (1973, 1976) and Roberts et al. (1980) which clearly demonstrate the inhibitory action of the dye, at appropriate concentrations, on the pinocytosis by 17.5-day yolk sac of ^{125}I-PVP (and ^{125}I-dBSA) is almost certainly due to the differences in serum concentrations of the culture medium used in these studies. Williams et al. (1976) routinely included 10% fetal calf serum in their culture medium but

reported preliminary findings that if the serum concentration was
increased to 50% the inhibitory action of trypan blue was decreased
compared with values from dye-free control experiments performed
with the same elevated concentration of calf serum in the medium. In
the present study, the culture medium comprises 100% homologous
serum, and seemingly this prevents totally the inhibitory action of
trypan blue, used at the same concentrations as by Williams et al.,
on pinocytosis. The implication is that free, and not protein-bound,
dye is the active inhibitor, and that in a culture medium of 100%
serum very little of the dye will be present in free form, when
applied at these concentrations. One obvious alternative to these
explanations of the different effects of trypan blue on the 17.5-day
yolk sac and the 9.5-11.5-day yolk sac is that the tissues function
quite differently at these different stages of gestation, and
consequently respond to trypan blue in a dissimilar manner. Assuming
that this is not the case, and the data of previous chapters would
indicate that it is not, it is clear that the composition of the plasma
transudate that bathes the extra-embryonic membranes during the period
of teratogenic susceptibility to trypan blue, and in particular its
protein concentration, is likely to be of critical importance to
the effect, if any, of trypan blue on yolk sac-mediated nutrition.
This subject is further discussed in Chapter 10.

CHAPTER SIX

THE EFFECT OF SURAMIN ON YOLK SAC-MEDIATED NUTRITION

6.1   Introduction

The trypanocidal drug suramin has many features in common with trypan blue. Although colourless and having no azo linkages, it does contain, in common with trypan blue, two polysulphonated naphthalene moieties (Figure 6.1) and has a very strong affinity for protein (Town et al., 1950; Muller and Wollert, 1976). Furthermore, suramin is taken up by cells, presumably by pinocytosis, and accumulates within lysosomes. Smeesters and Jacques (1968) demonstrated a lysosomal location of suramin by subcellular fractionation of liver homogenates, following injection of the drug into rats two days previously. Buys et al. (1978) inferred lysosomal storage of suramin from the electron microscopic appearances of liver Kupffer and endothelial cells from rats injected with the drug 24 hours previously. Perhaps of most relevance to the present study, Schultz (1970) observed that following injection of suramin into 11.5-day pregnant rats, no suramin could be detected in the embryo 12 hours later. In contrast, the yolk sac (and to a lesser extent the chorioallantoic placenta) contained significant quantities of suramin which, in view of the highly developed nature of the vacuolar system of these cells, might be expected to be in the lysosomes.

These properties of suramin led Lloyd and Beck (1969) to theorise that the drug might be teratogenic in rats and might act by a mechanism similar to that proposed for trypan blue, a view made all the more plausible when one considers that suramin is a potent inhibitor of a number of enzymes, including some lysosomal hydrolases. In the course of an extended study using suramin, Wills and Wormall and their colleagues (Town et al., 1950; Wills and Wormall, 1950) discovered that suramin was a potent inhibitor of hexokinase, succinic
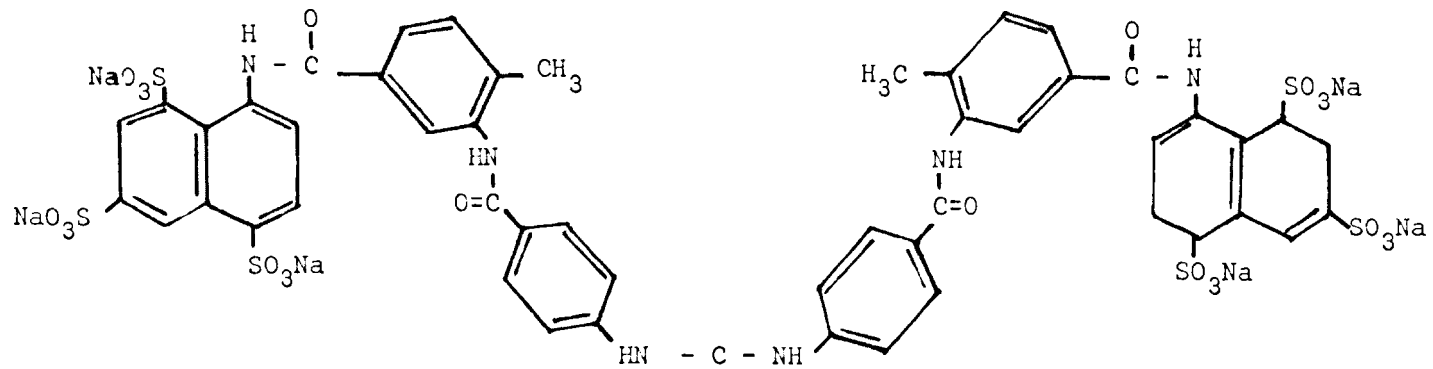
Figure 6.1   Structural formula of suramin

dehydrogenase, urease, cytochrome oxidase, trypsin, pepsin and a number of other enzymes. Smeesters and Jacques (1968) observed inhibition of lysosomal β-glycerophosphatase, β-N-acetylaminodeoxy-glucosidase and β-glucuronidase, and Schultz (1970) found a significant reduction in rat placental acid phosphatase activity in the presence of the drug. Additionally, Davies et al. (1971) demonstrated that isolated rat liver lysosomes, pre-loaded with suramin and ^{125}I-labelled albumin, on incubation in vitro released acid-soluble radioactivity more slowly than controls from which suramin was absent, a result since confirmed by Buys et al. (1973, 1978) and Korolenko et al. (1980, 1981).

However, there is some uncertainty surrounding the teratogenicity of suramin. Lloyd and Beck (1969) claimed an embryolethal, though not teratogenic, effect of suramin on rat embryos. These authors, it must be said, examined the surviving fetuses of suramin-treated mothers for external malformations only. Thus any malformed viscera would have gone undetected. Schultz and Neubert (in the discussion to Schultz, 1970) both claimed to have observed malformed fetuses as a result of injection of suramin into pregnant rats.

The present study has sought to establish whether or not suramin is teratogenic to rat embryos. Further experiments have concentrated on elucidating the effect of suramin on the nutritional properties of the visceral yolk sac established earlier, using the experimental protocol described in the last chapter.

6.2   Materials

Chemicals

Suramin BP   ICI Pharmaceuticals Division, Alderley

                                                      Park, Macclesfield, Cheshire

All other chemicals not previously listed were of analytical grade.

6.3   Methods

6.3.1   Teratogenic Testing of Suramin

The procedure was as described for trypan blue (5.3.3). Suramin was administered at a dosage of 250mg/kg.

6.3.2   Culture Experiments

The procedure was as described for trypan blue (5.3.4). In cultures which used as substrate ^{125}I-PVP, suramin was added to the culture medium to a final concentration of 25, 50, 100, 500, 1,000, 5,000 or 10,000 µg/ml. For subsequent experiments with ^{125}I-dBSA and ^{3}H-labelled serum proteins as substrates, suramin was added to a final concentration of 50 or 5,000 µg/ml.

6.4   Results

6.4.1   Teratogenic Testing of Suramin

The injection of a teratogenic dose (250 mg/kg) of suramin into pregnant rats at both 8.5 and 9.5 days of gestation resulted in the production of malformed fetuses (Table 6.1). In both the 8.5 and 9.5-day injected groups a high percentage (32% and 39% respectively) of the total number of implantations were resorbed. Although none of the surviving fetuses showed any external malformations, sectioning of the fetuses that had been fixed in Bouin's fluid revealed some abnormal visceral organs (Table 6.2). In the 8.5-day injected group 2 types of malformation affecting eyes and kidney were noted. Malformed kidneys were observed in one third of all surviving fetuses sectioned. In the 9.5-day injected group hydrocephalus and kidney defects were the most frequently observed malformations. In addition, one fetus had a megaureter and one fetus a grossly enlarged heart.

Nature and time of injection	No. of ♀ treated	No. of implantations (% resorptns)	Av. weight surviving fetuses (g)	External Abnormalities			No. of fetuses sectioned	No.(%) sectioned fetuses with anomalies	No.of fetuses stained with alizarin	No.(%) stained fetuses with anomalies
				No.	%surviving fetuses	% total implntns				
Suramin 8.5 days	8	78 (32)	2.70 ± 1.6*	0	0	0	36	13 (36)	17	0
Suramin 9.5 days	8	90 (39)	2.22 ± 0.4*	0	0	0	37	9(24)	18	0
Saline 8.5 days	4	45 (4)	$4.19 \pm .42$	0	0	0	28	0	15	0
Saline 9.5 days	4	44 (5)	$4.40 \pm .60$	0	0	0	30	0	12	0

* significantly different from saline-injected controls ($p < 0.001$)

Table 6.1 Effects on offspring of s.c. injection of suramin (250 mg/kg) or saline (0.9%) into pregnant rats. Details of method are given in text.

Day of injection of suramin	8.5	9.5
Abnormality		
Eye defects (anophthalmia or microphthalmia	3 (36)	0 (37)
Kidney (deformed)	12 (36)	4 (37)
Megaureter	0 (36)	1 (37)
Hydrocephalus	0 (36)	7 (37)
Heart defects	0 (36)	1 (37)

Table 6.2 Abnormalities of fetuses after suramin treatment of pregnant rats. Figures in brackets indicate the size of the population examined for that particular defect.

The average weight of surviving fetuses in both 8.5- and 9.5-day injected groups was significantly different ($p < 0.001$) from controls injected with 0.9% saline at corresponding days of gestation.

6.4.2 Culture Experiments

Viability of conceptuses exposed to suramin for a maximum period of 6 hours, was at least 95%. No developmental deviations of conceptuses were observed at harvesting. The protein contents at harvesting of yolk sacs and embryos exposed to suramin at concentrations of the drug equal to or less than 5,000 μg/ml for the last 6 hours of culture were not noticeably different from controls cultured in the absence of suramin. (Figure 6.2). At concentrations of suramin above 5,000 μg/ml the protein contents of yolk sacs and embryos were lower than controls, although the ratio of protein in each tissue whether or not suramin was present was approximately the same. When suramin was presented to conceptuses in culture between 36 and 42 hours of culture, the protein contents at harvesting of yolk sacs and embryos was lower than controls. Again the ratio of protein in each tissue was about the same (Figure 6.2).

When conceptuses were incubated for the last 6 hours of culture in the presence of ^{125}I-PVP (2.3 μg/ml) and various concentrations of suramin, there was no uptake of radioactivity into the embryo (Figure 6.3). At concentrations of 100 μg/ml or less, suramin neither stimulated nor inhibited the uptake of radioactivity by yolk sac. At suramin concentrations of greater than 100 μg/ml however, the uptake of radioactivity by yolk sac at harvesting was inhibited, the amount of inhibition being proportional to the concentration of suramin in the culture medium. In subsequent experiments using the radiolabelled
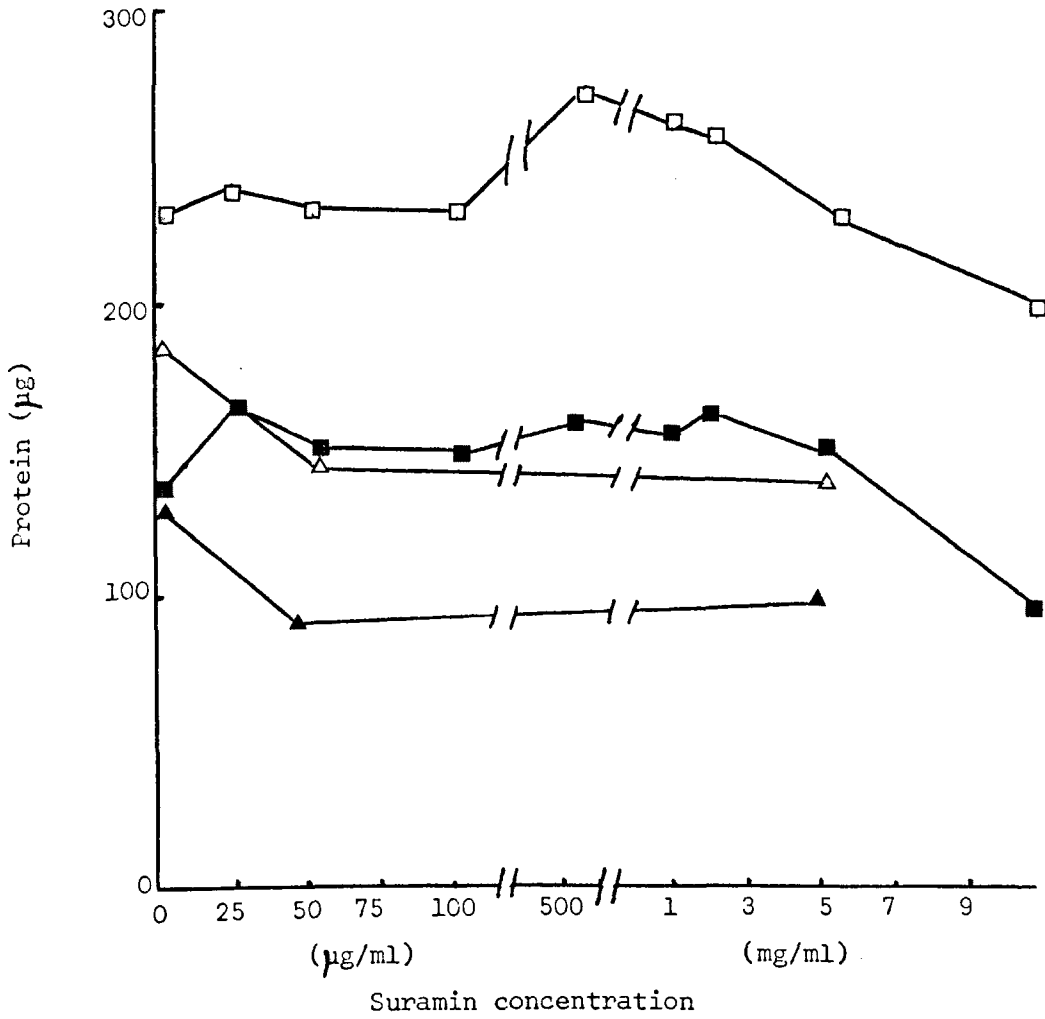
Figure 6.2 Protein contents of yolk sacs (filled symbols) and embryos (open symbols) after incubation of conceptuses in the presence of suramin for either the final 6 hours (squares) or the penultimate 6 hours (triangles) of culture. Values are means of at least 6 determinations.
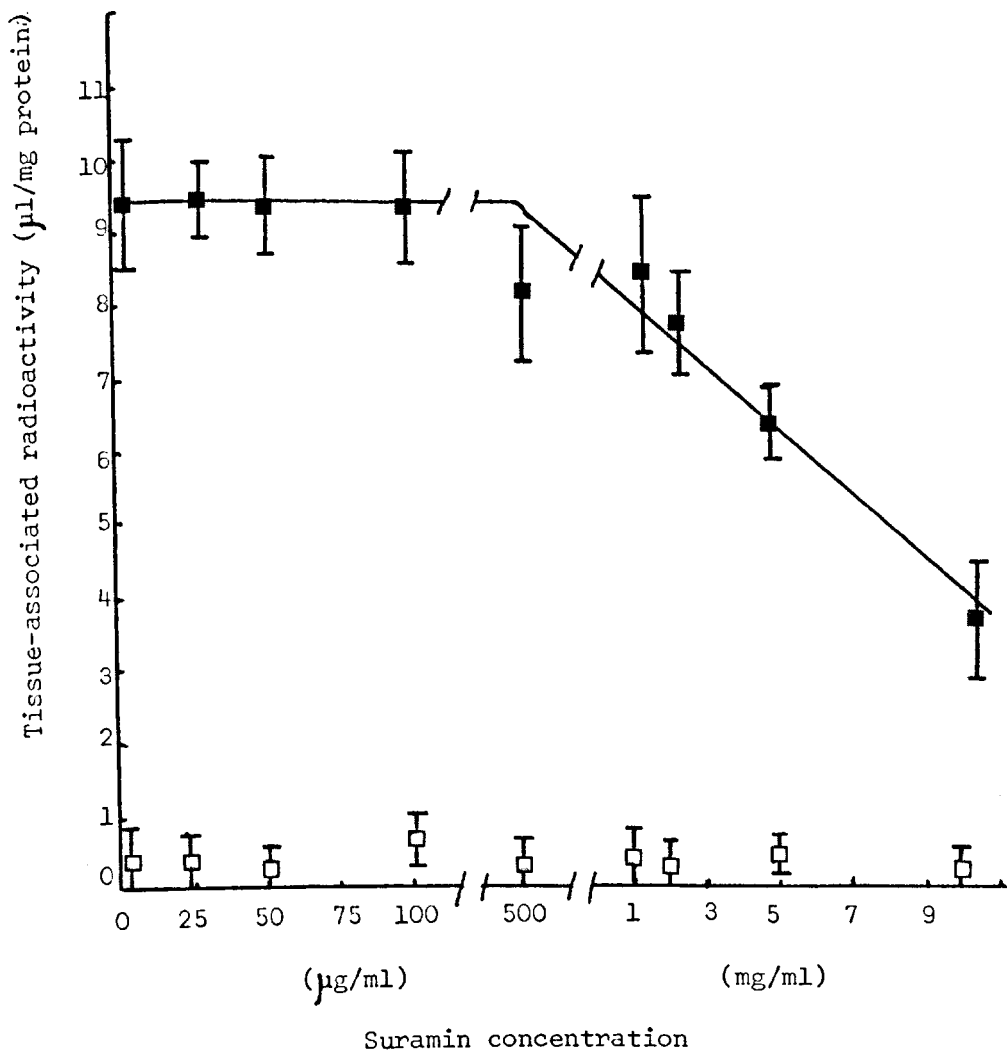
Figure 6.3 Radioactivity associated with the yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 μg/ml) and suramin for the final 6 hours of culture. Values are means (± S.D.) of at least 6 determinations.

substrates ^{125}I-dBSA and ^{3}H-labelled serum proteins, two concentrations of suramin were used, one of which (50 μg/ml) did not inhibit ^{125}I-PVP uptake, and another (5,000 μg/ml) which decreased ^{125}I-PVP uptake by 35%.

Following incubation of conceptuses with ^{125}I-dBSA (11 μg/ml) and suramin (50 or 5,000 μg/ml) for the last 6 hours of culture, little radioactivity was detected in the embryo at harvesting (Figure 6.4). The small quantity of radioactivity present in the embryo was all TCA-soluble. Suramin at a concentration of 50 μg/ml decreased the total quantity of radioactivity processed by the yolk sac to a level significantly below (p<0.01) controls. Of this radioactivity, a larger proportion (9%, 33% TCA-soluble) was associated with the yolk sac than in controls (6%, 43% TCA-soluble). Suramin at a concentration of 5,000 μg/ml caused a pronounced reduction in the total quantity of radioactivity processed by yolk sac (20% of control, p<0.001). The proportion of this radioactivity that was yolk sac-associated was again about 9%, though a very much smaller percentage (5%) was TCA-soluble.

When suramin, at the same concentrations, was present in culture between 36 and 42 hours before exposure of conceptuses for the final 6 hours of culture to ^{125}I-dBSA in suramin-free medium, a qualitatively similar pattern to that described above for radiolabel distribution in the conceptus was observed at harvesting (Figure 6.5). Quantitatively however, the degree of difference between experimental and control conceptuses was greater. Suramin at 50 ug/ml reduced the total clearance of radioactivity by yolk sac to about 42% (p<0.001) of control values of which 15% (42% TCA-soluble) was associated with the yolk sac, compared with a control value of 5% (58% TCA-soluble).
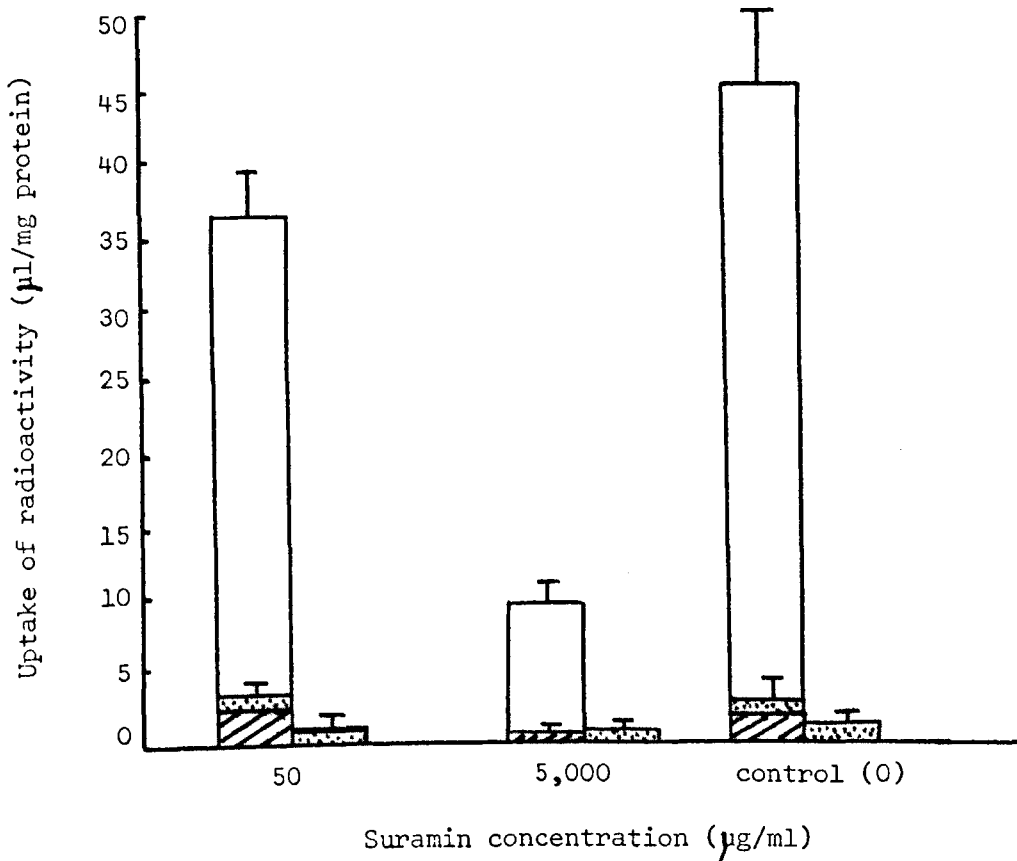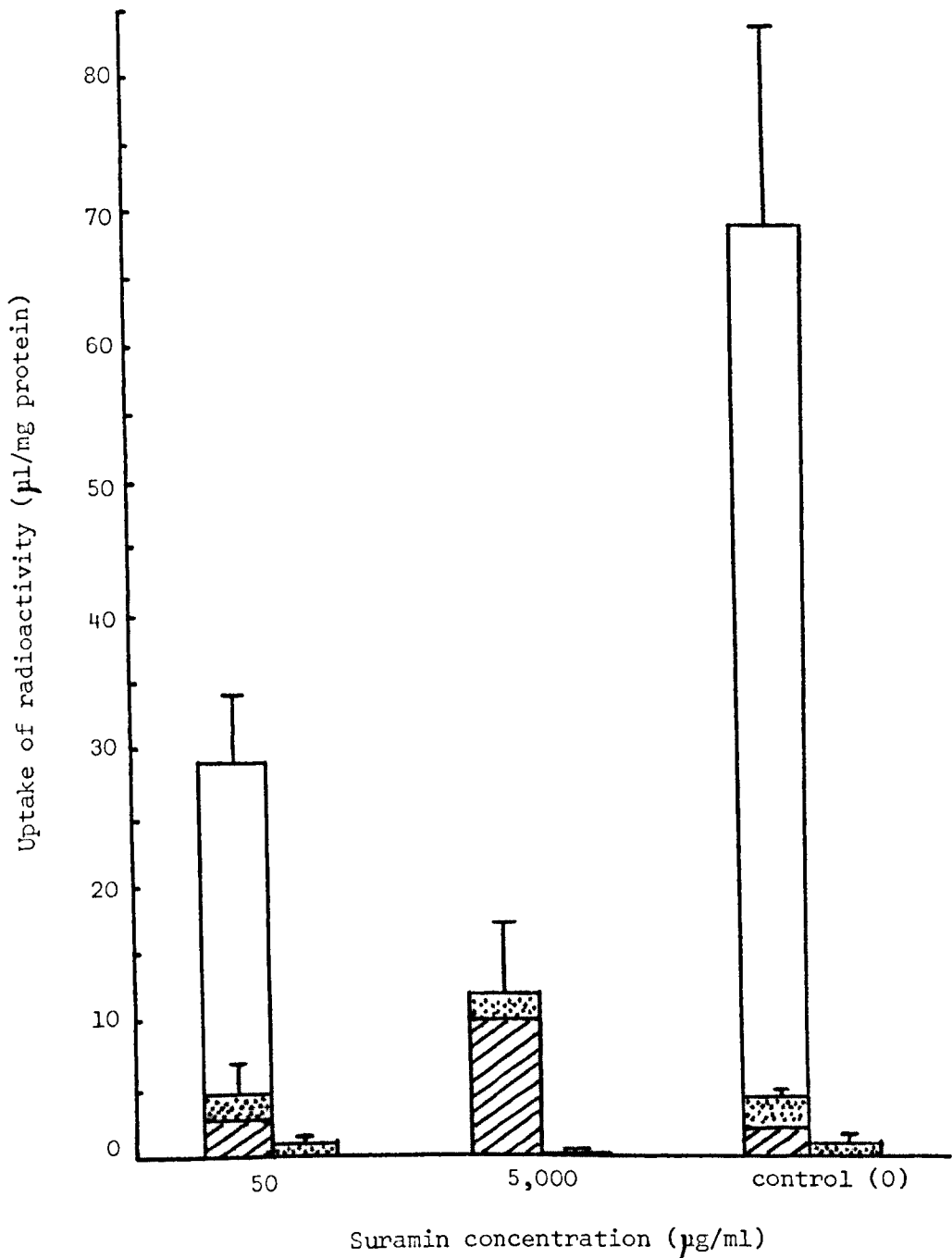
Figure 6.4 Total radioactivity taken up by the yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) and suramin for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▦ and TCA-insoluble ▨ are indicated. Values are means of at least 6 determinations.

Figure 6.5 Total radioactivity taken up by the yolk sac (each left
hand column) and embryo (each right hand column), and the
radioactivity associated with the yolk sac (total shaded area of
each left hand column) after incubation of conceptuses in the presence
of ^{125}I-dBSA (11 μg/ml) for the final 6 hours, and suramin for the
penultimate 6 hours of culture.The proportions of yolk sac- and
embryo-associated radioactivity that were TCA-soluble ▨ and TCA-
insoluble ▨ are indicated. Values are means ($\pm$ S.D.) of at least
6 determinations.

A small amount of radioactivity, all TCA-soluble, was associated with the embryo at harvesting. At a concentration of 5,000 μg/ml, suramin decreased the total quantity of radioactivity processed by the yolk sac to a mere 17% (p$<$0.001) of the control value. All of this radioactivity was retained within the yolk sac (about 10% TCA-soluble) and no radioactivity could be detected in the embryo.

The uptake of radioactivity by conceptuses incubated for the last 6 hours of culture in the presence of ^{3}H-labelled serum proteins and suramin (50 or 5,000 μg/ml), is shown in Figure 6.6. The presence of 50 μg/ml of suramin the culture medium did not affect the uptake or digestion of radiolabelled protein, or distribution of radio-activity in conceptuses, relative to controls. At 5,000 μg/ml, suramin decreased the total uptake of radioactivity by conceptuses to about 20% (p$<$0.001) of the control value. Of this radioactivity, approximately 52% was associated with the yolk sac (9% TCA-soluble) compared with a control value of 39% (30% TCA-soluble). The proportion of the total radioactivity taken up by conceptuses that was associated with the embryo was 29% in the experimental group and 43% in controls.

The loading of suramin (50 μg/ml) for 6 hours into the yolk sacs of conceptuses that were immediately transferred to a suramin-free medium containing ^{3}H-labelled serum proteins and cultured for a further 6 hours, did not result in a decrease in the uptake of radiolabel by conceptuses at harvesting, compared with controls (p$>$0.05)(Figure 6.7). However, the proportion of this radioactivity that was associated with the yolk sac at harvesting was significantly greater than in controls (p$<$0.01). Correspondingly, the embryo-associated radioactivity was decreased in the experimental group. Furthermore, the percentage TCA-soluble radioactivity in the yolk
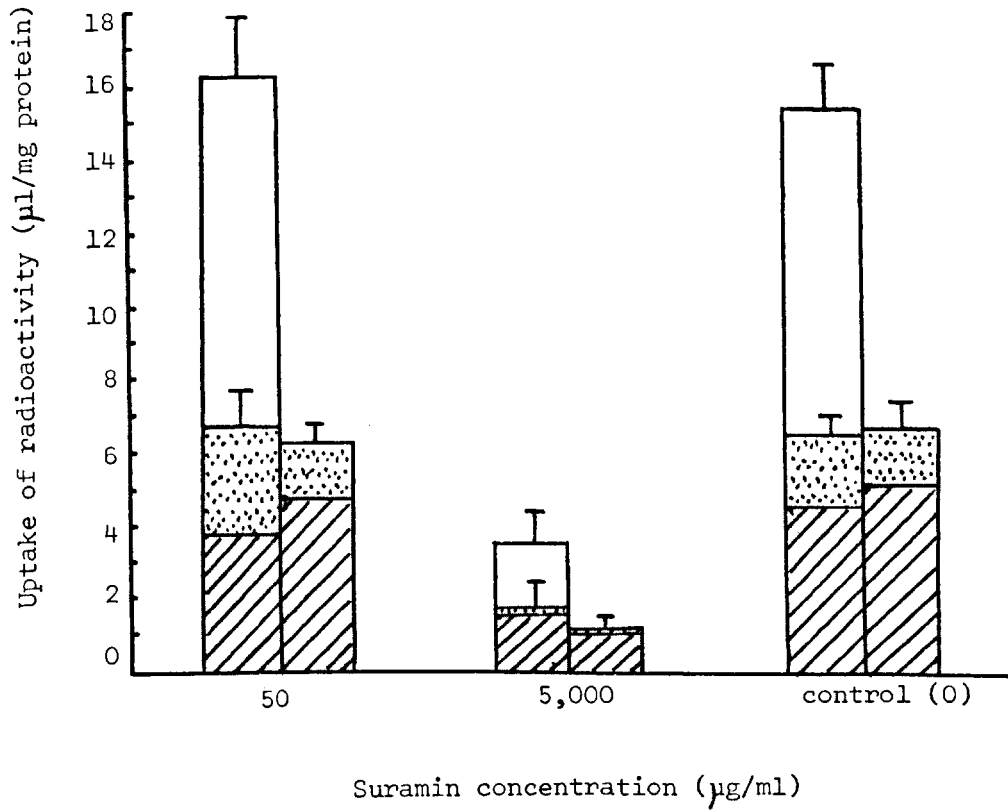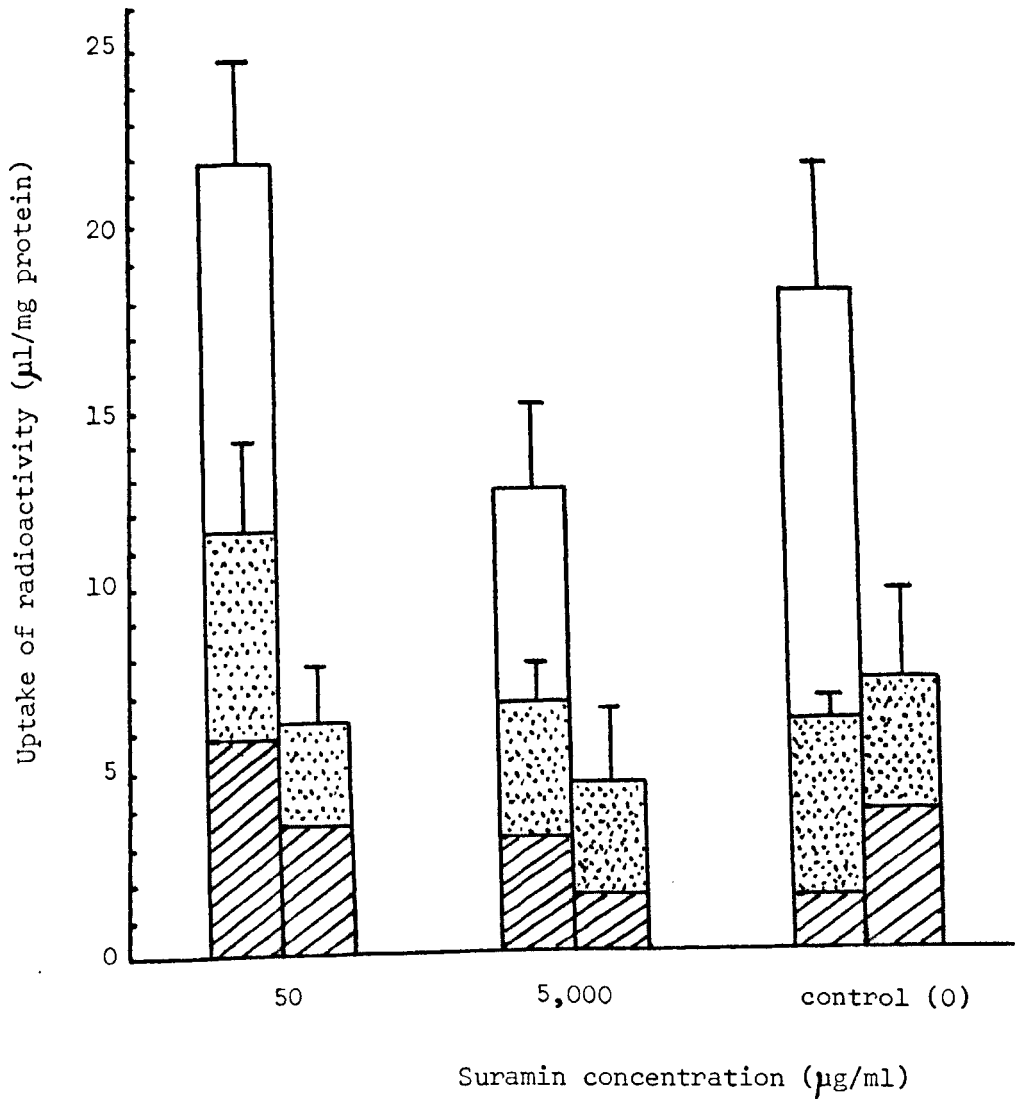
Figure 6.6 Total radioactivity taken up by the yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3\text{H}]$leucine-labelled serum proteins and suramin for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▨ and TCA-insoluble ▨ are indicated. Values are means (± S.D.) of at least 6 determinations.

Figure 6.7 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$leucine-labelled serum proteins for the final 6 hours, and suramin for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ⣿ and TCA-insoluble ⣿ are indicated. Values are means (± S.D.) of at least 6 determinations.

sac, 50%, was much less than in controls, 79%, indicative of inhibition of proteolysis. Under the same experimental regime, 5,000 µg/ml of suramin reduced total uptake of radioactivity by conceptuses to 60% of control values. Again experimental conceptuses possessed a higher proportion of this, compared with controls, within the yolk sac and also contained a higher percentage of yolk sac radioactivity in TCA-soluble form. These differences between experimental and control conceptuses were significant. (p < 0.01).

6.5   Discussion

The embryotoxicity and teratogenicity of suramin has not been fully reported in the literature. In this study, the limited investigation of the drug's effects on the developing embryo following maternal injection demonstrates that suramin is both embryolethal and teratogenic. In view of the drug's similarity in structure to trypan blue this property of suramin is not unexpected. In its mechanism of teratogenesis suramin could conceivably act on the yolk sac (where it has been shown to be localised - Schultz, 1970) by inhibiting the pinocytic ingestion of nutrients or by inhibiting the intralysosomal digestion of macro-molecular histiotroph. Another mechanism of action of suramin could be to inhibit the fusion of the nutrient-bearing pinosomes with the digestive lysosomes, a property of the drug that has been observed in mouse peritoneal macrophages (D'Arcy Hart and Young, 1975) and rat hepatocytes (Buys, 1976).

Suramin, at a concentration of 100 µg/ml or less, had no effect on the uptake of ^{125}I-PVP by yolk sac when both substances were present together in the culture medium. At higher concentrations of suramin,

uptake of ^{125}I-PVP by yolk sac was inhibited by an amount proportional to the concentration of suramin. In contrast to the effect of trypan blue, 'piggy-back' pinocytosis of ^{125}I-PVP at low concentrations of suramin was not observed. This is also in contrast to the recent finding (Pratten and Lloyd, 1981) that low concentrations of suramin stimulate ^{125}I-PVP uptake by rat peritoneal macrophages by a mechanism specific for that radiolabelled substrate. In the same report it was stated that the uptake and degradation of ^{125}I-dBSA was considerably decreased by suramin. It is interesting to note therefore that suramin, at a concentration (50 μg/ml) that does not inhibit pinocytosis of ^{125}I-PVP, markedly decreases the uptake and degradation of ^{125}I-dBSA by conceptuses. The further observation that this effect of suramin is enhanced when it is loaded into the yolk sac before exposure of conceptuses to ^{125}I-dBSA, suggests that the drug is acting at an intracellular locus, presumably within the vacuolar system. Buys et al. (1978) found that the isolated perfused rat liver, loaded with suramin in vivo, pinocytosed ^{125}I-dBSA, present in the perfusion medium, to a lesser extent than control liver containing no suramin. The reduced uptake of ^{125}I-dBSA observed in the present study must be specific for that protein since the uptake of ^{3}H-labelled serum proteins by conceptuses was not decreased by 50 μg/ml of suramin regardless of whether the drug was presented to conceptuses at the same time as or before the radiolabelled proteins. If suramin were acting only to inhibit the digestion of ^{125}I-dBSA, an explanation consistent with the observed increased in the proportion of total uptake of radioactivity that was associated with the yolk sac (and the increased

proportion of this radioactivity that was TCA-insoluble), then it would be expected that the total clearance of radiolabel would be unaltered by this concentration (50 μg/ml) of suramin, unless of course one postulates some kind of feedback mechanism to prevent the further uptake of substrate. Assuming the latter not to be the case, two explanations can be advanced to account for these observations. Either suramin is irreversibly bound to those sites on the plasma membrane to which ^{125}I-dBSA adsorbs and also inhibits intralysosomal proteolysis, or suramin is taken into cells within pinosomes and inhibits the normal fusion of the pinosome with a lysosome. This may or may not involve an inhibition of digestion of ^{125}I-dBSA.

Actively pinocytosing cells must possess the means to withdraw membrane from the vacuolar system and re-insert it into the plasma membrane. Normal fusion of incoming pinosomes with resident lysosomes probably affords the cell an opportunity to re-cycle membrane (Duncan and Pratten, 1977). If suramin were inhibiting pinosome-lysosome fusion, the cell would be denied this opportunity and as a result the rate of pinosome formation at the cell surface may be decreased. Inhibition of fusion would also separate pinocytosed ^{125}I-dBSA from digestive enzymes and hence explain the increase in proportion of the total radiolabel processed by the yolk sac cells that was associated with the yolk sac at harvesting, although this may also be due in part to direct enzyme inhibition by suramin. However, if suramin at this concentration is inhibiting fusion of intracellular vesicles it is evidently only able to do this in combination with ^{125}I-dBSA.

At a suramin concentration of 5,000 μg/ml (which inhibits ^{125}I-PVP uptake by about 30%) ^{125}I-dBSA uptake and digestion by yolk sac is decreased even further. When conceptuses were loaded with suramin prior

to incubation in a suramin-free medium containing ^{125}I-dBSA, degradation of substrate was completely abolished. All of the radioactivity taken up by the yolk sac remained within the tissue and nearly all of this was TCA-insoluble. The embryo contained no radioactivity.

That suramin (50 μg/ml) can inhibit intralysosomal proteolysis is demonstrated by the observation that when the drug was loaded into yolk sacs before incubation of conceptuses in a suramin-free medium that contained ^{3}H-labelled serum proteins, though the overall uptake of radiolabel, compared with controls, was unaltered, the proportion of the yolk sac radioactivity that was TCA-insoluble was considerably higher. Correspondingly the radioactivity in the embryo was decreased. Furthermore, these data provide powerful evidence that yolk sac proteolysis is a necessary step in the utilisation of exogenous protein by the conceptuses.

In contrast to experiments using ^{125}I-dBSA as radiolabelled substrate, the effect of suramin (5,000 μg/ml) upon the uptake of ^{3}H-labelled serum proteins by conceptuses was observed to be greater when the drug was present in the culture medium together with radiolabelled substrates than when it was loaded into yolk sacs before exposure to radiolabel, though under both regimes the total uptake of radiolabel by conceptuses was decreased and the proportion of this radioactivity that was associated with the yolk sac was the same (approximately 50%). Suramin therefore cannot be acting upon the uptake and digestion of ^{3}H-labelled serum proteins in the same way as it does with ^{125}I-dBSA. The fact that a very much larger proportion of yolk sac radioactivity was TCA-insoluble when conceptuses were exposed to drug and substrate at the same time indicates that under these conditions the drug is inhibiting proteolysis either directly or by preventing the confrontation of lysosomal enzymes

and substrates through an inhibition of fusion of pinosomes and lyso-somes. In the latter case membrane recycling would also be inhibited and might therefore explain also the very much lower total uptake of substrate. Why suramin should produce a greater effect on the uptake of ^{3}H-labelled serum proteins when presented to conceptuses at the same time as substrate, in contradistinction to the drug's effect on ^{125}I-dBSA uptake and digestion, is difficult to explain, particularly since the same concentration of serum protein exists in the culture medium used in both sets of experiments. Part of the explanation is likely to lie in the difference in affinity of the drug for denatured bovine albumin and native homologous serum proteins, as was proposed for trypan blue in the preceding chapter.

In interpreting the data of these experiments, it cannot be ruled out that suramin, at a concentration of 5,000 μg/ml, is toxic to the conceptus. Although at harvesting conceptuses exposed to this amount of the drug appeared healthy, it has been shown (Wesolowski et al., 1973) that levels of suramin required to inhibit the endocytosis of trypan blue by mouse Kupffer cells are also toxic to these cells.

In conclusion it can be stated that suramin is able to inhibit, probably by a combination of actions, the nutritional function of yolk sac and as a consequence reduce the flow of nutrients reaching the embryo. In considering a mechanism of teratogenic action of suramin, other biological effects of the drug such as the inhibition of complement systems (Fong and Good, 1973) and fibrinolysis (Moroz, 1977) should be taken into account. Only when such effects upon maternal metabolism to the well-being of the embryo have been evaluated will it be possible to fully appreciate the extent of the contribution made by induced yolk sac dysfunction to the mechanism of suramin's teratogenicity.

CHAPTER SEVEN

THE EFFECT OF SODIUM AUROTHIOMALATE ON YOLK SAC-MEDIATED NUTRITION

7.1   Introduction

The teratogenicity of the anti-rheumatic drug sodium aurothiomalate (Figure 1) has been demonstrated in rats (Lloyd and Beck, 1969; Kidston et al., 1971; Szabo et al., 1978a) and rabbits (Szabo et al., 1978b). A recent report (Rogers et al., 1980) has also described the teratogenic effects of the drug in a human.

Morphological studies have shown that sodium aurothiomalate localises in lysosomes. Norton et al. (1968) and Strunk and Ziff (1970) found characteristic granular particles concentrated in the lysosomes of synovial membrane and kidney glomerular epithelial cells of rats and humans injected with the drug. Sodium aurothiomalate has also been shown to be an inhibitor of a number of lysosomal enzymes. Persellin and Ziff (1966) observed that the drug inhibited, in vitro, acid phosphatase and β-glucuronidase activities of guinea pig peritoneal macrophages and suggested lysosomal enzyme inhibition as a mechanism for the anti-inflammatory action of the drug. Inhibition by sodium aurothiomalate of these two enzymes and 'cathepsin', obtained from rabbit liver lysosomes and human synovial fluid, has also been described (Ennis et al., 1968). An inhibitory action of the drug on lysosomal proteolysis was suggested (Davies et al., 1971) to account for a decreased release of TCA-soluble radioactivity observed when liver lysosomes isolated from rats injected with ^{125}I-labelled BSA and sodium aurothiomalate were incubated at $22^{\circ}C$ in osmotically-protected medium.

In the light of these studies, it was suggested (Lloyd and Beck, 1969) that the embryopathic effects of sodium aurothiomalate, injected into pregnant rats during the early organogenesis period of embryonic development, might be caused by a mechanism of action similar to the

$$\text{Au} - \text{S} - \underset{\underset{\text{H}}{|}}{\overset{\overset{\text{H}}{|}}{\text{C}}} - \text{C} \overset{\text{O}}{\underset{\text{O} - \text{Na}}{}}$$

$$\text{H} - \underset{\underset{\text{H}}{|}}{\overset{\overset{|}{}}{\text{C}}} - \text{C} \overset{\text{O}}{\underset{\text{O} - \text{Na}}{}}$$
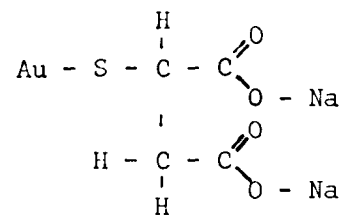
Figure 7.1 Structural formula of disodium aurothiomalate

one proposed for trypan blue (Beck et al., 1967b), a hypothesis that was strengthened by the finding (Kidston et al., 1971) that aurothiomalate accumulates in visceral yolk sac lysosomes following injection into pregnant rats between 7.5 and 11.5 days of gestation.

The present experiments were undertaken to examine the effects of sodium aurothiomalate on the already established nutritional function of the visceral yolk sac in cultured early organogenesis-stage conceptuses.

7.2   Materials

Chemicals

Disodium aurothiomalate (Myocrisin) was a generous gift of May and Baker Ltd., Dagenham, Essex.

All other chemicals used have been listed previously.

7.3   Methods

7.3.1 Teratogenic Testing of Sodium Aurothiomalate (Myocrisin)

The procedure was as described for trypan blue. Sodium aurothiomalate was administered at a dosage of 100 mg/kg.

7.3.2 Culture Experiments

The procedure was as described for trypan blue. In cultures that used ^{125}I-PVP as substrate, sodium aurothiomalate was added to the culture medium to a final concentration of 25, 50, 100, 200 or 500 µg/ml. For subsequent experiments with ^{125}I-dBSA and ^{3}H-labelled serum proteins as substrates, sodium aurothiomalate was added to a final concentration of 25 or 500 µg/ml.

7.4   Results

7.4.1 Teratogenic Testing of Sodium Aurothiomalate

Injection of sodium aurothiomalate into pregnant rats at either 8.5

or 9.5 days of gestation resulted in the production of malformed

fetuses at term (Table 7.1). Following injection at 8.5 days, a

high rate (53%) of fetal resorption was observed. Of the surviving

fetuses, 33% were abnormal, hydrocephalus and kidney malformation

being the most common anomalies (Table 7.2). The average weight of

fetuses surviving maternal treatment at 8.5 days was significantly

$(p < 0.001)$ below that of controls. Maternal injection at 9.5 days

resulted in a lower rate (30%) of fetal resorption but a higher

proportion of the total number of implantations were malformed. Again

hydrocephalus and kidney defects were the most common malformations

observed (Table 7.2) and fetuses similarly incurred a significant

$(p < 0.001)$ weight deficit, compared with controls.

7.4.2 Culture Experiments

The inclusion of sodium aurothiomalate in the culture medium during

either the final 6 hours, or the penultimate 6 hours of culture did

not affect the viability of conceptuses or cause developmental

deviations. When sodium aurothiomalate was present in culture for

the final 6 hours, the protein content at harvesting, of embryos

(Figure 7.2) was little affected. However in the yolk sac, an

increase in protein content was observed which was approximately

proportional to the concentration of sodium aurothiomalate in the

culture medium, up to concentrations of 100 μg/ml. Concentrations of

the drug above 100 μg/ml caused no further increase in the protein

level of the yolk sac. When sodium aurothiomalate was present in culture

between 36 and 42 hours, the protein contents of embryos and yolk sacs

Nature and time of injection	No. of ♀ treated	No. of implantations (% resorptns)	Av. weight surviving fetuses (g)	External Abnormalities			No. of fetuses sectioned	No.(%) sectioned fetuses with anomalies	No.of fetuses stained with alizarin	No.(%) stained fetuses with anomalies
				No.	%surviving fetuses	% total implntns				
Aurothiomal- ate, 8.5 dys	8	86 (53)	$3.18 \pm .33$*	1	2.50	1.16	27	9 (33)	13	0
Aurothiomal- ate, 9.5 dys	8	97 (30)	$2.36 \pm .70$*	1	1.47	1.03	46	17 (37)	22	0
Saline 8.5 days	4	45 (4)	$4.19 \pm .42$	0	0	0	28	0	15	0
Saline 9.5 days	4	44 (5)	$4.40 \pm .60$	0	0	0	30	0	12	0

* significantly different from saline-injected controls ($p < 0.001$)

Table 7.1 Effects on offspring of s.c. injection of sodium aurothiomalate (100 mg/kg) or saline (0.9%) into pregnant rats. Details of method are given in text.

Day of injection of aurothiomalate	8.5	9.5
Abnormality		
Encephalocoele	0 (40)	1 (68)
Hydrocephalus	5 (27)	13 (46)
Anophthalmia	0 (27)	2 (46)
Kidney (deformed)	6 (27)	3 (46)
Megaureter	2 (27)	1 (46)
Gonad (absent or deformed)	1 (27)	0 (46)

Table 7.2 Abnormalities of fetuses after sodium aurothiomalate treatment of pregnant rats. Figures in brackets indicate the size of the population examined for that particular defect.
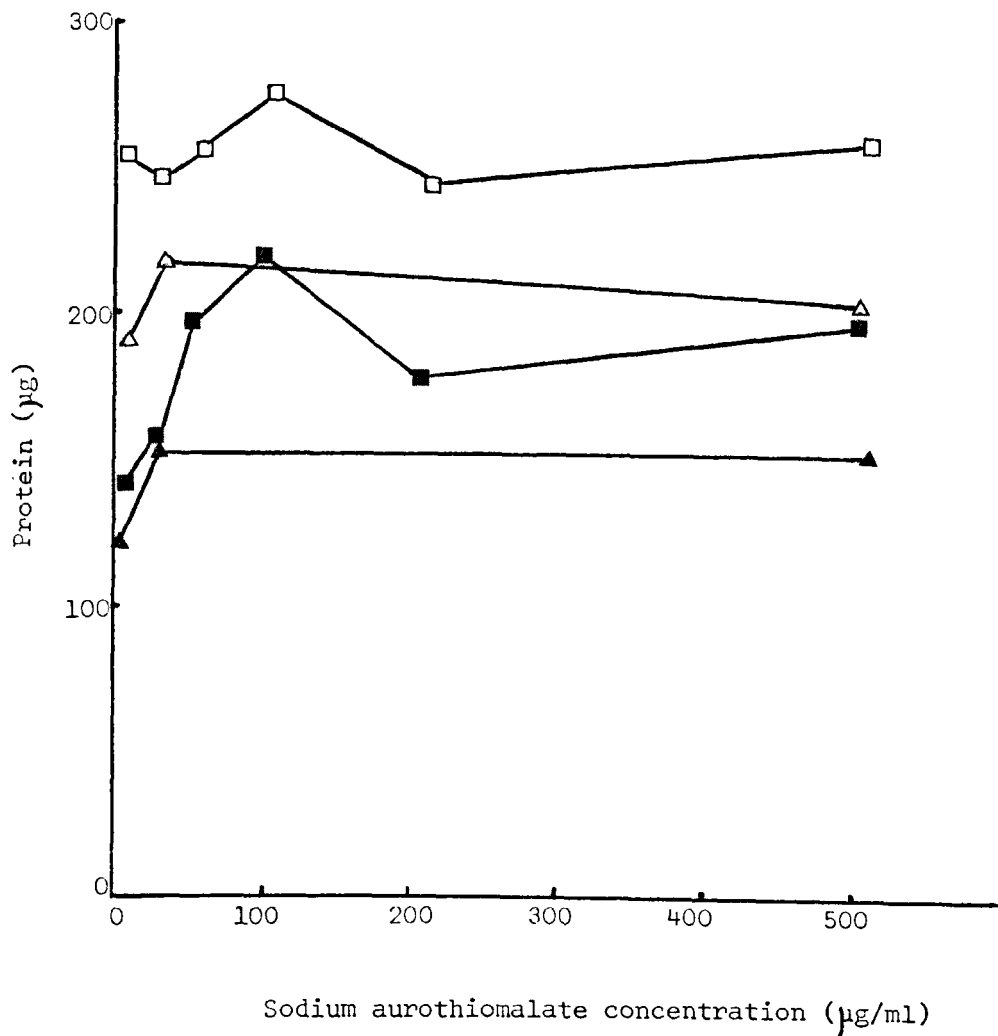
Figure 7.2 Protein contents of yolk sacs (filled symbols) and embryos (open symbols) after incubation of conceptuses in the presence of sodium aurothiomalate for either the final 6 hours (squares) or the penultimate 6 hours (triangles) of culture. Values are means of at least 6 determinations.

at harvesting varied similarly with concentration of the drug (Figure 7.2).

When sodium aurothiomalate, with ^{125}I-PVP, was present in culture for the final 6 hours, there was no radioactivity detectable in embryos at harvesting (Figure 7.3). In yolk sacs, the uptake of radioactivity was decreased, compared with the control value. This lower level of uptake was independent of the concentration of sodium aurothiomalate.

When ^{125}I-dBSA and sodium aurothiomalate were present in culture for the final 6 hours, little radioactivity, and all of it TCA-soluble, was detected in the embryos (Figure 7.4). The total amount of radiolabelled protein processed by yolk sacs was decreased, relative to the control. At a concentration of 25 μg/ml, sodium aurothiomalate reduced the total uptake by yolk sac to about 72%. However, the proportion that was associated with the yolk sac at harvesting was almost twice that seen in the control. The percentage of yolk sac-associated radioactivity that was TCA-soluble was also lower in conceptuses exposed to the drug. At a sodium aurothiomalate concentration of 500 μg/ml, the total uptake of radioactivity by yolk sac was reduced to 43% of the control value. This decrease was accompanied by an increase in the proportion that was yolk sac-associated, approximately 4 times the control figure, and a decrease in the percentage TCA-soluble radioactivity associated with the yolk sac (55% of the control value).

The effect of sodium aurothiomalate on the total uptake of radioactivity by yolk sacs (Figure 7.5) when the drug was present in culture between 36 and 42 hours and ^{125}I-dBSA for the final 6 hours, was qualitatively similar, though of smaller magnitude, to that described above. However, in contrast to those data, the drug did not alter the proportion of the
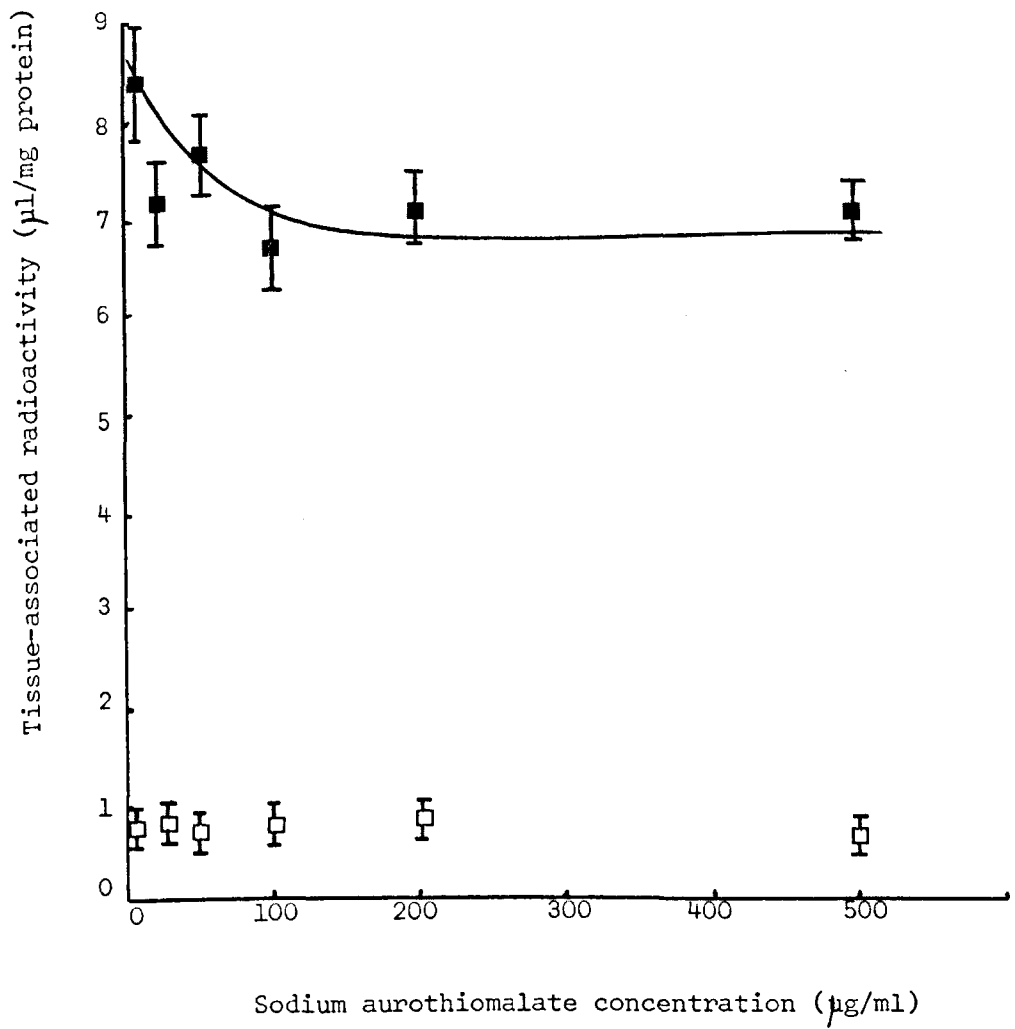
Figure 7.3 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 µg/ml) and sodium aurothiomalate for the final 6 hours of culture. Values are means (± S.D.) of at least 6 determinations.
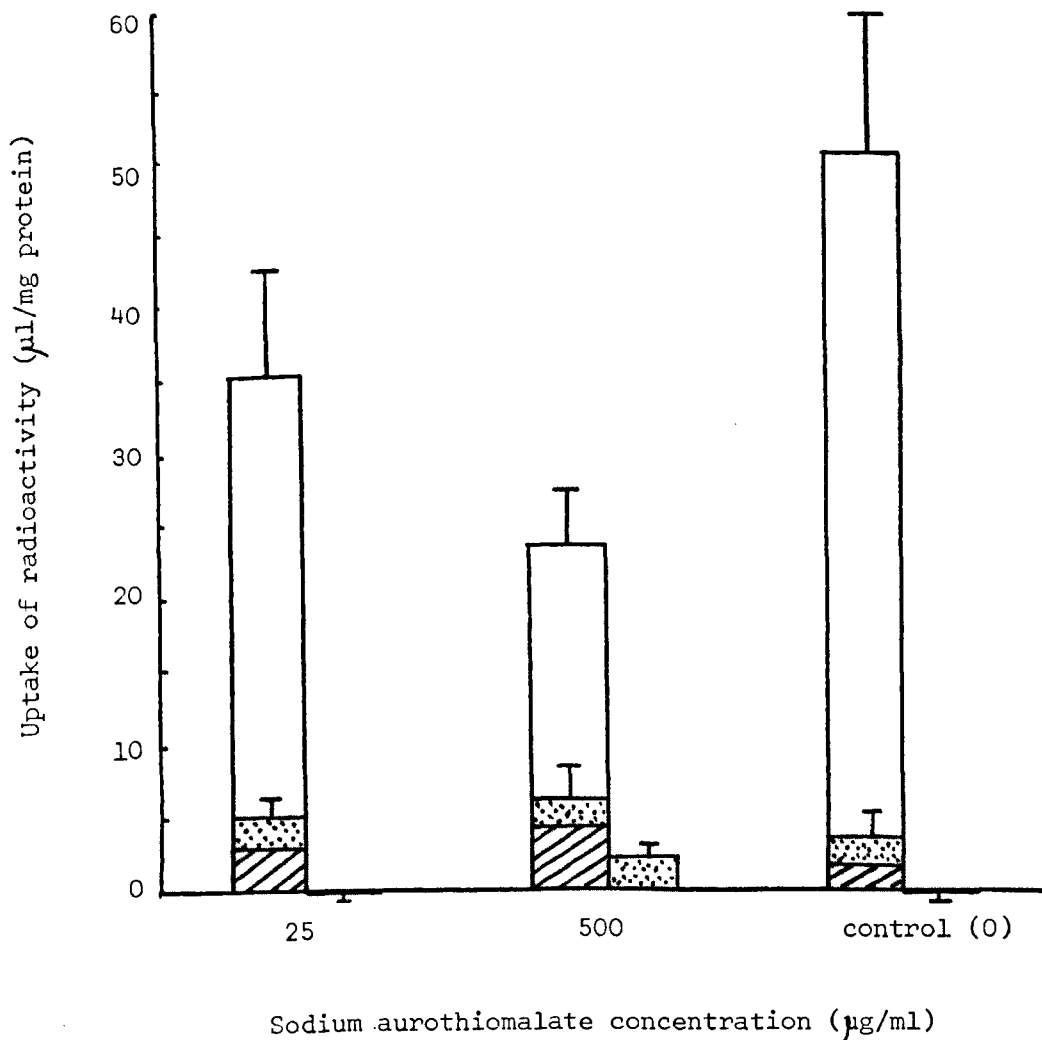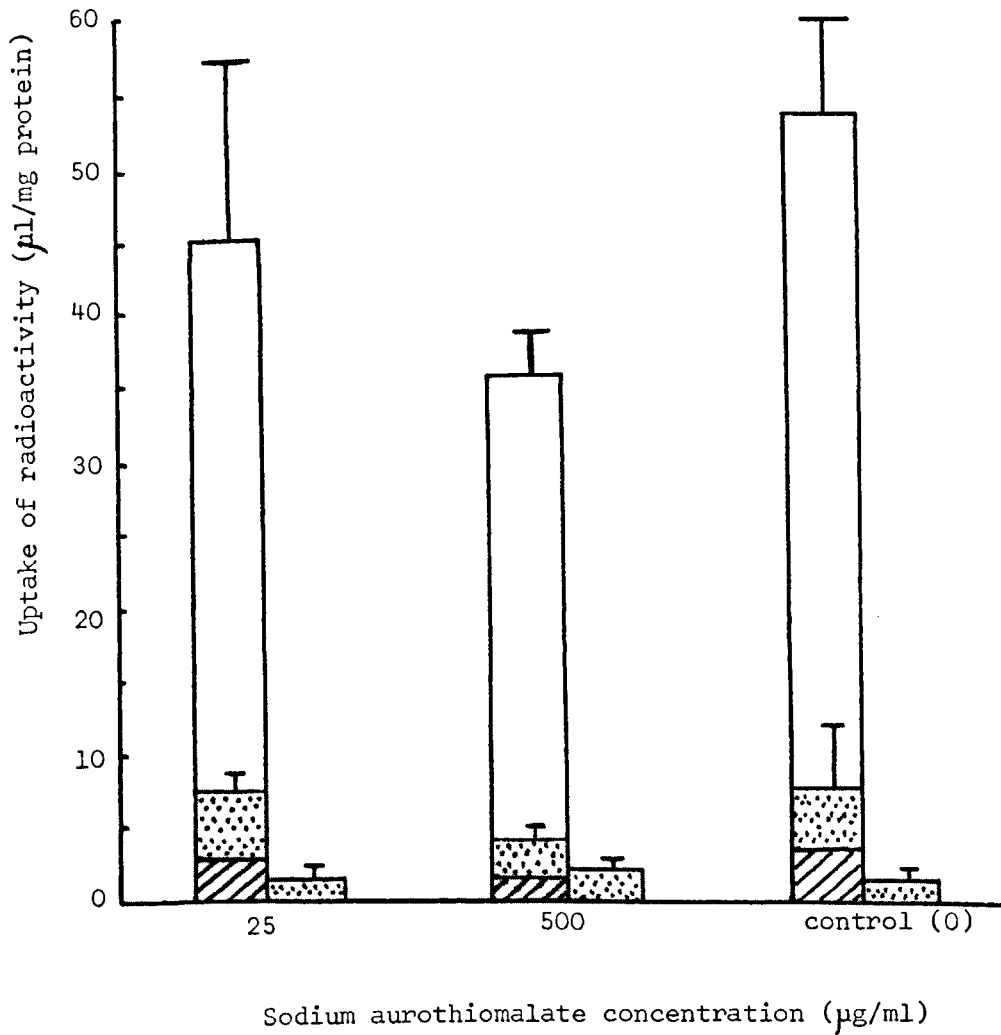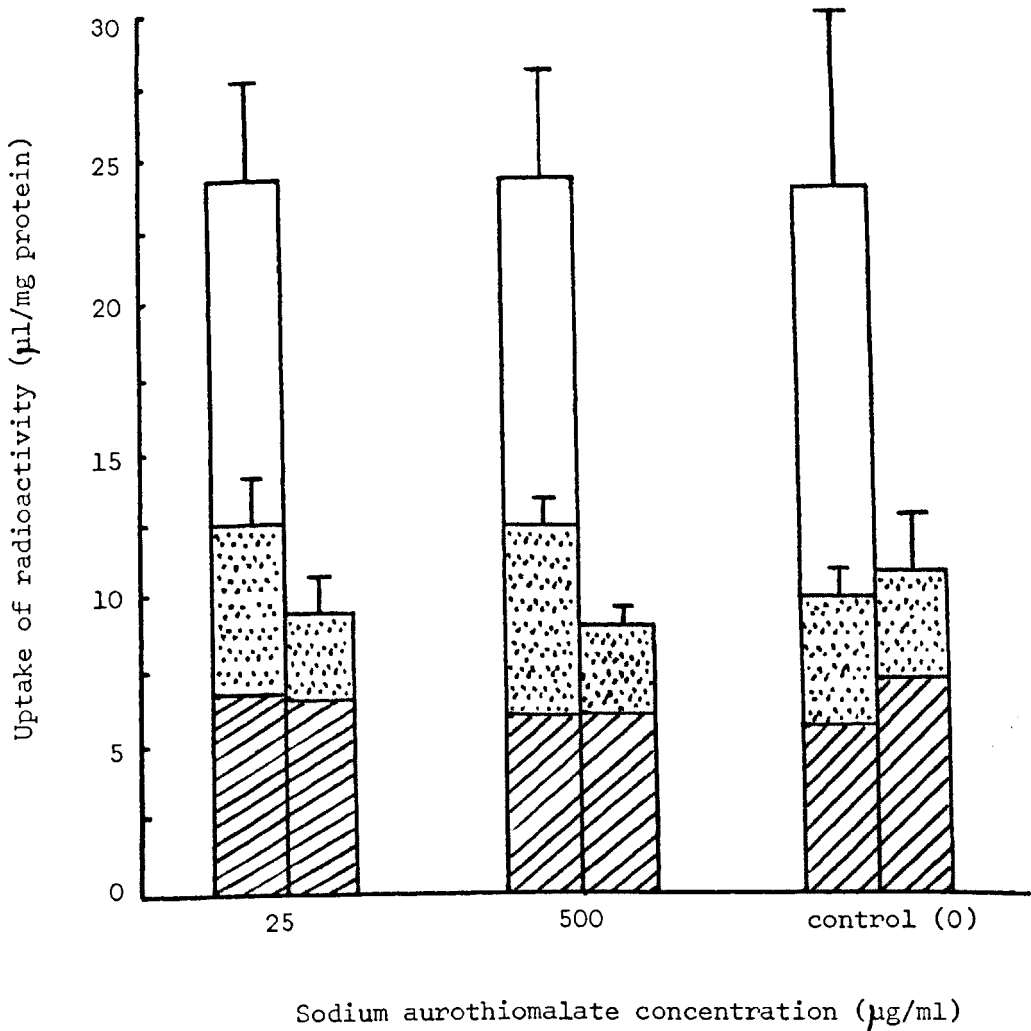
Figure 7.4 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml) and aurothiomalate for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble and TCA-insoluble are indicated. Values are means (± S.D.) of at least 6 determinations.

Figure 7.5   Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml) for the final 6 hours, and sodium aurothiomalate for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble [▨] and TCA-insoluble [▨] are indicated. Values are means (+ S.D.) of at least 6 determinations.
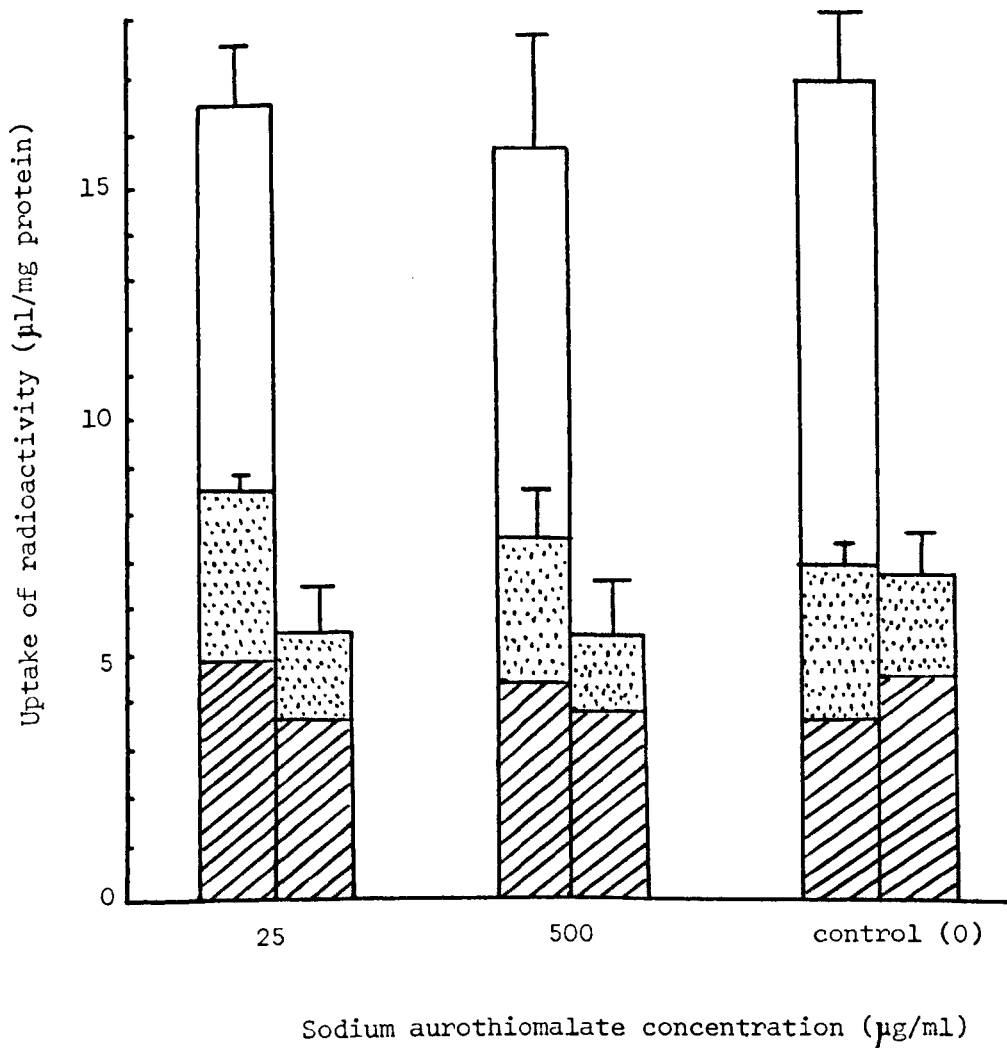
Figure 7.6 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$ leucine-labelled serum proteins and sodium aurothiomalate for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble and TCA-insoluble are indicated. Values are means ($\pm$ S.D.) of at least 6 determinations.

Figure 7.7 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$ leucine-labelled serum proteins for the final 6 hours, and sodium aurothiomalate for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▨ and TCA-insoluble ▨ are indicated. Values are means (± S.D.) of at least 6 determinations.

total radioactivity taken up that was, at harvesting, yolk sac-associated, or the percentage of the yolk-associated radioactivity that was TCA-soluble.

When sodium aurothiomalate was presented to conceptuses with ^{3}H-labelled serum proteins for the final 6 hours of culture, the uptake of radioactivity was not altered (Figure 7.6). There was however a slight, but significant (p < 0.02), increase in the proportion of this radioactivity that was associated with the yolk sac at harvesting, and a concomitant decrease in embryo-associated radioactivity. Essentially identical data were obtained (Figure 7.7) when the drug was present in culture from 36 to 42 hours, and the radiolabelled proteins from 42 to 48 hours.

7.5   Discussion

In agreement with previous reports (Kidston et al., 1971; Szabo et al., 1978a) aurothiomalate was found in the present study to be teratogenic in rats. As in these studies, the most common defects observed were hydrocephalus and kidney malformation.

The observation (Figure 7.2) that the protein content of yolk sac is increased following exposure to sodium aurothiomalate in culture, is most readily explained by an induced lysosomal storage of protein due to an inhibition of lysosomal proteolysis by the drug. Indeed, this is clearly demonstrated by the finding that, when $\left[^3H\right]$ leucine-labelled serum proteins were taken up by the conceptus, radioactivity was unevenly distributed between yolk sac and embryo in the presence of sodium aurothiomalate, a greater proportion being associated with the yolk sac (Figures 7.6 and 7.7). This must be borne in mind when considering the apparent inhibition of ^{125}I-PVP uptake into yolk sac caused by sodium aurothiomalate. Since, in the expression used throughout this study, uptake is inversely related to the quantity of tissue protein, an

increase in the amount of yolk sac protein through lysosomal storage, will artificially decrease the calculated figure of uptake. The relationship of the concentration of sodium aurothiomalate in the culture medium to protein content of yolk sacs (Figure 7.2), and to ^{125}I-PVP uptake by yolk sac (Figure 7.3), demonstrates that the decreased 'uptake' of radiolabel is entirely due to the elevated protein levels in the yolk sac, and not to an inhibition of pinocytosis. No inhibition of the uptake of ^{3}H-labelled serum proteins was observed. This is to be expected. The stored protein in yolk sac lysosomes caused by the action of sodium aurothiomalate is of course derived from the serum protein in the culture medium. When this serum protein is radiolabelled, radioactivity will be accumulated in direct proportion to the stored serum protein.

However, the decreased uptake of ^{125}I-dBSA by yolk sac in the presence of aurothiomalate cannot be wholly explained on a similar basis. Certainly, at the lower concentration of sodium aurothiomalate used, the decrease in the total uptake of radioactivity is largely due to the increased protein content of yolk sac. At the higher concentration of the drug though, the extent of inhibition of uptake (more than 50% of the control value) is much more than would be expected simply on the basis of increased yolk sac protein. The drug would seem therefore to be causing a true inhibition of the pinocytosis of ^{125}I-dBSA. This observation is in agreement with the finding of Kidston (1974) that pinocytosis of ^{125}I-dBSA is inhibited by aurothiomalate in the cultured 17.5-day rat yolk sac. Kidston (1974) also observed that the drug did not affect the uptake of ^{125}I-PVP by yolk sac. In the present study, aurothiomalate inhibited the uptake of ^{125}I-dBSA both when present in culture at the same time as the

substrate and when loaded into conceptuses before their exposure to radiolabel. Evidently, the drug's effects on pinocytosis are exerted from within the cell and are not explicable on the basis of a competition for the binding sites on the plasma membrane to which the substrate normally adsorbs, or by an interaction between drug and substrate which modifies the latter's affinity for such binding sites.

The reason why sodium aurothiomalate inhibits the pinocytosis by yolk sac of ^{125}I-dBSA but not ^{125}I-PVP is not clear, but may relate to the different modes of uptake of the two substrates, fluid-phase in the case of ^{125}I-PVP and adsorptive in the case of ^{125}I-dBSA. Silverstein et al. (1977) reported that the microtubule disaggregator cytochalasin B inhibited fluid-phase but not adsorptive pinocytosis by the mouse peritoneal macrophage. Hence, it would appear that the two types of uptake are not governed by exactly the same factors, and their different sensitivities to sodium aurothiomalate might be explained on that basis.

In addition to inhibiting the uptake of ^{125}I-dBSA, sodium aurothiomalate also inhibited the yolk sac intralysosomal degradation of the radio-labelled protein. However, inhibition only occurred when the drug was present in culture for the final 6 hours, and not when loaded into yolk sacs before their exposure to radiolabel. In this respect, the data resemble those obtained using trypan blue (Chapter 5). However, unlike trypan blue, sodium aurothiomalate inhibited the degradation of ^{3}H-labelled serum proteins in yolk sacs. Furthermore, this inhibition occurred regardless of whether the drug was added before, or together with, the radiolabelled substrates. Taken together, these observations would seem to indicate that pinosomes containing ^{3}H-labelled serum protein are able to fuse with heterolysosomes containing sodium

aurothiomalate that had been previously taken up by yolk sac, while pinosomes containing ^{125}I-dBSA are not. This explanation again infers that differences exist in the intracellular processing of substrates pinocytosed adsorptively (such as ^{125}I-dBSA) and substrates (such as ^{3}H-labelled serum proteins) that are pinocytosed to a large extent in the fluid phase. Until such differences are characterised, it is not possible to draw a more definite conclusion.

The findings of Ennis et al. (1968) that the inhibitory action of sodium aurothiomalate on lysosomal enzymes resembled the action of enzyme inhibitors known to act by sulphydryl binding, and could be reversed by the addition of cysteine to the incubation medium, led these workers to propose that the biological effects of the drug were due to its sulphydryl reactivity. In support of this claim, the inhibition by sodium aurothiomalate of mitogen-induced human lymphocyte proliferation, in vitro, has been shown (McCormack and Palmer, 1980) to be sulphydryl-dependent. In view of this property of the drug, it is to be expected that lysosomal thiol proteinases (such as cathepsins B and L) would be inhibited by aurothiomalate (Davies et al., 1971). Inhibition of thiol proteinases is, most likely, the cause of the induced lysosomal storage of ^{125}I-dBSA and ^{3}H-labelled serum proteins. However, sodium aurothiomalate binds strongly to native serum albumin (McQueen and Dykes, 1969) and probably enters yolk sac lysosomes, in vitro, in this bound form. Such binding will protect the yolk sac cells from too severe an inhibitory effect of the drug on lysosomal enzymes and would explain why the decrease in proteolysis, though significant, is only slight.

From the foregoing discussion, it is once again clear that the composition of the fluid surrounding the conceptus during early organogenesis in utero will be of critical importance in determining the extent of the effect that sodium aurothiomalate is going to exert upon the lysosomal digestion of macromolecular nutrient, and hence the induction of embryonic malformation. In considering the mechanism of aurothiomalate-induced teratogenesis, the possible effects of the drug on the maternal organism which may secondarily affect the development of the embryo must also be evaluated before any firm hypothesis can be advanced to explain the drug's teratogenic action. Even so, the data presented here provide firm support for the proposal that aurothiomalate's primary target in teratogenesis is yolk sac-mediated histiotrophic nutrition, specifically the inhibition of proteolysis in yolk sac lysosomes which consequentially reduces the supply of amino acids for protein synthesis in the embryo.

CHAPTER EIGHT

THE EFFECT OF ANTI-YOLK SAC ANTISERUM
ON YOLK SAC-MEDIATED NUTRITION

8.1   Introduction

In a long-running series of publications Brent and his associates
have reported the teratogenic effects and tissue distribution in rats
of maternally-administered heterologous antisera to various rat tissues.
The finding (Slotnick and Brent, 1966) that sheep anti-rat kidney and
sheep anti-rat placenta antisera, when administered to pregnant rats
at day 8 of gestation, localised strongly in the yolk sac but did
not reach the embryo, together with the proposal (Beck et al., 1967a)
that the yolk sac serves a nutritional function during early organo-
genesis, led to the suggestion (Lloyd, 1970) that the antisera caused
malformations by interfering with this function of the yolk sac in
the same way as proposed for trypan blue (Beck et al., 1967b). This
suggestion was strengthened further when it was discovered (Brent et
al., 1971) that antiserum raised in sheep against rat visceral yolk
sac was also teratogenic in rats, and showed a strong and lasting
localisation in the yolk sac cytoplasm. Additional evidence that
the yolk sac was the primary target in antiserum-induced terato-
genesis came from experiments by New and Brent (1972). Using a
whole-embryo culture technique they were able to demonstrate that the
yolk sac endoderm was uniquely sensitive to the yolk sac antibody
and that exposure of this membrane to the antibody caused growth
and developmental retardation of the embryo.

In an effort to understand the mechanism of teratogenic action of
antiserum better, the effect of a rabbit anti-rat yolk sac antiserum,
of established teratogenicity, on pinocytosis and proteolysis by the
early organogenesis-stage rat conceptus has been studied.

8.2   Materials

Chemicals

Rabbit Anti-Rat 18-day Visceral Yolk sac Antiserum was a generous gift of Dr. R. L. Brent, Thomas Jefferson University, Philadelphia, USA. Other chemicals not previously listed were of analytical grade.

8.3   Methods

8.3.1 Preparation of Normal Rabbit Serum

A single Californian rabbit was bled from the marginal vein of the ear. The ear was shaved of hair, swabbed in alcohol and heated with a lamp to dilate the marginal vein. An oblique cut was made across the vein with a scalpel and blood was allowed to drip into a plastic centrifuge tube and clot. The clotted blood was refrigerated overnight and then re-mixed and centrifuged at 2,000 g for 10 minutes. The clear supernatant serum was decanted and its protein content (approximately 70 mg/ml) was estimated by the method of Lowry et al. (1951), as described in Chapter 2.

8.3.2 Teratogenic Testing of Rabbit Anti-Rat Visceral Yolk Sac Antiserum

The procedure was as described for trypan blue (5.3.3). Antiserum was administered by intraperitoneal injection at a dosage of 100 mg serum protein/kg body weight. Normal rabbit serum was administered, in an identical manner and at the same dosage, to a control group of pregnant rats.

8.3.3 Culture Experiments

The procedure was as described for trypan blue (5.3.4). In cultures that used ^{125}I-PVP as substrate, antiserum was added to the culture

medium to a final concentration of 83, 167, 333, 1,667 or 3,333 µg
total serum protein/ml. For subsequent experiments with ^{125}I-dBSA
and ^3H-labelled serum proteins as substrates, antiserum was added to
a final concentration of 83 or 333 µg serum protein/ml. Control
cultures tested the effects of normal rabbit serum at concentrations
identical to those of antiserum employed. The effect of normal
rabbit serum on pinocytosis and digestion when present in culture
for only the last 6 hours, was investigated.

8.4   Results
8.4.1 Teratogenic Testing of Anti-Rat Yolk Sac Antiserum

Intraperitoneal injection of rabbit anti-rat yolk sac antiserum
(100 mg protein/kg) into pregnant rats at either 8.5 or 9.5 days
of gestation resulted in the production of malformed fetuses (Table 8.1).
Following injection of antiserum at 8.5 days, the number of resorption
sites visible at 20.5 days of gestation constituted 37% of the total
number of implantations. An exceedingly high proportion, 87%, of the
surviving fetuses were visibly externally malformed, and all of those
fetuses sectioned exhibited malformation. Of the 18 fetuses assessed
for skeletal development, 17 (94%) were malformed, though the single
fetus whose skeleton was normally developed had been observed to be
anophthalmic upon external examination. Hence all fetuses that
survived maternal antiserum injection were malformed.

The types of defects observed and their frequencies are listed in
Table 8.2. Anophthalmia and hydrocephalus were the most common mal-
formations noted and there was a high incidence also of absent or
malformed kidneys and gonads. Among the skeletal defects, agenesis
or malformation of the sternum was particularly common, while

Nature and time of injection	No. of ♀ treated	No. of implantations (% resorptions)	Av. weight surviving fetuses (g)	External Abnormalities			No. of fetuses sectioned	No.(%) sectioned fetuses with anomalies	No.of fetuses stained with alizarin	No.(%) stained fetuses with anomalies
				No.	%surviving fetuses	% total implntns				
Antiserum 8.5 days	8	87 (37)	2.18 ±.59*	48	87.27	55.17	37	37 (100)	18	17 (94)
Antiserum 9.5 days	8	84 (74)	3.65 ±1.59	6	27.27	7.14	16	14 (88)	6	6 (67)
Normal rab. serum, 8.5 d	4	39 (3)	3.73 ± .33	0	0	0	26	1 (4)	12	3 (25)
Normal rab. serum, 9.5 d	4	49 (2)	3.82 ± .38	0	0	0	32	1 (3)	16	1 (6)

* significantly different from normal rabbit serum-injected controls (p < 0.001)

Table 8.1 Effects on offspring of intraperitoneal injection of rabbit anti-rat visceral yolk sac antiserum (100 mg/kg) or normal rabbit serum (100mg/kg) into pregnant rats. Details of method are given in text.

Day of injection of antiserum	8.5	9.5
Abnormality		
Anophthalmia	53 (55)	10 (22)
Hydrocephalus	31 (48)	9 (16)
Kidney (absent or deformed)	13 (48)	1 (16)
Gonads (absent or deformed)	15 (48)	3 (16)
Exencephaly	1 (55)	0
Limb defects	1 (55)	0
Spina bifida	2 (55)	0
Ventral hernia	1 (55)	0
Facial defects	2 (55)	0
Tail defects	2 (55)	0
Jaw defects	2 (18)	0
Spinal defects	8 (18)	1 (6)
Absent sternebrae	17 (18)	4 (6)
Ribs (absent or deformed)	8 (18)	1 (6)

Table 8.2 Abnormalities of fetuses after rabbit anti-rat visceral yolk sac treatment of pregnant rats. Figures in brackets indicate the size of the population examined for the particular defect.

malformations of the spine and ribs were also frequent. Maternal injection of antiserum at 8.5 days of gestation significantly ($p < 0.001$) reduced the average weight of surviving fetuses, compared with normal rabbit serum-injected controls.

Following injection of antiserum into 9.5-day pregnant rats, a very high level (74%) of fetal resorption was recorded. Of the 22 surviving fetuses, all but 4 were malformed. Though the range of malformations observed was not as extensive as in the 8.5-day injected group, the relative frequencies of the malformations observed in the 9.5-day injected group were similar to those in the 8.5-day injected group. The average weight of fetuses surviving maternal injection of antiserum at 9.5 days of gestation was not significantly different from normal rabbit serum-injected controls ($p > 0.1$).

Control pregnant rats, injected with normal rabbit serum (100 mg protein/kg) at either 8.5 or 9.5 days of gestation, did not resorb their embryos to any significant extent, though a few fetuses were malformed. (Table 8.1).

8.4.2 Culture Experiments

The inclusion of anti-rat yolk sac antiserum in the culture medium during either the final 6 hours or the penultimate 6 hours of culture, did not affect the viability of conceptuses, which was at least 95%, or cause developmental deviations among cultured conceptuses. The protein contents at harvesting of yolk sacs and embryos of conceptuses incubated for the final 6 hours of the culture period (42-48 hours)

in a culture medium containing antiserum were decreased to a similar extent (Figure 8.1). The extent of the decrease was dependent on the concentration of antiserum protein in the culture medium. A decrease in protein content of conceptuses at harvesting was also observed when antiserum was present in culture between 36 and 42 hours (Figure 8.1). The inclusion of normal rabbit serum in the culture medium, at the same concentrations as were used for antiserum, did not alter the protein contents of conceptuses at harvesting (Figure 8.2).

When antiserum and ^{125}I-PVP (2.3 μg/ml) were present in the culture medium for the final 6 hours of culture, there was virtually no uptake of radioactivity by embryos at harvesting (Figure 8.3). The yolk sac however contained quantities of radioactivity that varied with the concentration of antiserum in the medium. Increasing the concentration of antiserum caused a progressive decrease in the uptake of radioactivity. The level of uptake by yolk sacs exposed to 167 μg/ml of antiserum was 57% of the control value. As the antiserum concentration was increased above 167 μg/ml, the uptake of radioactivity by the yolk sac was further decreased. At 3,333 μg/ml of antiserum (1.0 mg/ml of gamma globulin) the uptake of radioactivity by yolk sac was reduced to 40% of controls.

Interestingly, when conceptuses were exposed to antiserum concentrations of 83 μg/ml (which inhibited uptake to 69% of controls) and 333 μg/ml (which inhibited uptake to 56% of controls) for a 6 hour period (36 - 42 hours of culture) before being transferred to fresh medium containing ^{125}I-PVP (2.3 μg/ml) but no antiserum and cultured for a further 6 hours (42 - 48 hours of culture), the extent to which uptake of radioactivity by yolk sac was inhibited was virtually identical
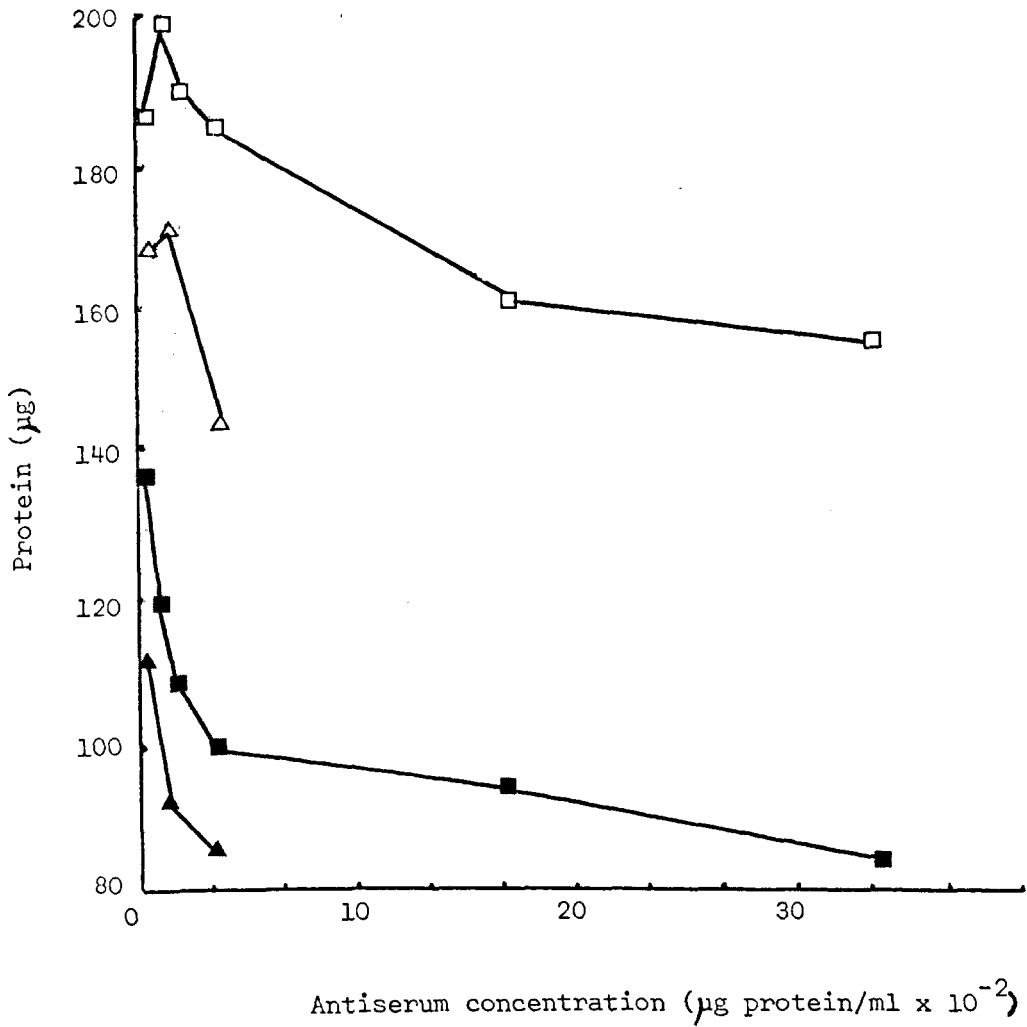
Figure 8.1 Protein contents of yolk sacs (filled symbols)
and embryos (open symbols) after incubation of conceptuses
in the presence of rabbit anti-rat visceral yolk sac
antiserum for either the final 6 hours (squares) or the
penultimate 6 hours (triangles) of culture. Values are
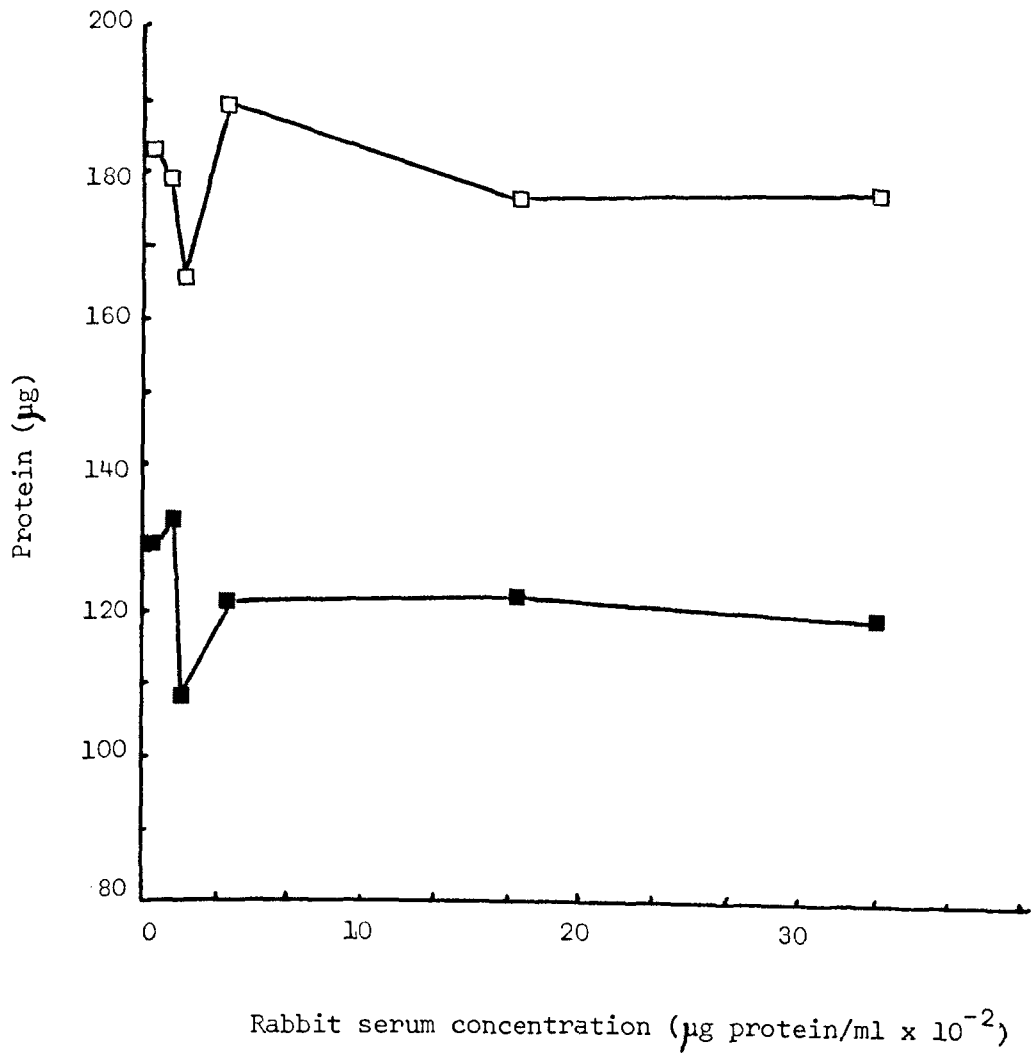means of at least 6 determinations.

Figure 8.2 Protein contents of yolk sacs (■) and embryos (□) after incubation of conceptuses in the presence of normal rabbit serum for the final 6 hours of culture. Values are means of at least 6 determinations.
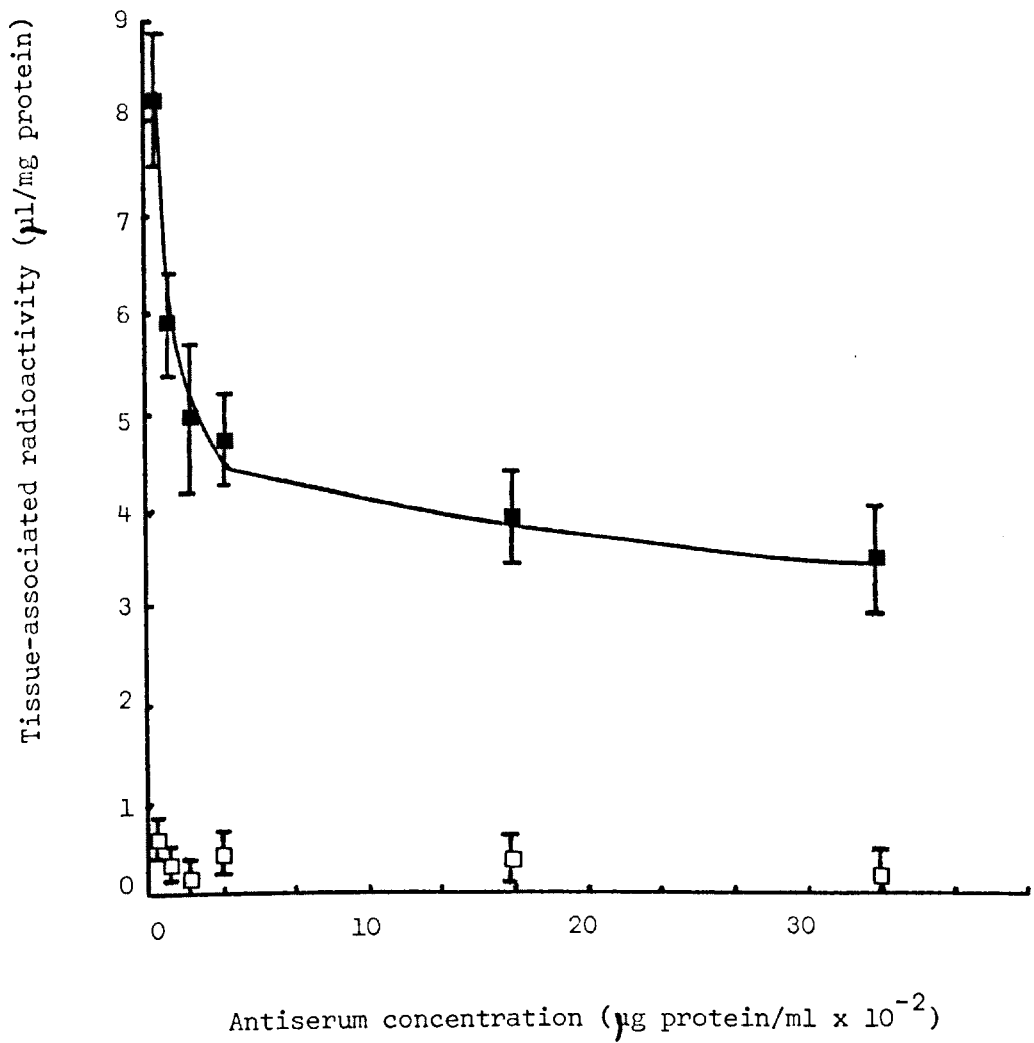
Figure 8.3 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 μg/ml) and rabbit anti-visceral yolk sac antiserum for the final 6 hours of culture. Values are means (± S.D.) of at least 6 determinations.

to that seen when antiserum and substrate were present in the culture medium at the same time (Figure 8.4). No uptake of radioactivity by embryos at harvesting was observed, but yolk sac uptake was inhibited to levels of 76% of controls at an antiserum concentration of 83 μg/ml (cf 69%) and 58% of controls at an antiserum concentration of 333 μg/ml (cf 56%).

In contrast to these data, antiserum (83 or 333 μg/ml) did not significantly affect (p > 0.05) the total uptake of radioactivity by conceptuses following their incubation for the final 6 hours of culture in a medium that contained both antiserum and ^{125}I-dBSA (11 μg/ml) (Figure 8.5). However, the proportion of the total radioactivity processed by the conceptus that was associated at harvesting with the yolk sac was significantly decreased (p < 0.001 at 83 μg/ml and p < 0.02 at 333 μg/ml), at both concentrations of antiserum, to less than half the control value. Furthermore, the proportion of yolk sac-associated radioactivity that was TCA-soluble was much lower in those conceptuses exposed to antiserum than controls. When antiserum was presented to conceptuses for 6 hours before a further 6 hour incubation in an antiserum-free medium containing ^{125}I-dBSA, almost identical results were obtained (Figure 8.6). Again little radio-activity, and all of it TCA-soluble, was detected in embryos at harvesting. Overall uptake of radioactivity by conceptuses was not different (p > 0.1) from controls, but the proportion of the total radioactivity processed that was associated with the yolk sac at harvesting was less than one half the control value (p < 0.001 at 83 μg/ml antiserum and p < 0.01 at 333 μg/ml antiserum). One difference between the two sets of data (Figures 8.5 and 8.6) is the
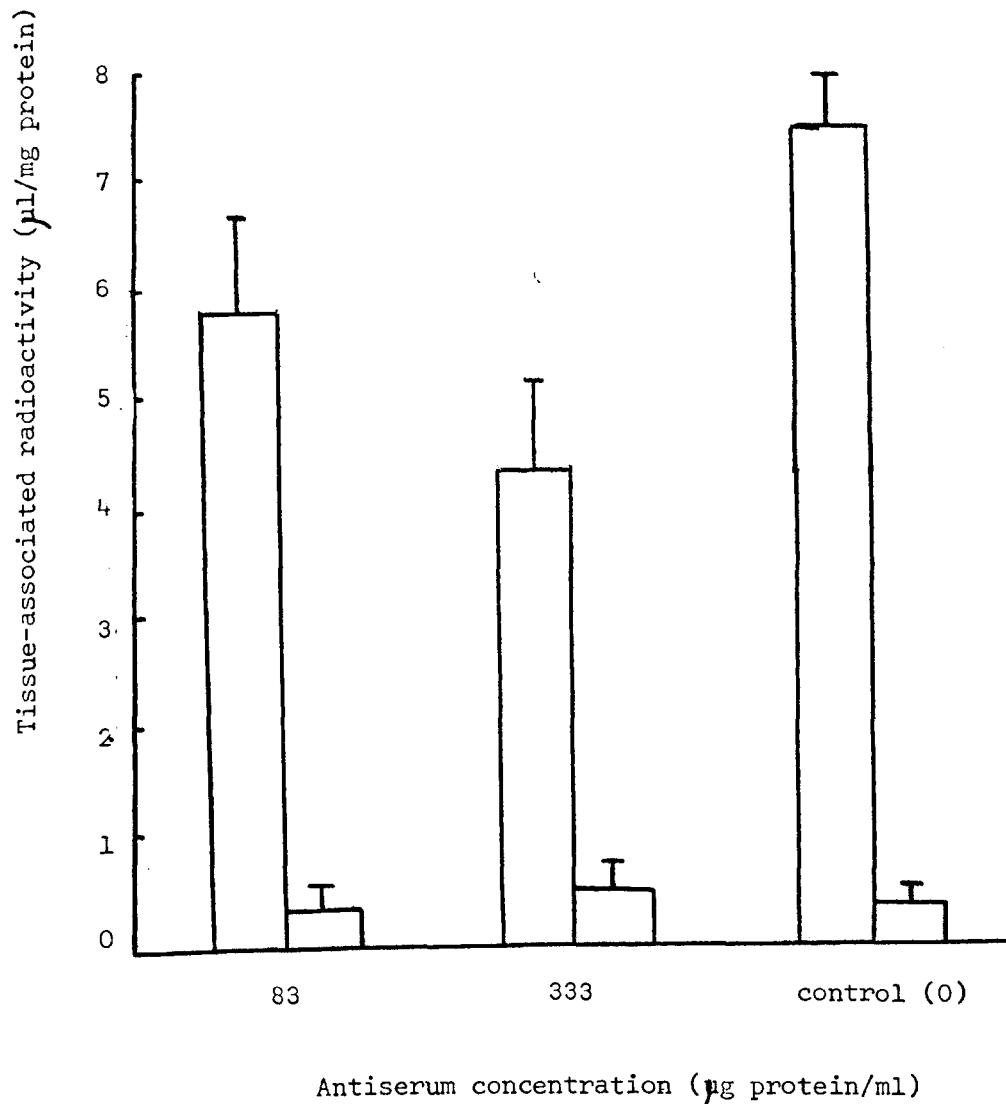
Figure 8.4 Radioactivity associated with yolk sac (each left hand column) and embryo (each right hand column) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 µg/ml) for the final 6 hours, and rabbit anti-visceral yolk sac antiserum for the penultimate 6 hours of culture. Values are means ($\pm$ S.D.) of at least 6 determinations.
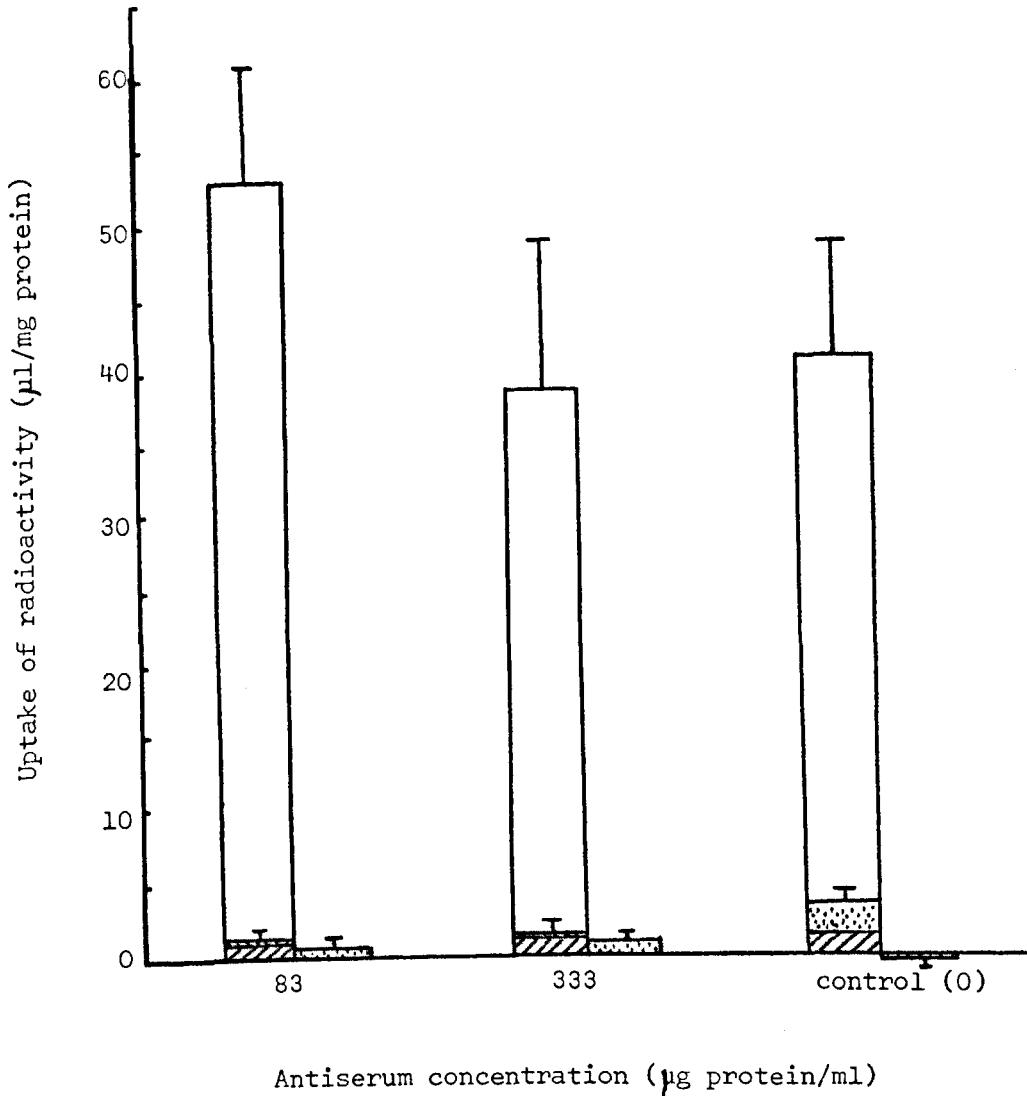
Figure 8.5 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml) and rabbit anti-visceral yolk sac antiserum for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble and TCA-insoluble are indicated. Values are means ($\pm$ S.D.) of at least 6 determinations.
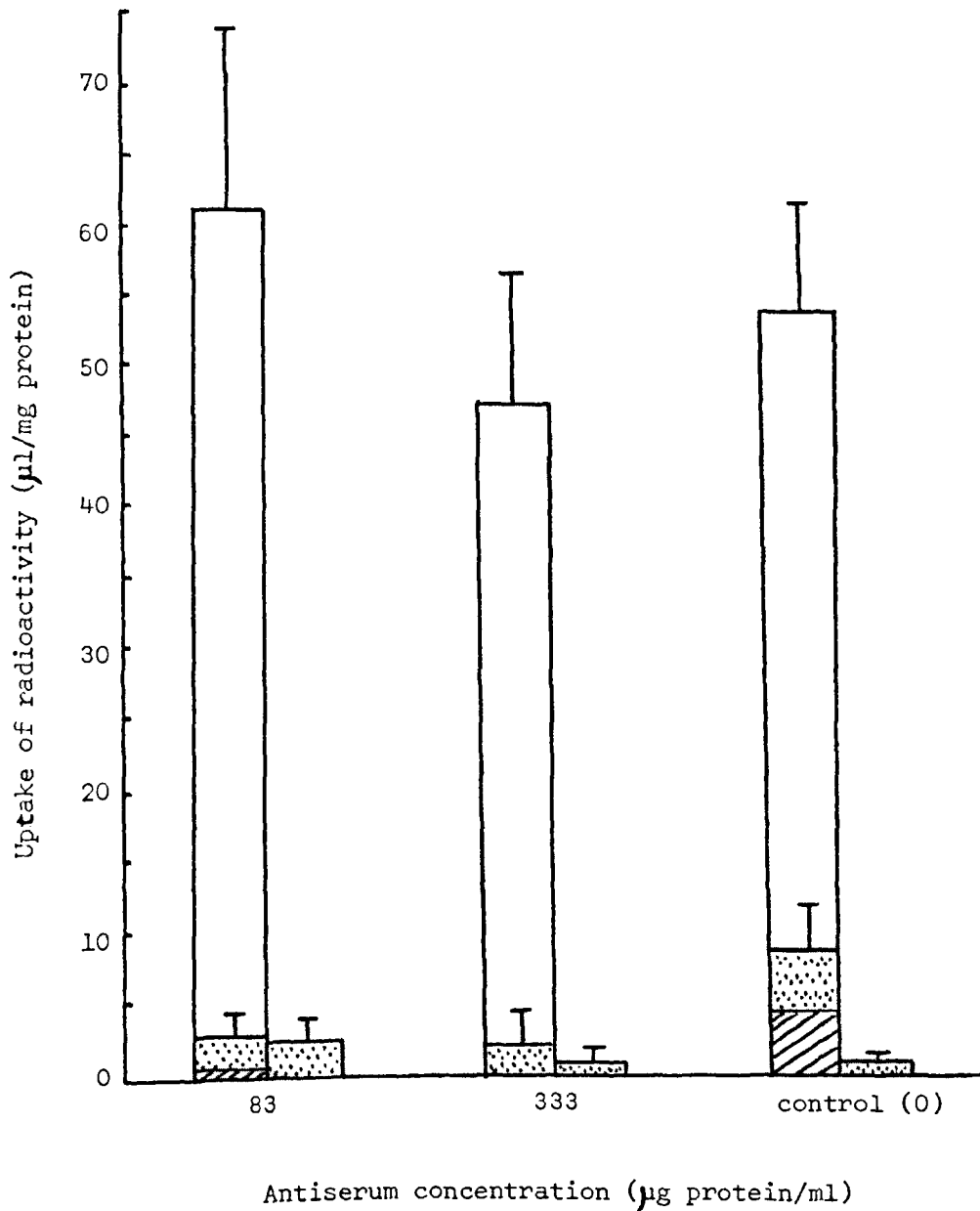
Figure 8.6   Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) for the final 6 hours, and rabbit anti-visceral yolk sac antiserum for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble [::::] and TCA-insoluble [////] are indicated. Values are means ($\pm$ S.D.) of at least 6 determinations.

high percentage of TCA-soluble radioactivity that was retained in the yolk sac at harvesting when conceptuses were exposed to antiserum before radiolabel as compared to the low TCA-soluble yolk sac-associated radioactivity when conceptuses were exposed to antiserum and radiolabel at the same time. However, it would be unwise to place too much weight on this observation since the standard deviations of the mean percent TCA-soluble radioactivity in the yolk sac were very large.

The uptake of radioactivity by conceptuses following simultaneous exposure for the final 6 hours of culture to antiserum (83 or 333 $\mu g/ml$) and ^3H-labelled serum proteins, is shown in Figure 8.7. At the lower antiserum concentration, the uptake and digestion of radiolabel within conceptuses was not noticeably different from controls. At the higher concentration of antiserum, total uptake of radioactivity into the conceptus was reduced to 62% of the control value, but the distribution of this radioactivity between yolk sac and embryo and the percentage radioactivity that was TCA-soluble, was not altered. When antiserum was loaded into conceptuses before exposure to ^3H-labelled serum proteins, almost identical data were obtained (Figure 8.8). Antiserum at a concentration of 83 $\mu g/ml$ produced no difference in the levels and distribution of radioactivity in conceptuses at harvesting compared with controls. At a concentration of 333 $\mu g/ml$, total incorporation of radiolabel into the conceptus was decreased to 55% of the control value. However the distribution of radiolabel and the proportion that was TCA-soluble was not altered.

The inclusion of normal rabbit serum in the culture medium for the final 6 hours of culture, at the concentrations employed with rabbit anti-rat yolk sac antiserum, together with either ^{125}I-PVP (2.3 $\mu g/ml$)(Figure 8.9),
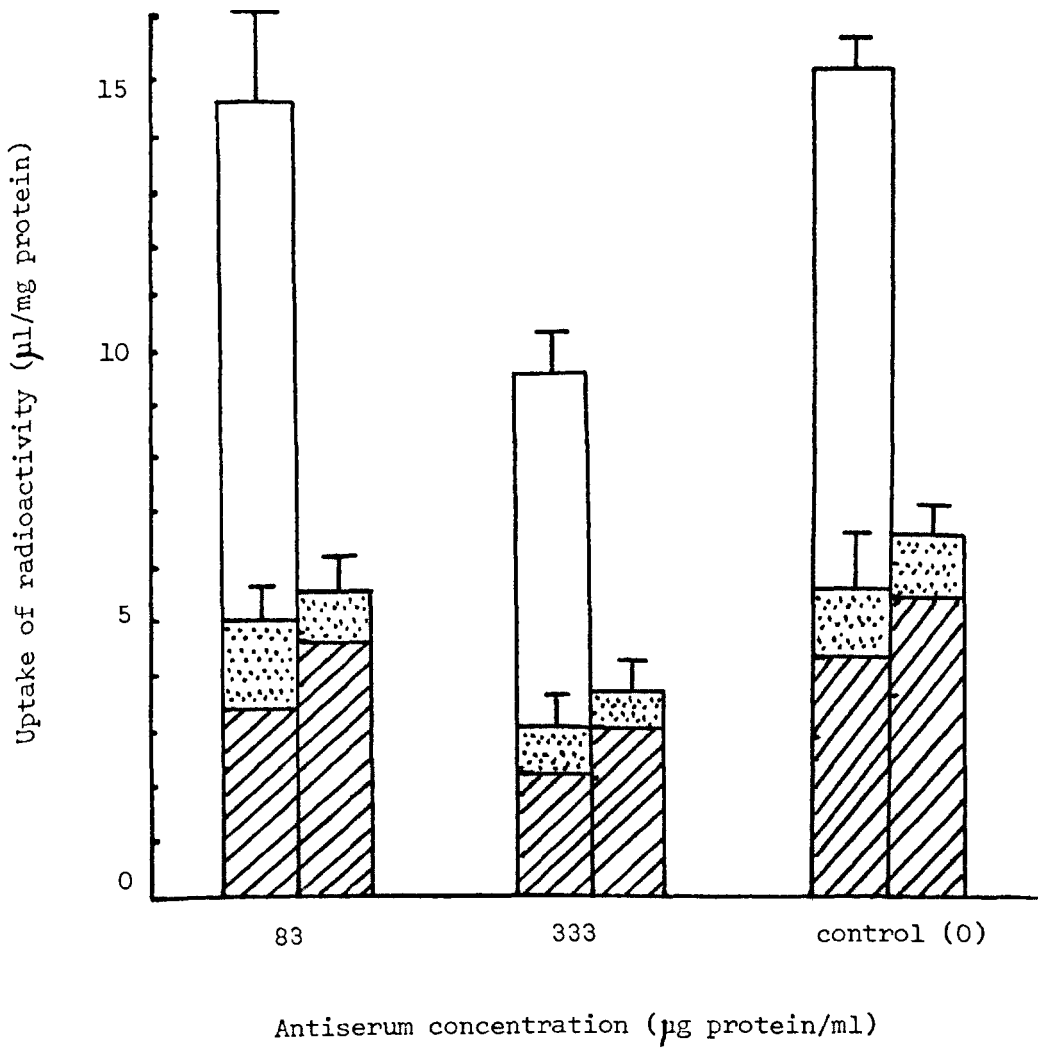
Figure 8.7 Total radioactivity taken up by yolk sac (each left
hand column) and embryo (each right hand column), and the
radioactivity associated with the yolk sac (total shaded area
of each left hand column) after incubation of conceptuses in the
presence of $[^3H]$ leucine-labelled serum proteins and rabbit anti-
visceral yolk sac antiserum for the final 6 hours of culture.
The proportions of yolk sac- and embryo-associated radioactivity
that were TCA-soluble [▨] and TCA-insoluble [▨] are indicated.
Values are means ($\pm$ S.D.) of at least 6 determinations.

Figure 8.8 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$ leucine-labelled serum proteins for the final 6 hours, and rabbit anti-visceral yolk sac antiserum for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble [⠿] and TCA-insoluble [⧄] are indicated. Values are means (± S.D.) of at least 6 determinations.

Figure 8.9 Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of ^{125}I-PVP (2.3 μg/ml) and normal rabbit serum for the final 6 hours of culture. Values are means (± S.D.) of at least 6 determinations.
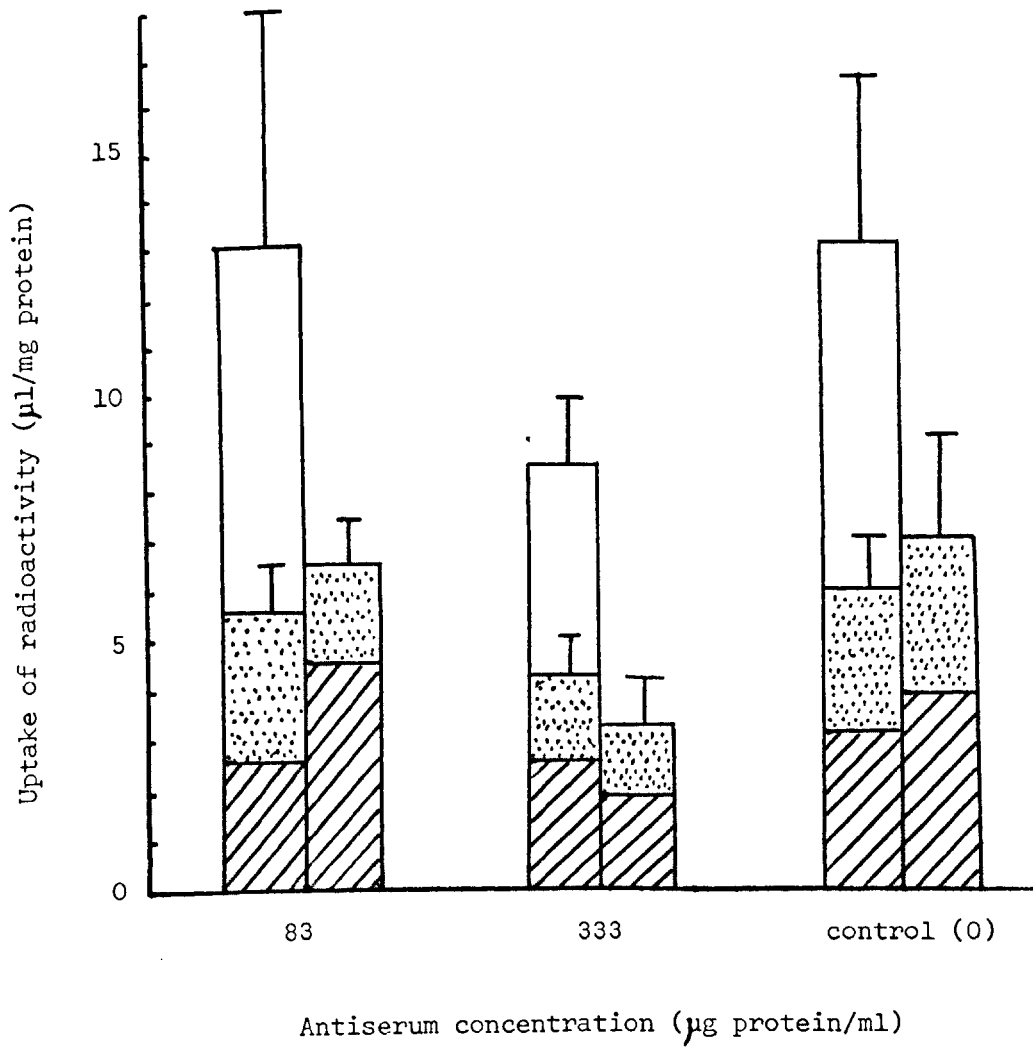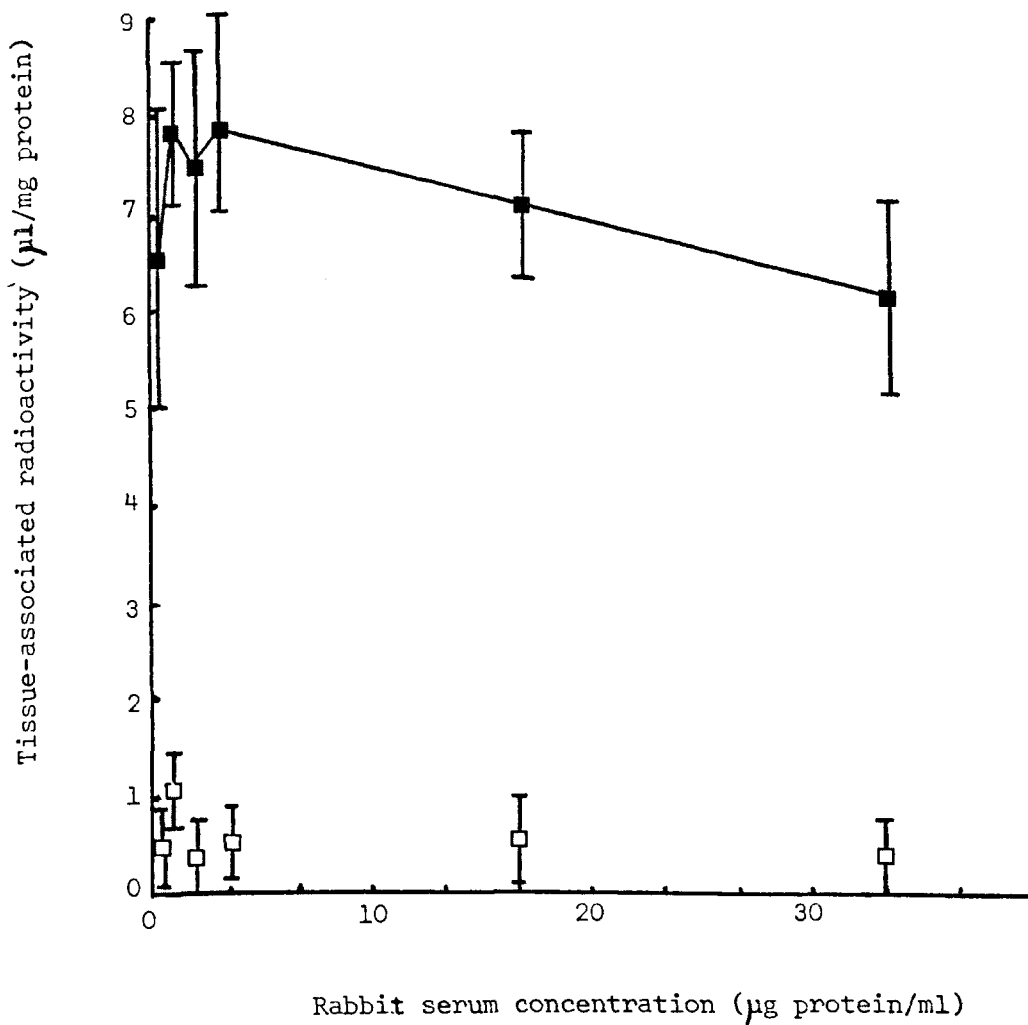
Figure 8.10 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) and normal rabbit serum for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▨ and TCA-insoluble ▨ are indicated. Values are means ($\pm$ S.D.) of at least 6 determinations.
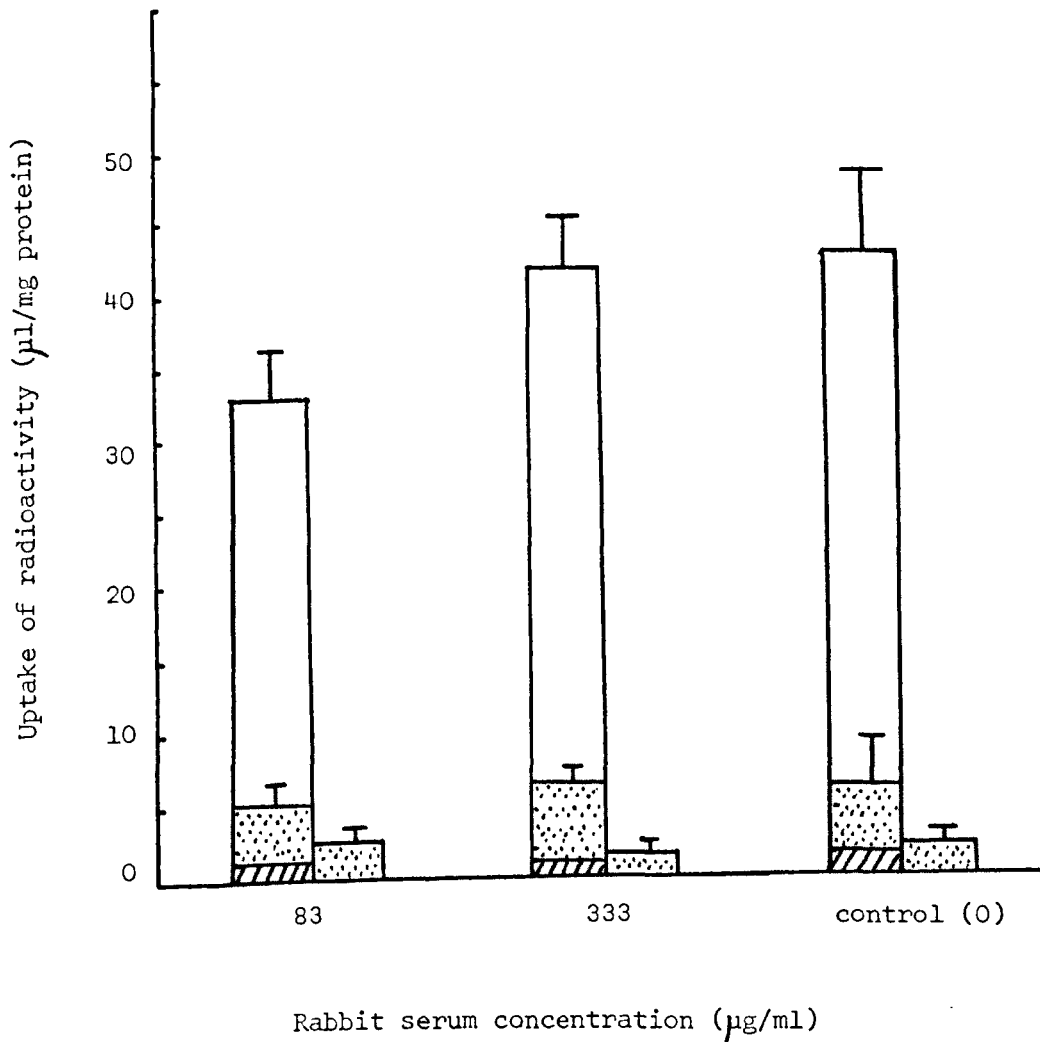
Figure 8.11 Total radioactivity taken up by yolk sac (each left
hand column) and embryo (each right hand column), and the
radioactivity associated with the yolk sac (total shaded area of
each left hand column) after incubation of conceptuses in the
presence of $[^3H]$ leucine-labelled serum proteins and rabbit serum
for the final 6 hours of culture. The proportions of yolk sac-
and embryo-associated radioactivity that were TCA-soluble 🔲
and TCA-insoluble 🔲 are indicated. Values are means ($\pm$ S.D.) of
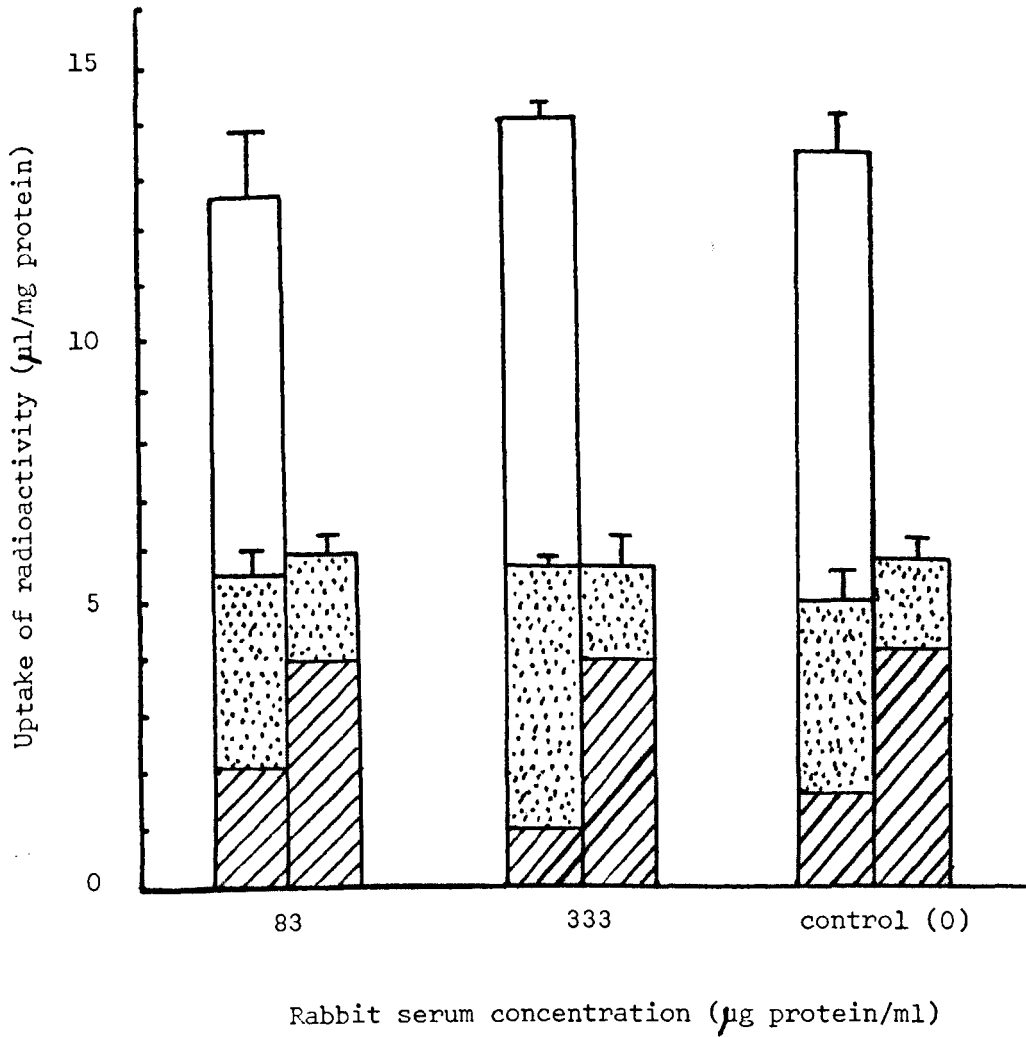at least 6 determinations.

125_{I}-dBSA (11 µg/ml)(Figure 8.10) or 3_{H}-labelled serum proteins (Figure 8.11), did not lead to any apparent differences in the levels of radioactivity either associated with or processed by conceptuses, when compared with controls that were not exposed to normal rabbit serum.

8.5   Discussion

The teratogenicity in rats of heterologous antisera raised against various rat tissues has frequently been reported in the literature. Brent et al. (1961) and Barrow and Taylor (1971) described the teratogenic effects in rats of anti-rat kidney antiserum obtained from rabbits. In subsequent studies, rabbit anti-rat placenta antiserum (Brent, 1967; Barrow and Taylor, 1971), sheep anti-rat visceral yolk sac antiserum (Brent et al., 1971) and rabbit anti-rat Reichert's membrane antiserum (Jensen et al., 1975) were shown to be teratogenic in rats. With the exception of the anti-Reichert's membrane antiserum, these antisera have all been observed by immunofluorescence to localise in the same tissues of the pregnant rat, particularly the kidney and the visceral yolk sac, indicating that they share a number of common antigens (Pressman and Korngold, 1957; Slotnick and Brent, 1966; Brent and Johnson, 1967).

The teratogenicity and embryolethality of rabbit anti-rat visceral yolk sac antiserum, obtained using yolk sacs from a different population of rats to the one used in the present study, is clearly evident from the data (Tables 8.1 and 8.2). The spectrum and incidence of the defects observed agreed with those previously reported (Brent et al., 1971).

The observations that antisera did not localise in the embryo (Slotnick and Brent, 1966) suggested that the teratogenic effect was due either to an interference with the normal function of extra-embryonic membranes, specifically the yolk sac, or to immunological disease of the mother which secondarily affected the embryo. This latter possibility merited investigation since it had been shown (Brent,1971) that both anti-kidney and anti-visceral yolk sac antiserum induced maternal nephritis. However this possible mechanism of action was made much less plausible by the finding (Leung et al., 1974) that two separate pools of antibodies raised against rat kidney in rabbits, were responsible for teratogenicity and nephrotoxicity, and that only the teratogenic antibodies localised in the visceral yolk sac. The visceral yolk sac's claim as the site of primary teratogenic action was further strengthened by the observations (New and Brent, 1972) that when sheep anti-rat visceral yolk sac antibody was added to the culture medium in which 9.5 or 10.5 day rat conceptuses were developing so that only the visceral yolk sac endoderm was directly exposed to antibody, the antibody caused retarded growth and development of the embryos. When, however, the antibody was injected into the amniotic cavity so that the embryo was directly exposed to it, or into the extra-embryonic coelom which exposed the mesodermal surface of the yolk sac to antibody, little or no effect on embryonic development was noted.

In the present study, the effect of anti-visceral yolk sac antiserum on the nutritional properties of the yolk sac was investigated. Anti-visceral yolk sac antiserum, added to the culture medium with ^{125}I-PVP for the last 6 hours of culture, decreased the uptake of radioactivity by conceptuses. The decrease was dose-dependent at antiserum concentrations

of 167 ug/ml or less, but approached a maximum level as the antiserum concentration was increased. The "levelling off" of inhibition of pinocytosis at higher concentrations of antiserum would seem to indicate that pinocytosis cannot be totally abolished by antiserum. When the antiserum was loaded into conceptuses before exposure to ^{125}I-PVP, almost identical inhibition of uptake of radiolabel was observed. The effect of antiserum on the protein contents of yolk sacs and embryos at harvesting was qualitatively similar to that described for uptake of ^{125}I-PVP. A noticeable reduction in conceptus protein levels at low concentrations of antiserum was observed. As the antiserum concentration was raised, the protein levels were reduced further but the decrease was less marked.

In contrast to these observations, the antiserum had no effect on the uptake of ^{125}I-dBSA by conceptuses, regardless of whether it was presented to conceptuses before, or together with, the radiolabelled substrate. However, although the total amount of radioactivity processed by conceptuses was unaffected by antiserum, the proportion that at harvesting was associated with the yolk sac was reduced. This suggests that the antiserum in some inexplicable way enhances the rate of release of ^{125}I-labelled digestion products from the tissues, an explanation that is also consistent with the observed reduced proportion of the yolk sac-associated radioactivity that was TCA-soluble.

It is interesting to compare these data with those of Goetze et al. (1975) who found that 19-day rat yolk sac, incubated in vitro for 20 minutes with goat anti-rat placenta antibody, showed a decreased tissue-associated radioactivity following a further 20 minute incubation in antibody-free medium containing ^{125}I-labelled human serum albumin. These workers ascribed this observation to an

inhibition of pinocytosis of the radiolabelled protein. However, since in this study the release of TCA-soluble radioactivity from the yolk sac into the medium was not monitored, a rapid release of digestion products from the yolk sac as postulated here would have gone unnoticed, and hence an erroneous conclusion may have been drawn. Furthermore, these workers used an exceedingly high concentration of antibody (30 mg/ml) which is approximately the equivalent of 100 mg antiserum protein/ml of culture medium, compared with the highest concentration used in the present study of 333 μg/ml. It is quite likely that the use of such a large concentration of antibody would result in a general cytotoxic effect on the yolk sac that would inevitably reduce pinoctyosis, rather than a specific effect of the antiserum on pinocytosis.

When the effect of antiserum on the utilisation of ^{3}H-labelled serum proteins by conceptuses was examined, three notable facts emerged. The first of these was that 83 μg/ml of antiserum in the culture medium, a concentration that reduced the uptake of ^{125}I-PVP to 69% of controls, had no effect on the uptake or distribution of radiolabel within the tissues of the conceptus. At a higher concentration of antiserum, 333 μg/ml, the uptake of radiolabel was reduced to 62% of control levels, which is very similar to the degree of inhibition of uptake by conceptuses of ^{125}I-PVP (56% of controls). The reason why the lower concentration of antiserum should not affect the uptake of ^{3}H-labelled serum proteins but markedly reduce the uptake of ^{125}I-PVP, while the higher concentration of antiserum affects the uptake of these substrates to more or less the same extent, is not clear, particularly when one considers that the total protein of conceptuses at harvesting is reduced by antiserum (83 μg/ml). The second point of note is that

when uptake of radioactivity by conceptuses was decreased, the distribution of the smaller quantity of radiolabel between the yolk sac and embryo was unaltered, indicating that degradation of radiolabelled proteins was not inhibited by antiserum and that inhibition of yolk sac pinocytosis consequentially reduces the uptake of radiolabel into the embryo by the same amount, providing further evidence of the mediating role of the yolk sac in histiotrophic nutrition. Thirdly, the effect of antiserum on pinocytosis of ^{3}H-labelled serum proteins by conceptuses was qualitatively and quantitatively the same regardless of when the antiserum was present in the culture medium. Indeed this has been an impressive feature throughout these studies of antiserum and possibly provides a clue to the mode of action of antiserum. By the use of immunofluorescent techniques, it has clearly been demonstrated (Brent et al., 1971) that anti-yolk sac antibody localises within the yolk sac and persists there, for periods much longer than do non-specific heterologous gamma globulins (Brent, 1971). This suggests that the anti-visceral yolk sac antibody is able to resist degradation by proteolytic enzymes. Since the antiserum caused the same degree of inhibition of pinocytosis when it was added to the culture medium at the same time as substrate and when it was added before the substrate, the teratogenic gamma globulin of the antiserum must be rapidly equilibrated with the specific process or processes that are being inhibited, and as a consequence decrease pinocytosis. Furthermore, inhibition must be maintained at the same level over at least a 12 hour period.

The failure of the antiserum at a low concentration to inhibit the uptake by conceptuses of ^{3}H-labelled serum proteins, and at both a high and a low concentration the uptake of ^{125}I-dBSA is difficult to

explain. However, since the total tissue protein was reduced at harvesting by antiserum treatment during culture, although the uptake of these substrates per unit protein of yolk sac is not affected, the absolute uptake by the whole yolk sac will be less.

In none of the experiments described could it be demonstrated that yolk sac intralysosomal digestion of protein was being inhibited. This conclusion is reinforced by the observation that the protein contents of both yolk sacs and embryos of conceptuses exposed to antiserum in culture were decreased in a similar way. An inhibition of yolk sac proteolysis would have led to an accumulation, rather that a reduction, of protein in the yolk sac.

There are a number of ways in which the antiserum could be acting to inhibit pinocytosis. The pinocytic process requires both energy and cytoskeletal involvement (Silverstein et al., 1977) and is known to be sensitive to inhibitors of energy metabolism and cytoskeleton disaggregators. Conceivably, antibodies directed against glycolytic or respiratory enzymes or cytoskeletal proteins could affect pino-cytosis. One problem here however is that it is likely that gamma globulin (antibody) is pinocytosed by yolk sac and is subsequently incorporated into heterolysosomes. It is therefore difficult to envisage how the antibody would gain access to cytosolic or mito-chondrial enzymes, although Franke et al. (1975) have demonstrated by electron microscopy that goat anti-rat placenta antibody both labilises lysosomal membrane and causes a reduction in the number of microvilli on the apical surface of yolk sac endodermal cells. A cytotoxic effect of antiserum and complement has also been described, in mouse yolk sac (Billington et al., 1976).

Alternatively, anti-visceral yolk sac antibody could be acting, to inhibit pinocytosis, at the plasma membrane. The demonstration (Schneider et al., 1978b) that membrane recycling between lysosomes and plasma membrane can carry with it anti-plasma membrane antibody explains how an intralysosomal material can continue to affect an event at the plasma membrane. It should be added however that the presence in culture of anti-plasma membrane antibody was observed not to affect the uptake of other immunoglobulins into fibroblasts (Schneider et al, 1978a).

In conclusion, it can be said that the mechanism of teratogenic action of anti-yolk sac antiserum involves an interference of the yolk sac-mediated histiotrophic nutrition of the embryo, through the inhibition of the pinocytic ingestion of macromolecular nutrients by the yolk sac. This may constitute the primary defect in antiserum-induced teratogenesis, or may be a single manifestation of a more general toxic effect of antiserum on the yolk sac.

CHAPTER NINE

THE EFFECT OF LEUPEPTIN ON YOLK SAC-MEDIATED NUTRITION

9.1   Introduction

The peptide leupeptin is one of a number of similar substances that
have been isolated from culture filtrates of Streptomycese (see review by
Umezawa and Aoyagi, 1977). This group of peptides share the char-
acteristic of being inhibitors of certain groups of proteolytic enzymes,
though the specificity of each inhibitor is not precisely the same.
Leupeptin is a tripeptide of the structure acetyl- (or propionyl-)
L-leucyl-L-leucyl-L-arginal (Figure 9.1), though substitution of
isoleucine or valine for leucine has been observed (Kondo et al., 1969;
Kawamura et al., 1969). Among the enzymes that leupeptin is known to
inhibit are the lysosomal cysteine proteinases cathepsin B (Huisman
et al., 1974) and cathepsin L (Kirschke et al., 1977). Leupeptin does
not however inhibit the aspartic proteinase cathepsin D (Kirschke
et al., 1977).

On the basis of this enzyme-inhibitory action, leupeptin has been demon-
strated to inhibit the degradation of intracellular protein in Reuber H35
hepatoma cells (Knowles and Ballard, 1976), isolated rat hepatocytes
(Hopgood et al., 1977; Seglen, 1978; Solheim and Seglen, 1980;
Ose et al., 1980; Berg et al., 1981; Tolleshaug and Berg, 1981),
perfused rat liver (Dunn et al., 1979; Ward et al., 1979), perfused
rat heart (Ward et al., 1979), cultured fetal mouse heart (Libby
et al., 1979) and cultured 17.5-day rat yolk sac (Knowles et al., 1981).

In the present experiments, the effects of leupeptin upon pinocytosis
and proteolysis by the yolk sac of the early organogenesis-stage
conceptus in vitro has been studied. An effect of leupeptin on the
yolk sac-mediated pathway of protein utilisation by embryos, due to
an inhibition of proteolysis in yolk sac lysosomes, would be expected
to cause a reduction in the flow of nutrient amino acids to the embryo,

```
CH₃-CO-NH-CH-CO-NH-CH-CO-NH-CH-CHO
            |          |          |
            CH         CH        (CH₂)₃
            |          |          |
            CH-CH₃     CH-CH₃     NH
            |          |          |
            CH₃        CH₃        C=NH
                                  |
                                  NH₂
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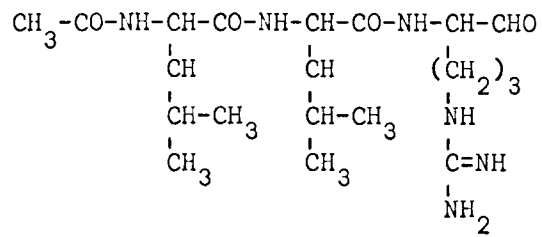
Figure 9.1 Structural formula of
leupeptin (acetyl-L-leucyl-L-leucyl-
L-arginal)

and as a consequence this might result in teratogenesis. A toxic effect of leupeptin on cultured 9.5-11.5-day conceptuses has been demonstrated (F. Beck, personal communication), but whether leupeptin is teratogenic to rat embryos following maternal injection is not known. In this chapter, a limited study of the teratogenicity in rats of maternally-administered leupeptin is described.

9.2   Materials

Chemicals

Leupeptin   The Peptide Center, Protein Research
                                             Foundation, Osaka, Japan.

All other chemicals used have been listed in previous chapters.

9.3   Methods

9.3.1 Teratogenic Testing of Leupeptin

The procedure was as described for trypan blue (5.3.3). Leupeptin was administered at a dosage of 50 mg/kg.

9.3.2 Culture Experiments

The procedure was as described for trypan blue (5.3.4). In cultures that used ^{125}I-PVP as substrate, leupeptin was added to the culture medium to a final concentration of 25, 50, 100, 200 and 500 μg/ml. For subsequent experiments with ^{125}I-dBSA and ^{3}H-labelled serum proteins as substrates, leupeptin was added to a final concentration of 25 or 200 μg/ml.

9.4   Results

9.4.1 Teratogenic Testing of Leupeptin

Injection of leupeptin (50 mg/kg) into pregnant rats at either 8.5 or 9.5 days of gestation resulted in the production of malformed fetuses

(Table 9.1). Following injection at 8.5 days, a resorption rate at term of 24% was observed. Of the surviving fetuses, more than 50% were visibly externally malformed, and the proportion of fetuses that were subsequently sectioned that showed malformations was 73%. The most common defects observed in this group (Table 9.2) were anophthalmia, microphthalmia, hydrocephalus and kidney malformation. Maternal injection at 9.5 days gave rise to a high incidence of resorption (48%) but a low malformation rate (Table 9.1). Only 3 of the 25 surviving fetuses were externally malformed and inspection of these surviving fetuses for visceral and skeletal anomalies showed no further malformations. The average weight of fetuses of both 8.5- and 9.5-day injected groups was significantly (p < 0.001) lower than control fetuses from pregnant rats injected with saline at the corresponding stages of gestation.

9.4.2 Culture Experiments

The viability of conceptuses exposed in culture to leupeptin for a period of 6 hours, either from 36 to 42 hours or from 42 to 48 hours, did not differ from that in cultures in which leupeptin was absent from the culture medium, and was at least 95%. Furthermore, no developmental deviations were noted among conceptuses exposed to leupeptin during culture.

At concentrations of leupeptin of 500 μg/ml or less, present in the culture medium for the final 6 hours, the protein contents at harvesting of yolk sacs were approximately twice the control value while the protein contents of embryos were progressively reduced by an increasing concentration of leupeptin, to about 70% of controls (Figure 9.2). When leupeptin (25 or 200 μg/ml) was present in culture between 36 and

Nature and time of injection	No. of ♀ treated	No. of implantations (% resorptns)	Av. weight surviving fetuses (g)	External Abnormalities			No. of fetuses sectioned	No.(%) sectioned fetuses with anomalies	No.of fetuses stained with alizarin	No.(%) stained fetuses with anomalies
				No.	%surviving fetuses	% total implntns				
Leupeptin 8.5 days	4	51 (24)	3.45 ±1.1*	21	53.85	41.18	26	19 (73)	13	0
Leupeptin 9.5 days	4	48 (48)	3.32 ±.42*	3	8.00	4.17	17	2 (12)	8	0
Saline 8.5 days	4	45 (4)	4.19 ±.42	0	0	0	28	0	15	0
Saline 9.5 days	4	44 (5)	4.40 ±.60	0	0	0	30	0	12	0

* significantly different from saline-injected controls (p< 0.001)

Table 9.1 Effects on offspring of s.c. injection of leupeptin (50 mg/kg) or saline (0.9%) into pregnant rats. Details of method are given in text.

Day of injection of leupeptin	8.5	9.5
Abnormality		
Anophthalmia	22 (39)	2 (25)
Hydrocephalus	9 (21)	0
Kidney (deformed)	9 (21)	0
Facial defects	1 (39)	0
Limb defects	0	1 (25)
Tail defects	0	1 (25)

Table 9.2   Abnormalities of fetuses after leupeptin treatment of pregnant rats.Figures in brackets indicate the size of the population examined for the particular defect.
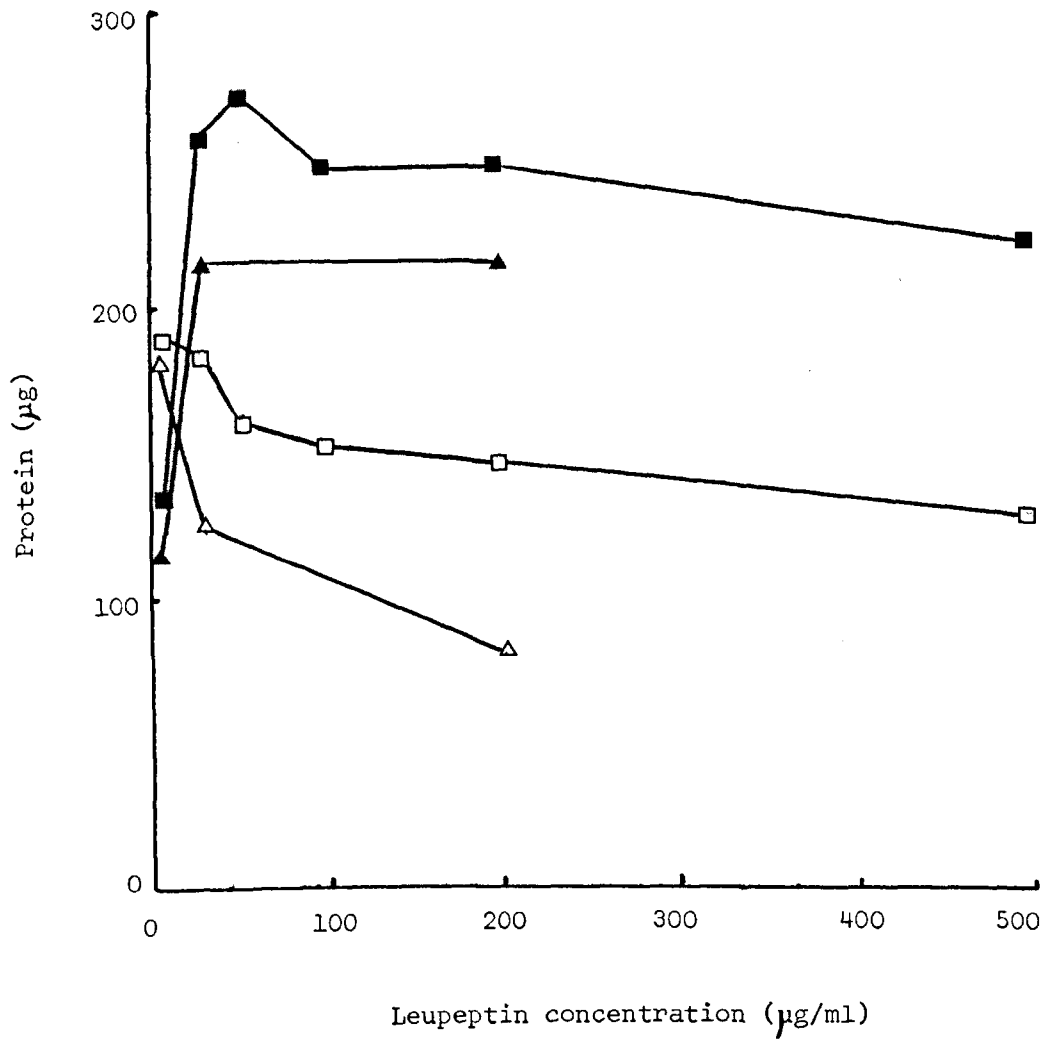
Figure 9.2 Protein contents of yolk sac (filled symbols) and embryo (open symbols) after incubation of conceptuses in the presence of leupeptin for either the final 6 hours (squares) or the penultimate 6 hours (triangles) of culture. Values are means of at least 6 determinations.

42 hours, the yolk sac protein content was again approximately twice the control value, but the embryo protein content fell to about 46% of the control value, at the highest concentration of leupeptin used (Figure 9.2).

When leupeptin was present with ^{125}I-PVP (2.3 µg/ml), for the final 6 hours of culture, there was no uptake of radioactivity into embryos by harvesting (Figure 9.3). In the yolk sac, the uptake of radioactivity was apparently decreased to between 56% and 72% of the control value. This decrease was independent of leupeptin concentration.

Exposure of conceptuses for the last 6 hours of culture to leupeptin (25 or 200 µg/ml) and ^{125}I-dBSA (11 µg/ml) resulted by harvesting in little radioactivity being associated with embryos (Figure 9.4). All of this radioactivity was TCA-soluble. At both leupeptin concentrations used, the total amount of radioactivity processed by conceptuses was decreased to a level approximately one half of the control. By far the greater proportion of this radioactivity was retained within the yolk sac; 77% following a leupeptin concentration of 25 µg/ml and 94% at a leupeptin concentration of 200 µg/ml (Figure 9.4). At either leupeptin concentration, yolk sac-associated radioactivity at harvesting was more than 95% TCA-insoluble. In controls only 12% of the total radioactivity processed by conceptuses was yolk sac-associated at harvesting, of which 65% was TCA-insoluble.

When leupeptin was present in culture between 36 and 42 hours, and ^{125}I-dBSA was present from 42 hours to harvesting, embryos at harvesting contained little radioactivity, all TCA-soluble (Figure 9.5). Total radioactivity processed by conceptuses exposed to leupeptin was slightly less than one half the control value. As before, a very high proportion
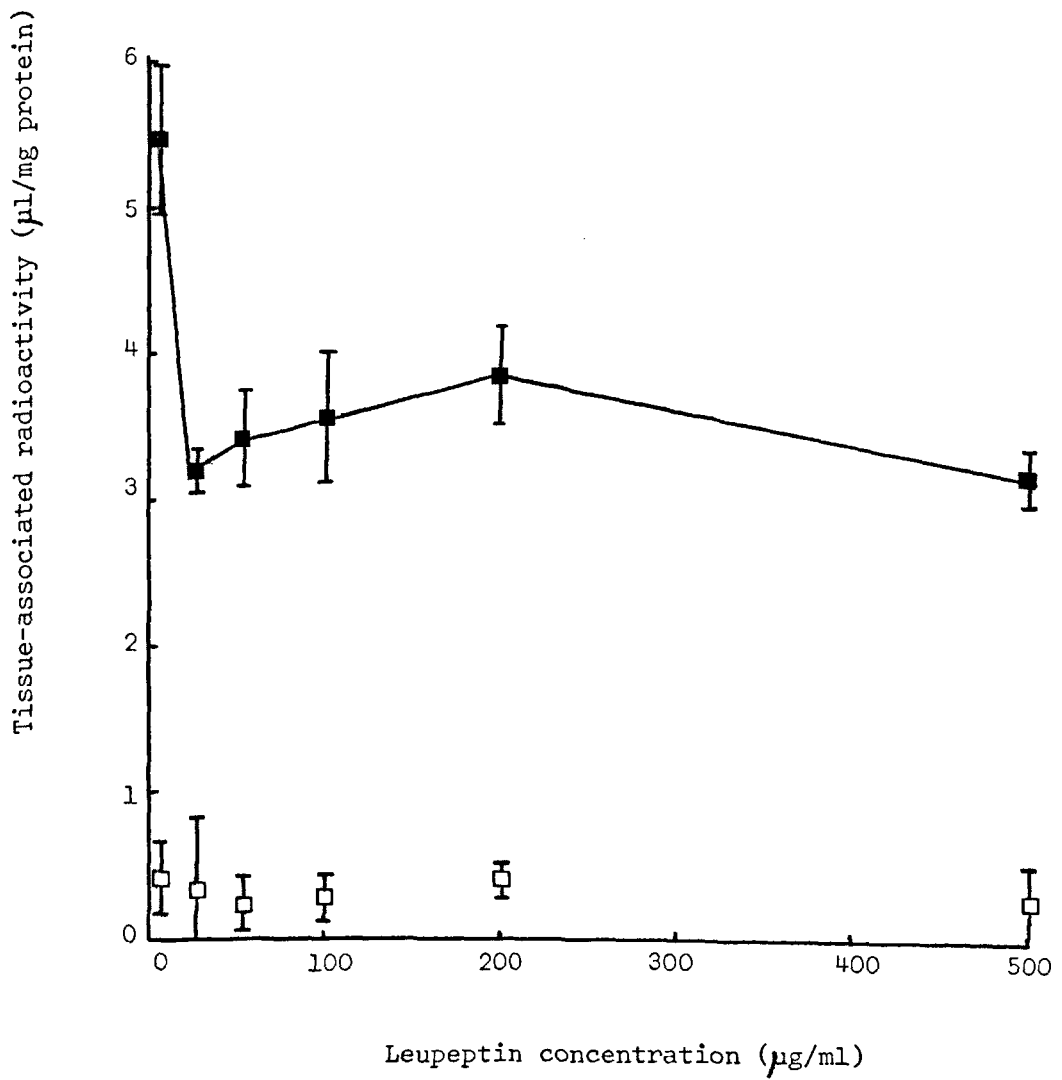
Figure 9.3 Radioactivity associated with yolk sac (■) and
embryo (□) after incubation of conceptuses in the presence of
^{125}I-PVP (2.3 µg/ml) and leupeptin for the final 6 hours of
culture. Values are means (± S.D.) of at least 6 determinations.

Figure 9.4 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 µg/ml) and leupeptin for the final 6 hours of culture. The proportions of yolk sac-associated and embryo-associated radioactivity that were TCA-soluble [shaded] and TCA-insoluble [hatched] are indicated. Values are means (+ S.D.) of at least 6 determinations.

60

50

Uptake of radioactivity (μl/mg protein)

40

30

20

10

0

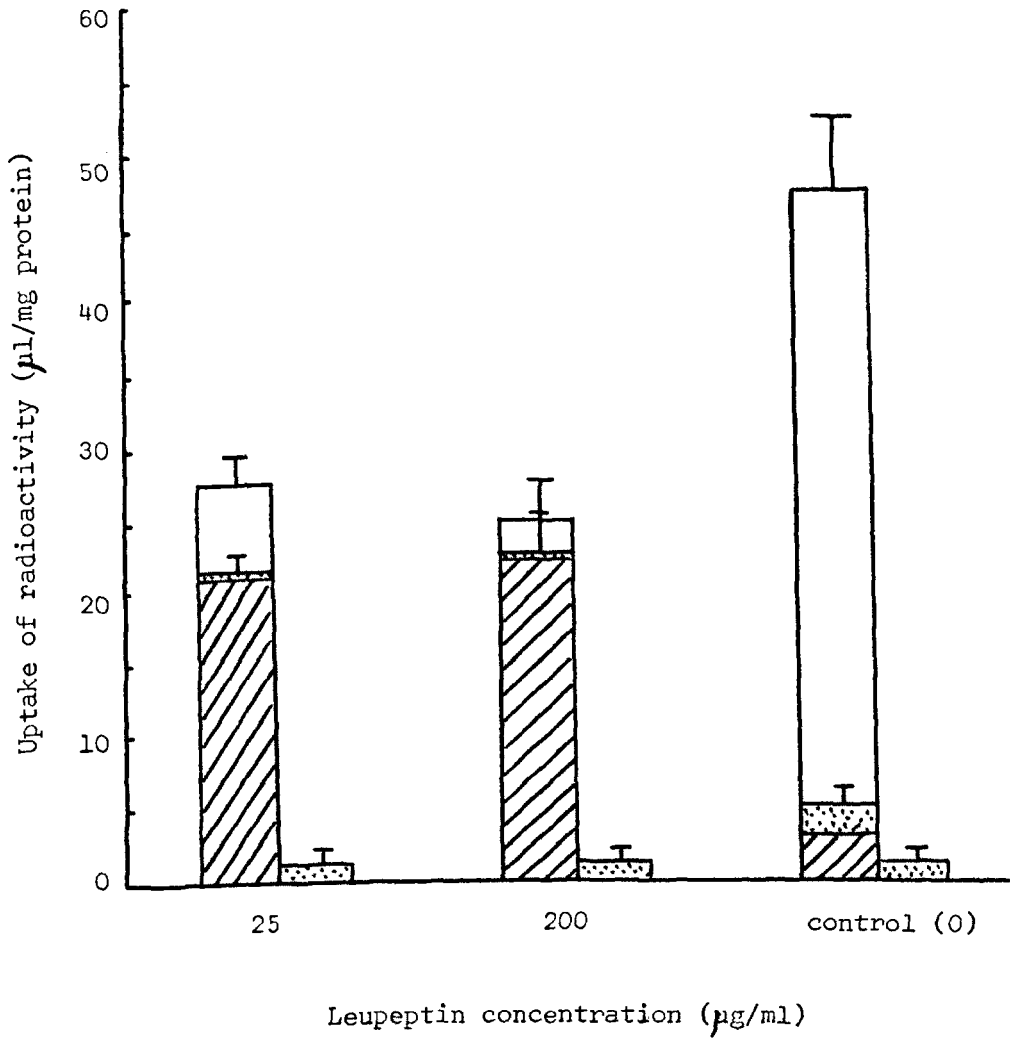25   200   control (0)

Leupeptin concentration (μg/ml)

Figure 9.5 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of ^{125}I-dBSA (11 μg/ml) for the final 6 hours, and leupeptin for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble ▨ and TCA-insoluble ▨ are indicated. Values are means (± S.D.) of at least 6 determinations.

of this radioactivity remained associated with the yolk sac at harvesting;
80% following a leupeptin concentration of 25 µg/ml and 91% at a
leupeptin concentration of 200 µg/ml. More than 90% of the yolk sac-
associated radioactivity was TCA-insoluble. In controls, only 16% of
the total radioactivity processed by conceptuses was yolk sac-associated
at harvesting and of this 47% was TCA-insoluble.

The uptake of radioactivity by conceptuses, cultured for the final 6 hours
in serum containing ^{3}H-labelled proteins and leupeptin did not differ
much from the control value (Figure 9.6). However, the distribution of
radioactivity between yolk sac and embryo was affected by leupeptin,
a greater proportion being associated with the yolk sac. At a
leupeptin concentration of 25 µg/ml, 82% of the total radioactivity
in the conceptus was yolk sac-associated while at a leupeptin concentration
of 200 µg/ml, 90% of the radioactivity was yolk sac-associated. In both
cases the proportion of the yolk sac-associated radioactivity that was
TCA-insoluble was more than 90%. In controls, only 39% of the total
radioactivity in the conceptus was yolk sac-associated (70% TCA-insoluble).
Interestingly, the proportion of the decreased levels of radioactivity
in embryos exposed to leupeptin was more than 90% TCA-insoluble
(controls less than 80%).

When conceptuses were exposed to leupeptin (25 or 200 µg/ml), present
in culture from 36 to 42 hours, before being transferred to serum
containing ^{3}H-labelled serum proteins, little effect on the uptake
of radioactivity by conceptuses at harvesting was observed (Figure 9.7).
At 25 µg/ml, leupeptin increased the proportion of radioactivity that
was yolk sac-associated to 89%, compared with a control value of 47%.
At 200 µg/ml of leupeptin, the yolk sac-associated radioactivity constituted
97% of the total radioactivity of the conceptus. At both concentrations
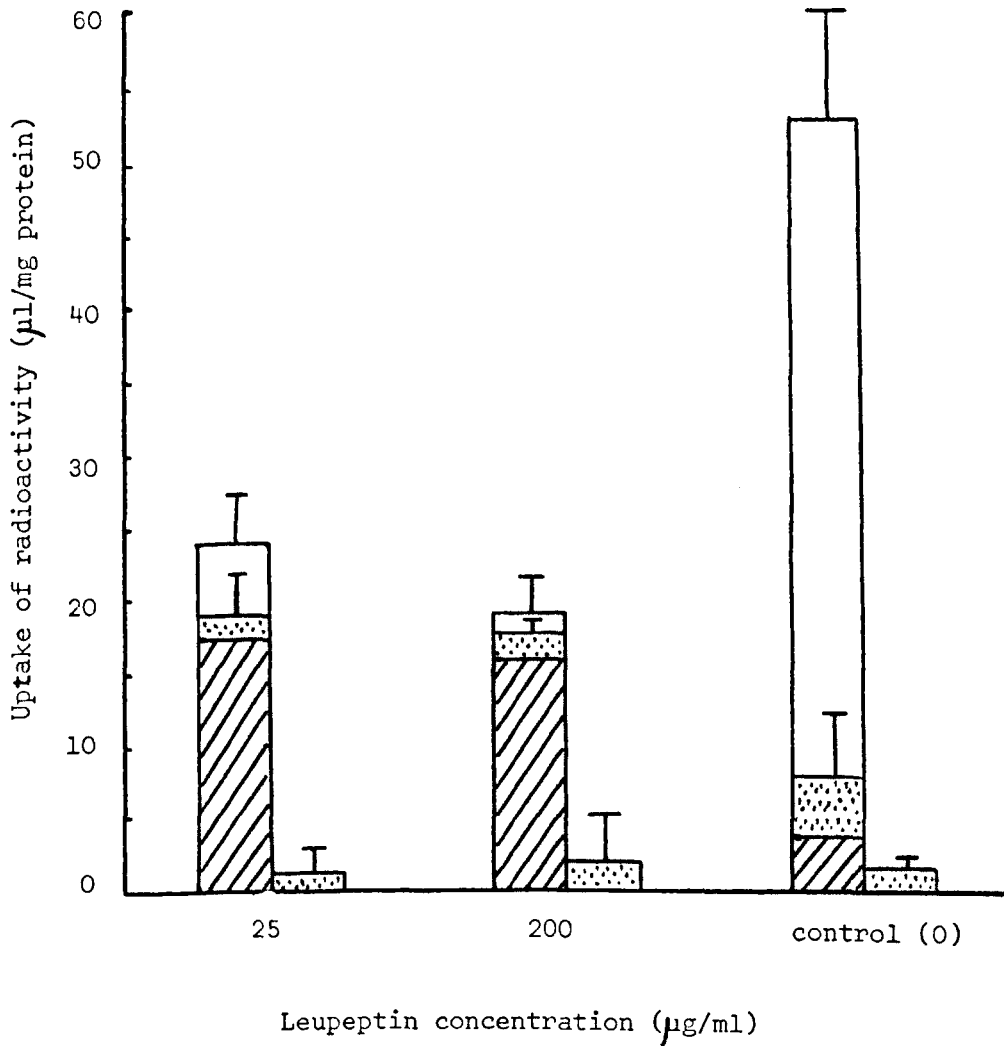
Figure 9.6 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of [^{3}H]leucine-labelled serum proteins and leupeptin for the final 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble [image] and TCA-insoluble [image] are indicated. Values are means (+ S.D.) of at least 6 determinations.
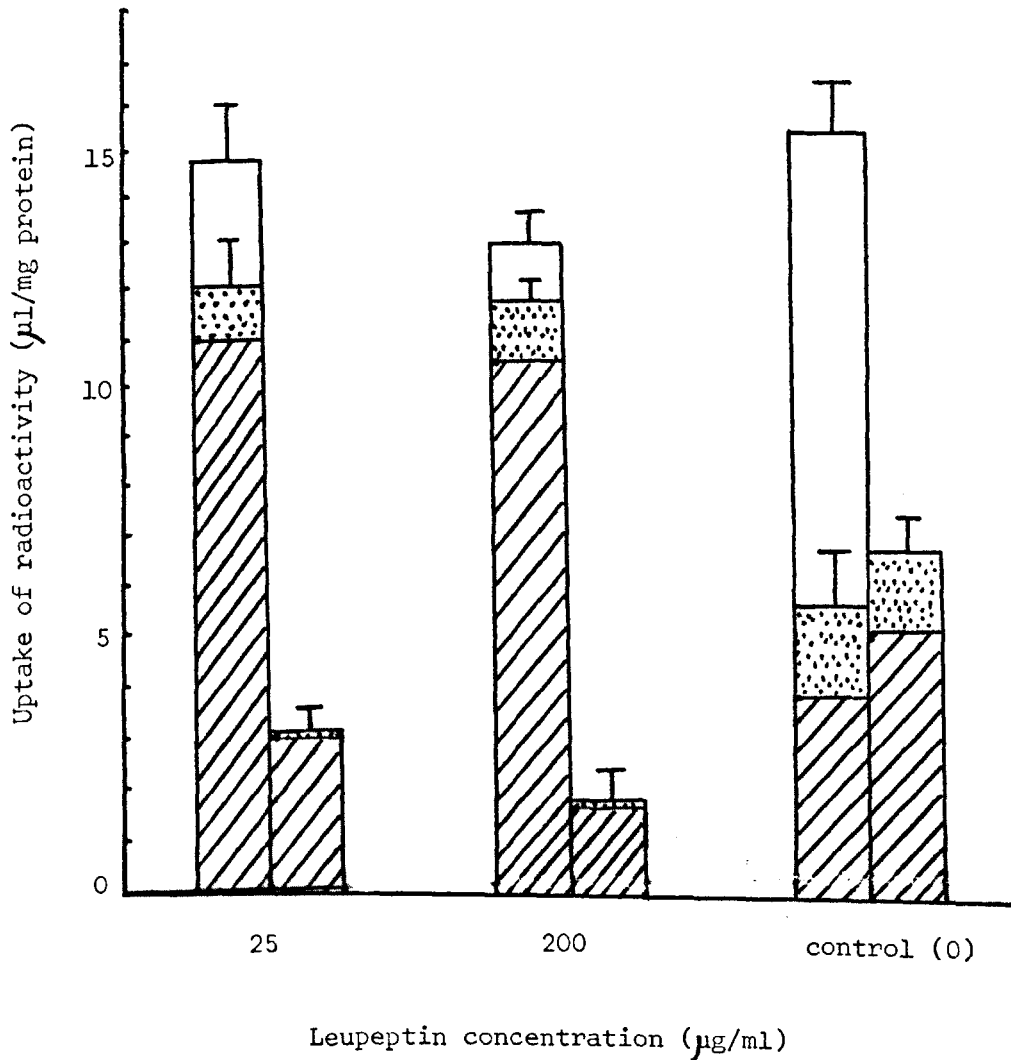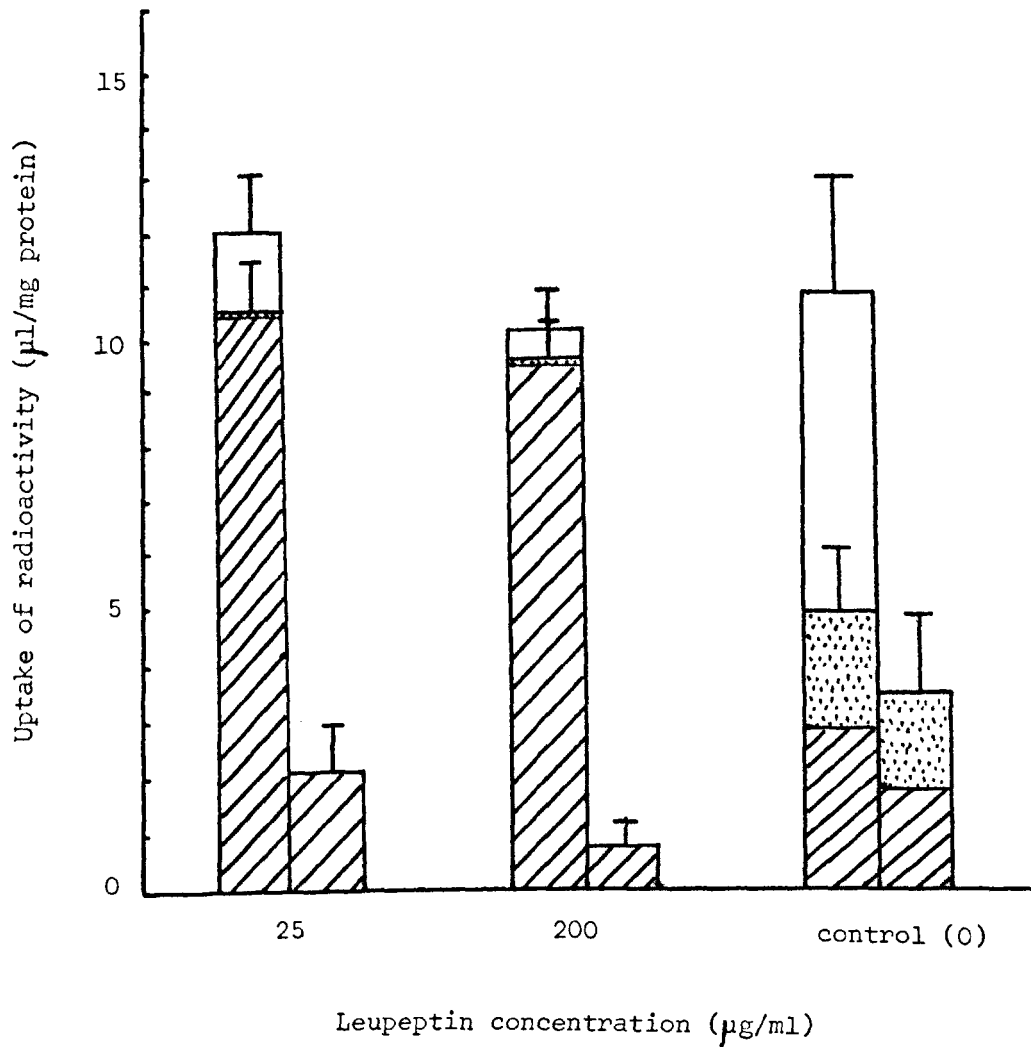
Figure 9.7 Total radioactivity taken up by yolk sac (each left hand column) and embryo (each right hand column), and the radioactivity that was associated with the yolk sac (total shaded area of each left hand column) after incubation of conceptuses in the presence of $[^3H]$ leucine-labelled serum proteins for the final 6 hours, and leupeptin for the penultimate 6 hours of culture. The proportions of yolk sac- and embryo-associated radioactivity that were TCA-soluble [▦] and TCA-insoluble [▨] are indicated. Values are means (± S.D.) of at least 6 determinations.

of leupeptin employed, more than 98% of the yolk sac-associated radioactivity was TCA-insoluble (control yolk sac-associated radioactivity 57% TCA-insoluble) and all the embryo-associated radioactivity was TCA-insoluble (control embryo-associated radioactivity 65% TCA-insoluble).

9.5   Discussion

The teratogenicity of leupeptin in rats has not previously been fully reported. Umezawa and Aoyagi (1977), quoting unpublished data of Matsuzaki, claimed that following oral administration of 200 mg/kg of leupeptin to pregnant rats daily from the seventh to the fourteenth days of pregnancy abnormal growth of bones occurred in 4 out of 200 fetuses, and that subcutaneous injection of 50 mg/kg of leupeptin into pregnant rats on the seventh and eighth days, or ninth and tenth days of pregnancy, inhibited the growth of bone of fetuses and induced abnormalities of the skull and sternum. In the present experiments, the results of a limited study of the effects on embryos of leupeptin, injected into pregnant rats at 8.5 or 9.5 days of gestation, clearly demonstrate that leupeptin is both teratogenic and embryolethal. Furthermore, the data indicate that, in common with trypan blue, leupeptin is rather more teratogenic when administered to mothers at 8.5 days of gestation than at 9.5 days. As with other teratogens that act during organogenesis, the most common defects elicited by leupeptin were anophthalmia, microphthalmia and hydrocephalus. In contrast to the report of Umezawa and Aoyagi (1977), no defects of the skeleton were observed.

The presence of leupeptin in culture for 6 hours caused a marked alteration in the protein contents of yolk sacs and embryos at harvesting. Compared with controls, leupeptin caused a doubling of

the protein contents of yolk sacs and a decrease in the protein contents of embryos. Since leupeptin is known not to affect protein synthesis (Seglen, 1978; Libby et al., 1979), the only interpretation of these data is that leupeptin induces storage of pinocytosed protein in the lysosomes of the yolk sac through an inhibition of proteolysis. This in turn would reduce the supply of amino acids available for the synthesis of embryonic proteins and cause a decrease in the total protein content of embryos. Such an interpretation is confirmed by the observations that when conceptuses were cultured for the final 6 hours in a serum whose proteins were $\left[^3H\right]$ leucine-labelled, the presence of leupeptin in culture, either from 36 to 42 hours, or from 42 hours to harvesting, caused a large proportion, always greater than 80%, of the total radioactivity in the conceptus to be retained within the yolk sac.

The uptake of radioactivity by yolk sacs when conceptuses were exposed to ^{125}I-PVP and leupeptin for the final 6 hours of culture, was decreased to levels of between 70% and 55% of the control, though there was no relationship between the decrease in uptake of radio-activity and the concentration of leupeptin in the culture medium. It is noteworthy that the increase in protein contents of yolk sacs at harvesting, following exposure to leupeptin for the final 6 hours of culture, was also independent of the leupeptin concentration. In the calculation of uptake of radioactivity (see Chapter 2), uptake is expressed per mg protein content of tissue. Since an increase in the protein content of the yolk sac, not through growth, but through lyso-somal storage of protein would not cause a corresponding increase in the quantity of ^{125}I-PVP associated with that tissue, the apparent decrease in ^{125}I-PVP uptake by yolk sacs in the presence of leupeptin

is entirely due to the elevated protein level of the yolk sac caused by the leupeptin, and not to an inhibition of pinosome formation. In other words, the quantity of radioactivity per yolk sac was not affected. Of course, with ^{3}H-labelled serum proteins as substrates, no decrease in uptake of radioactivity by yolk sac would be observed since it is these proteins that are being accumulated in the lysosomes of the yolk sac. Consequently, the amount of radioactivity in the yolk sac will increase in proportion to the quantity of protein accumulated. The uptake of ^{125}I-dBSA, present in culture for the final 6 hours, when conceptuses were incubated in the presence of leupeptin for 6 hours, either from 36 to 42 hours, or 42 to 48 hours of culture, was decreased to roughly the same extent as ^{125}I-PVP uptake. Similarly, this decreased uptake can be wholly attributed to the rise in protein content of the yolk sac as a result of exposure to leupeptin.

Taken together, these data demonstrate that at the concentrations used, leupeptin does not affect the rate of pinocytosis in the early organogenesis-stage yolk sac. This is consistent with previously reported observations (Berg et al., 1981; Knowles et al., 1981) which showed that leupeptin at either 50 µg/ml or 150 µg/ml, did not inhibit the uptake of ^{125}I-labelled asialoglycoproteins by isolated rat hepatocytes or ^{125}I-dBSA by the cultured 17.5-day rat yolk sac.

The degradation of pinocytosed ^{125}I-dBSA and ^{3}H-labelled serum proteins by yolk sac was strongly inhibited by leupeptin. This is evidence by the observations that leupeptin added to the culture medium either before or together with the radiolabelled proteins caused a very large proportion of the pinocytosed radiolabel to remain associated with the yolk sac. Generally, this proportion was slightly more when conceptuses were exposed to leupeptin before radiolabel, in contrast

to the findings of Knowles et al. (1981) who found that yolk sacs, following their exposure to leupeptin in vitro, quickly recovered their ability to degrade ^{125}I-dBSA on re-incubation in leupeptin-free medium. Furthermore, always more than 90% of the yolk sac-associated radioactivity was insoluble in TCA. This large inhibition of the degradation of pinocytosed protein, as a result of treatment with leupeptin, is in agreement with previous reports (Dunn et al., 1979; Ose et al., 1980; Berg et al., 1981; Knowles et al., 1981; Tolleshaug and Berg, 1981). In view of the findings (Huisman et al., 1974; Kirschke et al., 1977) that leupeptin is a powerful inhibitor of the lysosomal cysteine proteinases cathepsins B and L, but not of the lysosomal aspartic proteinase cathepsin D, the most likely explanation of these results is that the inhibition of the degradation of ^{125}I-dBSA and ^{3}H-labelled serum proteins by 9.5-11.5-day yolk sac is effected by direct action of leupeptin on degradative enzymes. There are two corollaries of such an explanation. Firstly, leupeptin gains access to lysosomes, either by pinocytosis or by permeation of the plasma and lysosomal membranes. Secondly, the digestion of ^{125}I-dBSA and ^{3}H-labelled serum proteins in visceral yolk sac is due largely to the action of cysteine proteinases. This latter proposal receives strong support from the observation (Huisman et al., 1974) that an inhibition by leupeptin of more than 95% of cathepsin B activity resulted in a strongly decreased rate of degradation of serum albumin by rat liver lysosomal extracts. Knowles et al. (1981) observed that leupeptin caused more than 75% of ^{125}I-dBSA, taken up by cultured 17.5-day yolk sac, to remain associated with the tissue after a 3-hour incubation, compared with a control value of only 22%. These authors concluded that cysteine proteinase(s) must be rate-limiting for the degradation of pinocytosed albumin. In neither of these studies did pepstatin,

a microbial peptide that inhibits cathepsin D (Umezawa and Aoyagi, 1977), influence the rate of degradation of albumin, indicating that in these tissues cathepsin D is of little importance in albumin degradation.

In addition to inhibiting the breakdown of ^{125}I-labelled asialofetuin by isolated rat hepatocytes, leupeptin was also considered (Berg et al., 1981; Tolleshaug and Berg, 1981) to inhibit the fusion of pinosomes and lysosomes in these cells. Subcellular fractionation studies showed that undegraded ^{125}I-labelled asialofetuin accumulated both in heterolysosomes and in an organelle of lower density (presumed by these authors to be a pinosome). A similar mechanism of action of leupeptin could be operating in the early organgenesis-stage yolk sac. However, the overall effect of leupeptin, an increase in the yolk sac-associated radiolabelled protein and a decrease in the embryo-associated radioactivity, would remain the same.

The efficacy of leupeptin in inhibiting the yolk sac-mediated pathway of protein utilisation by the early organogenesis-stage conceptus may be important in the teratogenic mechanism of leupeptin. However, other factors must be considered before any firm conclusions can be drawn. For example, some of the proteins present in the plasma transudate that bathes the conceptus in utero may not, unlike those chosen in the present in vitro studies, be degraded by cysteine proteinases to any important degree and hence would not be affected by leupeptin. Furthermore, by inhibiting cysteine proteinases, leupeptin may secondarily be acting upon other cellular enzymes and proteins whose normal turnover is dependent on the action of lysosomal proteinases, and whose accumulation in cells may be deleterious. It has recently been found (Kominami et al., 1981a, b) that leupeptin, administered to rats, labilised membranes of liver lysosomes as

evidenced by the increased activities of a range of lysosomal enzymes in the cytosol of liver cells, and the greater sensitivity of isolated lysosomes to osmotic shock. A similar action of leupeptin on the lysosomes of the visceral yolk sac endodermal cells might produce a more general toxic effect on these cells, which in turn might adversely affect the embryo. Alternatively, leupeptin may be able to penetrate directly to the embryo and produce a toxic effect there, the action of the peptide on yolk sac lysosomal proteolysis being of only secondary importance. Finally, an effect of leupeptin on the mother, which secondarily affects the embryo, cannot be ruled out, although Beck (personal communication) has noted an embryotoxic effect of leupeptin in vitro, indicating that it is able to act on the developing conceptus independently of the maternal organism.

CHAPTER TEN

GENERAL DISCUSSION

10.1 Histiotrophic Nutrition

Beck et al. (1967a) proposed that the visceral yolk sac fulfilled an important nutritional function during the early organogenesis period of rat embryogenesis. The evidence for this proposal is, as emphasised in the General Introduction to this Thesis, of a rather circumstantial and limited nature. In Chapters 2, 3 and 4 of the present study, experiments designed to test critically the hypothesis of Beck et al. have been described. The data from these experiments provide, in my mind, conclusive evidence that the yolk sac performs a critical nutritional function during early organogenesis by a mechanism entirely concordant with that proposed. Although these data have already been discussed in preceding Chapters, it is appropriate here to consider them in a broader context and to indicate specific areas of the nutritional scheme in which our knowledge is still incomplete.

Figure 10.1 represents diagrammatically the data of Chapters 2, 3 and 4. The visceral yolk sac endoderm pinocytoses macromolecules from the extracellular fluid and incorporates them into heterolysosomes in the apical regions of the cell. The observations that non-digestible macromolecules, ^{125}I-PVP and $[^{3}H]$ dextran, are accumulated in the yolk sac and do not gain access to the embryo, suggest that the yolk sac is the only tissue of the early organogenesis-stage conceptus which macromolecules can enter. This suggestion is strengthened by the finding that ^{125}I-dBSA, a more physiological substrate, does not penetrate intact to the embryo, but is taken into the yolk sac by pinocytosis. The further observation that ^{125}I-dBSA is degraded in the yolk sac lysosomes demonstrates this tissue's ability to hydrolyse proteins, an essential pre-requisite according to the model (Figure 10.1).
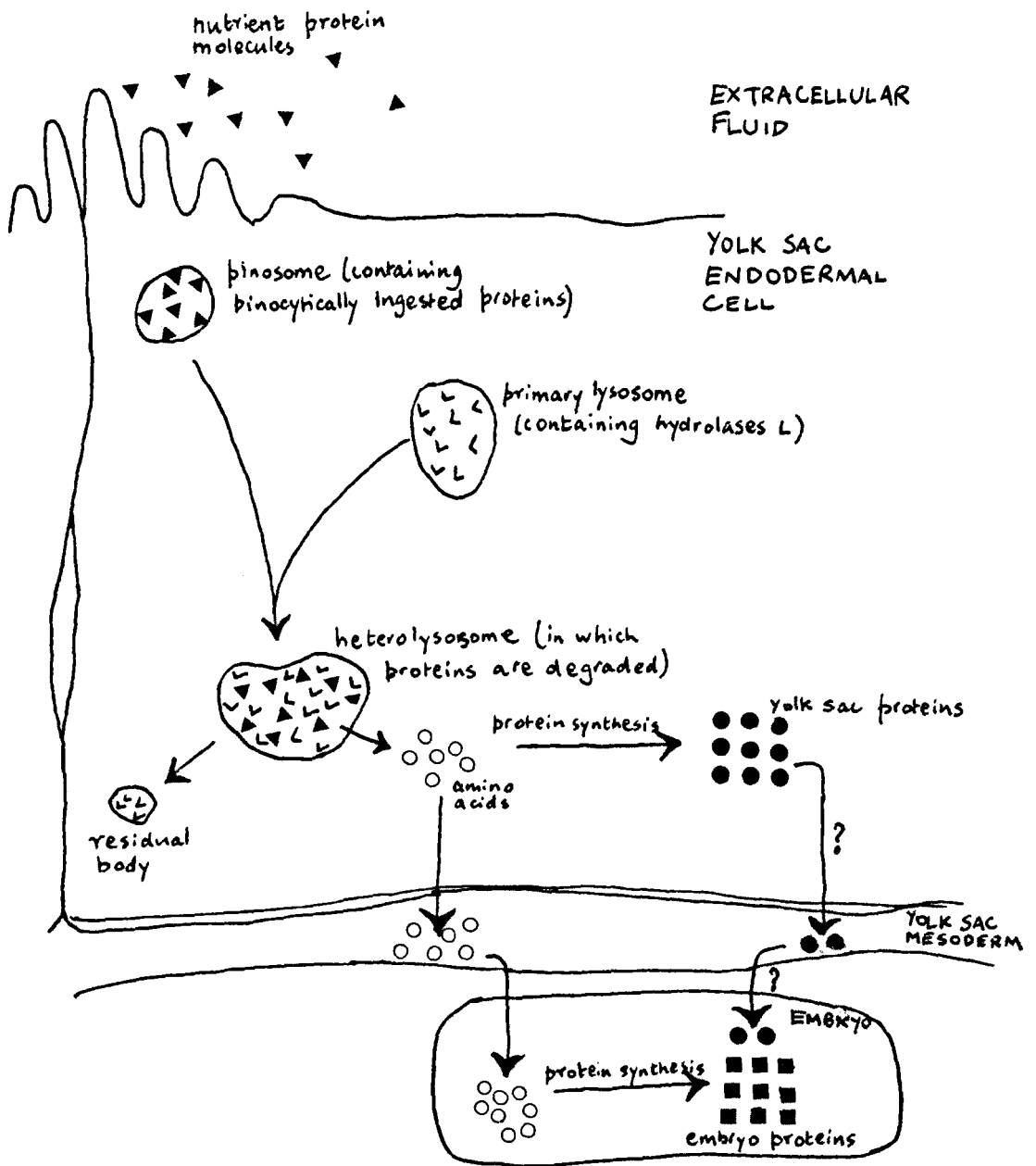
Figure 10.1 Proposed pathway of yolk sac-mediated protein
nutrition of early organogenesis-stage rat embryo.

However, it was suggested (Section 3.5) that yolk sac intralysosomal hydrolysis of ^{125}I-dBSA, a heterologous protein, might simply be a mechanism to de-toxify a potentially harmful foreign substance and that homologous proteins, abundantly present in the culture serum _in vitro_, and in the fluid surrounding the conceptus _in utero_ (vide infra), traversed the yolk sac in vesicles that did not fuse with lysosomes and hence were not degraded but were delivered intact to the embryo. There are reports in the literature which demonstrate that passage of intact homologous proteins across the rat yolk sac does occur. Mayersbach (1958) showed that fluorescent-labelled homologous serum proteins, injected into the maternal blood during the last third of gestation, could be traced through the visceral yolk sac endodermal cells and into the fetus. Brambell and Halliday (1956) and Anderson (1959) observed a transfer of gamma-globulins across the visceral yolk sac from the mother to the fetus. Masters et al. (1969) confirmed this finding and also observed that radiolabelled albumin and transferrin were transmitted to the fetus in small quantities during the last 3 days of gestation. It is interesting to note too the observations of Schultz (1966) who found that a small but significant amount of yeast invertase a foreign protein, was transferred from mother to fetus as early as day 12 of gestation. It was unclear however whether this protein was transmitted through the yolk sac or the chorioallantois. In other rodent species, the transfer of maternal proteins to the fetus by a route through the yolk sac has also been reported. During the second half of gestation, the guinea pig fetus received intact homologous (Anderson and Leissring, 1961; Leissring and Anderson, 1961) and heterologous (King and Enders, 1970) proteins that had been injected into the mother. Shortly after implantation of the mouse embryo, maternal immunoglobulins

were detected in the cells and fluids of the embryo (Bernard et al., 1977). This latter study is of particular significance since, in contrast to the others cited, it demonstrates that intact maternal protein crosses the yolk sac during early organogenesis, a time during which yolk sac-mediated histiotrophic nutrition is presumed to be of greatest importance. It should be added though that only a very small fraction of the considerable quantity of maternal protein taken into the yolk sac cells actually found its way into the embryo, and besides, the transfer of gamma globulins into the embryo or fetus at any stage of gestation is unlikely to be of nutritional importance, but rather would serve to endow the fetus with some immunity in preparation for the immediate post-natal period when the newborn's own immune system is yet poorly developed.

In the present study, radiolabel, derived from homologous serum proteins or haemoglobin present in the culture medium, was detected in large amounts and associated with many different proteins in the tissues of the embryo and the yolk sac. From the kinetics of uptake of radiolabel into the conceptus and the time-dependent ratio of TCA-soluble to -insoluble radioactivity in the tissues, it was argued (Section 4.5) that very little, if any, of the radioactivity in the embryo was due to uptake of intact radiolabelled proteins present in the culture medium, but that it was derived from digestion of pinocytosed radio-labelled proteins in the yolk sac lysosomes. Hence the hypothesis that the uptake and digestion of ^{125}I-dBSA by the yolk sac is an action specifically directed against foreign proteins cannot be sustained; it is clearly an example of a general response by yolk sac to most, probably all, proteins to which this tissue is exposed, and which forms the basis of the yolk sac's nutritional role during early organogenesis. Furthermore, if the passage of intact homologous protein

to the embryo were the essence of histiotrophic nutrition, it would be expected that an absence of such homologous protein from the environment of the conceptus would prevent normal growth and development of the embryo. The findings that early organogenesis-stage conceptuses can be cultured in human (Chatot et al., 1980) and fetal calf (Fisher, 1981) serum, clearly indicates the non-toxic nature of heterologous proteins. One final piece of evidence that demonstrates unequivocally that rat serum proteins do not enter the embryo intact comes from the observations (reported in Chapter 9) that the lysosomal proteinase inhibitor leupeptin, added to cultures with radiolabelled serum proteins, effectively blocked the uptake of radioactivity into the embryo and caused a concomitant accumulation of undigested radiolabelled protein within the yolk sac.

The mechanism of utilisation of protein by the early rat conceptus outlined here, contains striking parallels to that proposed for the chick embryo (Hassell and Klein, 1971; Klein, 1975). The chick yolk sac is divided into two zones, the area pellucida, proximal to the developing embryo, and the area opaca, distal to the embryo. It is the area opaca that has been observed to ingest and degrade the radiolabelled egg white protein $\left[^{14}C\right]$ ovalbumin, present in the medium in which chick embryo explants were cultured. The digestion products, amino acids, were utilised in the synthesis of embryonic proteins. The uptake of $\left[^{14}C\right]$ ovalbumin by the area pellucida or the embryo itself, was not observed.

A feature of this mechanism, and equally of that proposed in the present study for the rat conceptus, is that the site of synthesis of embryonic protein is unknown. There are two possibilities. The amino acids produced by proteolysis in the yolk sac either diffuse, or are transported,

into the embryo and are there incorporated into newly synthesised proteins, or they are utilised for synthesis of embryonic protein in the yolk sac, and then transported into the embryo. The latter possibility is perhaps more likely once the vitelline circulation has been established (around gestational-day 10.5). The 15-day rat yolk sac is able to synthesise alphafetoprotein (Gitlin and Boesman, 1967), as is the 12-day mouse yolk sac (Dziadek and Adamson, 1978; Dziadek, 1978), the 15-day rabbit yolk sac (Branch and Wild, 1972) and the chick yolk sac (Gitlin and Kitzes, 1967). Furthermore, the visceral yolk sac endoderm of the chick embryo has been identified as the exclusive site of serum protein synthesis (Young et al., 1980). Whether mammalian, in particular the rat, yolk sac is able to synthesise proteins during early organogenesis is unknown, except in the mouse yolk sac in which alphafetoprotein synthesis does not occur before the 12th day of gestation (Dziadek and Adamson, 1978), well after the formation of a functional chorioallantoic placenta. Further work is required before this aspect of yolk sac-mediated protein nutrition is more fully understood.

That the yolk sac can serve an important and elaborate function in the protein nutrition of the early rat conceptus, and presumably in nutritional mechanisms involving other classes of biological macromolecules, is not in reasonable doubt, and the finding of Cockroft (1979), confirmed in the present study, that early organogenesis-stage conceptuses grow and develop normally in vitamin- and glucose-supplemented dialysed serum clearly demonstrates that exogenous proteins alone are perfectly adequate in supplying the embryo with amino acids. What is less certain however is whether such a process is of quantitative significance to the developing conceptus _in utero_. It was demonstrated

in Chapter 4 that L-leucine successfully competed with $[^3H]$ leucine, derived from digestion in the yolk sac of $[^3H]$ leucine-labelled serum proteins, for incorporation into embryonic proteins. It is clear therefore that the ratio of protein to free amino acid in the environment of the conceptus will be of critical importance in determining the extent to which proteolytically-derived amino acids are utilised in embryonic protein synthesis. In an electron microscopical study, Merker and Villegas (1970) examined the nature of the fluid bathing the rat conceptus during early organogenesis. These workers described a periembryonal sinus which surrounds the conceptus and which contains sluggishly-moving maternal blood, delivered to this region by the decidual blood vessels. In such a relatively static medium, free amino acids would quickly be used up and not replaced by fresh blood, and in any case, the concentration of free amino acid in rat plasma is more than 100 times less than protein-bound amino acid (Long, 1961). In view of this, proteins present in the periembryonal sinus are likely to be of maximum importance in the provision of amino acid precursors for the synthesis of embryonic proteins, and it is concluded that the visceral yolk sac, by the mechanism demonstrated _in vitro_, is of paramount significance in furnishing the early organogenesis-stage rat conceptus with such amino acids _in utero_.

10.2   Histiotrophic Nutrition as a Target for Teratogens

The early organogenesis period of rat embryonic development is the time both when yolk sac-mediated histiotrophic nutrition is at its height, and the embryo is maximally sensitive to teratogenic insult (Wilson, 1973). The nutritional mechanism would appear therefore to be a likely and vulnerable target for the actions of some teratogens (Lloyd et al., 1974), and there are many reports in the literature (reviewed

by Giroud, 1968, and Hurley, 1977) of embryotoxicity and teratogenesis resulting from nutritional deficiency.

Brent and Franklin (1960) showed that clamping the uterine blood vessels of 9.5-day pregnant rats effectively blocked the uptake of maternally-injected macromolecules by the visceral yolk sac. Clamping for periods of between only 30 minutes and 3 hours induced embryo resorption and malformation, thus demonstrating a correlation between reduced histiotrophic nutrition and teratogenesis. Conceivably, any substance capable of inhibiting pinocytosis and/or proteolysis, processes here shown to be essential for the utilisation of exogenous protein by the conceptus during early organogenesis, is also a potential teratogen. To exert a direct action on yolk sac-mediated protein nutrition, the teratogen must be present, in an active form, in high enough concentration in the maternal plasma that bathes the conceptus during the susceptible period.

Three teratogens employed in the present study, trypan blue, suramin and sodium aurothiomalate, have all been proposed to exert their teratogenic action by disturbing yolk sac nutritional function. All three substances localise in the yolk sac lysosomes following their pinocytic uptake by yolk sac cells, and also bind avidly to plasma proteins (see Chapters 5, 6 and 7 for references). In the case of trypan blue, it is the free dye molecule, and not the protein-bound form, that is considered to be active in inhibiting pinocytosis (Williams et al., 1976), and hence teratogenesis. The failure of trypan blue in the present study to inhibit the rate of pinosome formation suggests that insufficient free dye was present in the culture medium, most of it being bound to serum proteins. It is

predicted that elevation of the total trypan blue concentration in the culture medium would eventually result in a high enough concentration of free trypan blue to inhibit pinocytosis. Similarly, a very high concentration of trypan blue in the plasma surrounding the conceptus in utero would be necessary to saturate the binding capacity of plasma proteins and enable sufficient free trypan blue to inhibit pinocytosis by the yolk sac.

Suramin appeared to affect protein utilisation by cultured conceptuses in two ways, by inhibiting pinocytosis of protein by the yolk sac, and also by inhibiting the fusion of pinosomes with lysosomes, thus preventing proteins from being digested by lysosomal enzymes. It is perhaps significant, in view of the foregoing discussion of trypan blue, that high concentrations of suramin were necessary to produce this effect.

Sodium aurothiomalate affected the protein nutrition of cultured conceptuses by inhibiting the yolk sac intralysosomal degradation of pinocytosed serum proteins. The consequence of this action was to reduce the amount of amino acids available for protein synthesis in the embryo and, as discussed in Chapter 7, this probably constitutes the mechanism of teratogenic action of aurothiomalate.

The effects of anti-visceral yolk sac antiserum in inhibiting pinocytosis by the yolk sac (Chapter 8) and leupeptin, a potent inhibitor of lysosomal proteolysis (Chapter 9), were clear cut. The powerful action exerted by these teratogens on protein utilisation by conceptuses most likely represents their mode of teratogenesis.

Substances that produce their teratogenic effect by inhibiting pinocytosis by the visceral yolk sac would cause a deficiency in the amount of pinocytosed substrate reaching the conceptus tissues. Proteins would be the most important, if not the only, macromolecules in short supply. It is known that in vitro, glucose can satisfy the energy requirements of the conceptus (Shepard et al., 1970; Tanimura and Shepard, 1970; Cockroft, 1979; Sanyal, 1980). Given that rat plasma contains little polysaccharide but a relative abundance of free glucose, and that glucose is able to permeate cell membranes and does not rely on pinocytosis for its entry into cells, inhibition of pinocytosis by the yolk sac is unlikely to affect the energy metabolism of conceptuses. The observation (Cockroft, 1979) that folic acid, an important coenzyme in nucleic acid biosynthesis, is essential for normal development of conceptuses in vitro, together with the facts that folic acid deficiency in vivo is teratogenic (reviewed by Hurley, 1977) and insignificant amounts of nucleic acid are present in plasma, demonstrates that conceptuses are able to synthesise their own nucleic acids and are not dependent on an exogenous source. Nucleic acid metabolism too therefore will not be directly affected by inhibitors of pinocytosis by yolk sac. Inhibition of yolk sac intralysosomal proteolysis would of course result in embryonic protein malnutrition. A second important effect of proteolysis inhibition might be that deficiencies of certain essential amino acids occur. It is interesting to note that trypan blue-treated pregnant mice, given a diet supplemented with the essential amino acid L-threonine, produced significantly fewer offspring with exencephaly than controls receiving no L-threonine supplement in their diets (Zawoiski, 1980). Supplementation of the diet with other, non-essential, amino acids failed to produce this effect.

From the results of the experiments described in Chapters 5 to 9 of this Thesis, it can be concluded that yolk sac mediated histiotrophic nutrition is, in all probability, a target for certain teratogens.

10.3   Whole-Embryo Culture

Throughout this study extensive use has been made of the whole-embryo culture technique developed by New and associates (New et al., 1973, 1976a). This technique, along with its variations (reviewed by New, 1978), has provided mammalian embryologists with an invaluable tool for the investigation of various aspects of embryonic development. Through the use of such culture methods important advances have been made in the study of teratogenic mechanisms (New and Brent, 1972; Morriss and Steele, 1974; Steele et al., 1974; Cockroft and New, 1975; Kochhar, 1975; New, 1976, 1978; Cockroft and Coppola, 1977, Deuchar, 1979; Mirkes et al., 1981; Sanyal et al., 1981), morphological and functional development of the embryo (Deuchar, 1971; Payne and Deuchar, 1972; Steele and New, 1974; Robkin et al., 1976; Morriss and New, 1979) and aspects of embryonic metabolism and biochemistry (Cockroft, 1979; Sanyal, 1980; Shepard et al., 1970; Tanimura and Shepard, 1970). It is hoped that the present study has made a significant and worthwhile contribution to the rapidly expanding corpus of knowledge of early mammalian embryology that has been due largely to the advent of simple and reliable culture techniques.

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