J. Embryol. exp. Morph. 73, 307–315, 1983
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Evidence that protein ingested by the rat visceral yolk sac yields amino acids for synthesis of embryonic protein

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SUMMARY

[^{3}H]Leucine-labelled haemoglobin was prepared from rat reticulocytes incubated in the presence of [^{3}H]leucine.

Conceptuses from 9·5-day pregnant rats were incubated *in vitro* for 48 h, with [^{3}H]leucine-labelled haemoglobin present for the final 12, 8, 4, 2 or 0·5 hours. Radioactivity accumulated in visceral yolk sac and in embryonic tissue. When exposure to labelled haemoglobin was for only a short period before harvesting, all the radioactivity found in the embryo and most of that found in the visceral yolk sac was trichloroacetic acid-soluble (i.e. associated with free amino acid rather than with protein). After longer exposures the proportion of radioactivity that was acid-soluble decreased to minimum values of about 20 %.

SDS-polyacrylamide gel electrophoresis of the protein-associated radioactivity in visceral yolk sac and embryo was performed. After exposure to labelled haemoglobin for 1 h only prior to harvesting, the yolk sac contained a single peak of radioactivity coincident in mobility with haemoglobin. The embryo contained no protein-associated radioactivity. After exposure to labelled haemoglobin for 12 h, many protein bands in both yolk sac and embryo were radiolabelled.

Thus a single radiolabelled protein pinocytically captured by the visceral yolk sac can give rise to the presence of many labelled proteins in embryo and visceral yolk sac. These results indicate that the source protein underwent proteolytic digestion and that the amino acids generated were re-utilized for protein synthesis in both embryonic and visceral yolk-sac cells.

INTRODUCTION

Pinocytosis of proteins by the epithelial cells of the rodent visceral yolk sac has been implicated in the transmission of immunoglobulins to the foetus (Wild, 1975) and in the protein nutrition of the embryo (Freeman, Beck & Lloyd, 1981). Digestion of incoming protein within yolk-sac lysosomes is seen as crucial to the latter mechanism, but in the former the intact protein is transmitted.

Experiments were reported recently (Freeman *et al.* 1981) in which early organogenesis-stage rat conceptuses were cultured *in vitro* in the presence of

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[^{3}H]leucine-labelled rat serum proteins. Radioactivity was subsequently found in the embryonic tissue, as well as in the visceral yolk sac, and some of it was trichloroacetic acid-insoluble, i.e. associated with macromolecules. Since other experiments, using ^{125}I-labelled polyvinylpyrrolidone, suggested that the embryo cannot itself capture intact macromolecules, it was concluded that transfer of [^{3}H]leucine from culture medium to embryo was mediated by the visceral yolk sac. Other data, particularly the ratios of acid-soluble (i.e. low molecular weight) and acid-insoluble radioactivity after different periods of exposure to [^{3}H]leucine-labelled serum proteins and the failure of ^{125}I-labelled protein to be passed intact to the embryo, indicated that the supply route involved pinocytic uptake of [^{3}H]leucine-labelled protein by the visceral yolk sac, its digestion within that tissue's lysosomes and the transfer of the [^{3}H]leucine to the embryo for use in protein synthesis.

Proof that proteins available to the conceptus can be digested to amino acids which are then incorporated into embryonic protein could be obtained by using a single protein rather than a mixture such as the [^{3}H]leucine-labelled serum proteins used by Freeman *et al.* (1981). If the protein is transmitted intact, only the one radiolabelled protein should be found in the embryo. If digestion is an obligatory intermediate step, several labelled proteins should be found. We have therefore investigated the labelled proteins found in the embryo after culture in the presence of [^{3}H]leucine-labelled rat haemoglobin.

MATERIALS AND METHODS

Preparation of [^{3}H]leucine-labelled rat haemoglobin

[^{3}H]Leucine-labelled haemoglobin was isolated from rat reticulocytes that had been incubated for 1 h in the presence of [^{3}H]leucine. The method was based on that described by Chandler & Ballard (1978).

Reticulocytosis was induced in male adult Wistar rats by daily subcutaneous injection for 5 consecutive days of 1 % phenylhydrazine at a dosage of 1 mg phenylhydrazine per 100 g body weight. After resting the animals for a further 2 days, blood was collected from the aortae of anaesthetized animals, using a 10 ml syringe and a serum needle that had been filled with heparin solution (Boots Co. Ltd., Nottingham) to prevent clotting of the blood. Approximately 7 ml of blood was withdrawn from each animal. The blood collected from two animals was pooled.

Into each of several 50 ml centrifuge tubes was placed 30 ml of 'reticulocyte washing solution', prepared by dissolving in 500 ml of distilled water 4·24 g NaCl, 0·187 g KCl, 0·153 g $MgCl_2 .6H_2O$ and 0·229 g N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES). Blood (2 ml) was added to each tube, and the mixture centrifuged at 2000 ***g*** for 3 min at 4 °C. After centrifugation the washing solution was decanted and the washing procedure repeated twice more.

Each washed reticulocyte pellet was dispersed in 10 ml of 'reticulocyte incubation medium' containing 100 μCi of L-[4,5-^{3}H]leucine (Radiochemical Centre, Amersham, Bucks, U.K.; preparation TRK 510; 130–190 Ci per mmol). The incubation medium comprised 349 mg NaCl, 18·7 mg KCl, 15·3 mg $MgCl_2 .6H_2O$, 222 mg TES, 49·5 mg glucose, and 3·9 mg $Fe(NH_4)_2SO_4 .6H_2O$ dissolved in 50 ml of distilled water. The suspension was incubated at 37 °C for 1 h, shaking at 5 min intervals throughout. Following incubation, reticulocytes were washed a further three times as described above, with the exception that centrifugation was at 2500 ***g*** for 5 min. The pelleted reticulocytes were haemolysed by adding 2 ml of distilled water and freezing and thawing through four cycles. The free haemoglobin was separated by centrifuging the haemolysate at 3000 ***g*** for 45 min at 4 °C. The clear supernatant was decanted and dialysed overnight at 4 °C against distilled water. Following dialysis less than 1 % of the total radioactivity of the haemoglobin solution was soluble in 6·7 % (w/v) trichloroacetic acid (TCA). Radiolabelling of haemoglobin was confirmed by eluting portions of the dialysed solution from a Sephadex G-75 column with 0·9 % saline. Haemoglobin and radioactivity were eluted together.

Cultures of 9·5-day rat conceptuses in the presence of [^{3}H]leucine-labelled haemoglobin

The techniques were as described by Freeman *et al.* (1981), except that a culture medium of 50 % heat-denatured homologous serum, 50 % Hanks balanced salt solution (Flow Laboratories, Irvine, Scotland) was used. The following criteria of normal development were routinely applied to all conceptuses after culture: a yolk-sac diameter of 3–4 mm, presence of a heart-beat and vitelline circulation, normal axial rotation to the dorsally convex position, the presence of the forelimb buds, neural tube closure, somite number (22–27) and protein content (120–240 μg). As judged by these criteria, conceptuses developed as successfully in 50 % as in 100 % heat-denatured homologous serum. All cultures were of 48 h duration. [^{3}H]Leucine-labelled haemoglobin solution (0·05 ml per ml of culture medium) was added to cultures at a chosen time-point between 12 and 0·5 h before harvesting.

Separation by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of radiolabelled proteins of conceptuses after culture in the presence of [^{3}H]leucine-labelled haemoglobin

Conceptuses were incubated for either the last 12 h or the last 1 h of a 48 h culture period in the presence of [^{3}H]leucine-labelled haemoglobin. At harvesting and after washing in three changes of Hanks, yolk sacs and embryos from each group were separately pooled and homogenized in 0·25 M-NaOH. On complete solubilization, tissue solutions were neutralized by adding an equal volume of 0·25 M-HNO_3. Solutions were then dialysed overnight at 4 °C, and prepared for electrophoresis by adding 0·2 ml fractions to 0·1 ml of a solution comprising

6% SDS and 30% 2-mercaptoethanol in 0·01 M-sodium phosphate buffer (pH 7·1), and heating the mixture at 100 °C for 5 min. Bromophenol blue (0·005 ml of a 0·25 % w/v solution) was added to each tissue solution to act as front marker. After electrophoresis according to the method of Fehrnstrom and Moberg (1977) in 7·5 % gels using a sodium phosphate buffer system, the gel was immediately sliced into 1·8 mm-thick portions. Each slice was individually homogenized in 0·5 ml of distilled water and the homogenate added to 4·5 ml of Lumagel scintillant (Lumac Systems Inc. Basle, Switzerland) and counted for total radioactivity. Background radioactivity was approx. 20 c.p.m.

RESULTS

Uptake of radioactivity by yolk sacs and embryos

Figure 1 shows that, in conceptuses incubated in the presence of [^{3}H]leucine-labelled haemoglobin, the uptake of radioactivity into both yolk sac and embryo increased in proportion to the duration of exposure to radiolabelled protein. The percentage of tissue radioactivity that was TCA soluble is shown in Fig. 2. Following short periods of exposure to radiolabelled protein, most of the radioactivity found in the yolk sac, and all of that in the embryo, was TCA soluble. On longer exposures, the percentages of yolk-sac and embryo radioactivity that were TCA soluble steadily fell to minimum values of 20–25 %.

The TCA-soluble radioactivity in the culture medium did not increase during the incubation period, indicating that digestion products of [^{3}H]leucine-labelled

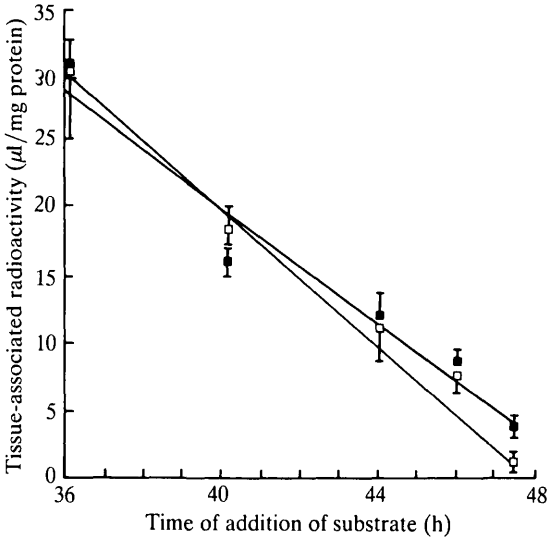

Fig. 1. Radioactivity associated with yolk sac (■) and embryo (□) after incubation of conceptuses in the presence of [^{3}H]leucine-labelled haemoglobin. Each point represents the mean (±S.D.) of at least six determinations. Levels of uptake of radiolabel are expressed as clearances (see Freeman *et al.* 1981).

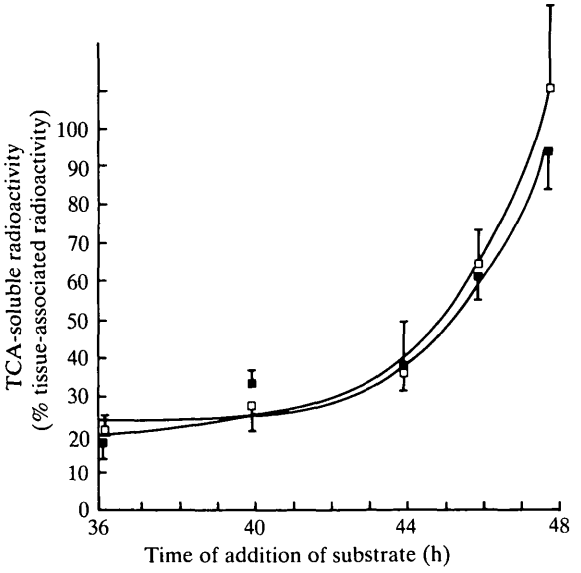

Fig. 2. Percentage of tissue radioactivity soluble in TCA after incubation of conceptuses in the presence of [^{3}H]leucine-labelled haemoglobin. Each point represents the mean (±S.D.) of at least six determinations. (■) Yolk sac, (□) embryo.

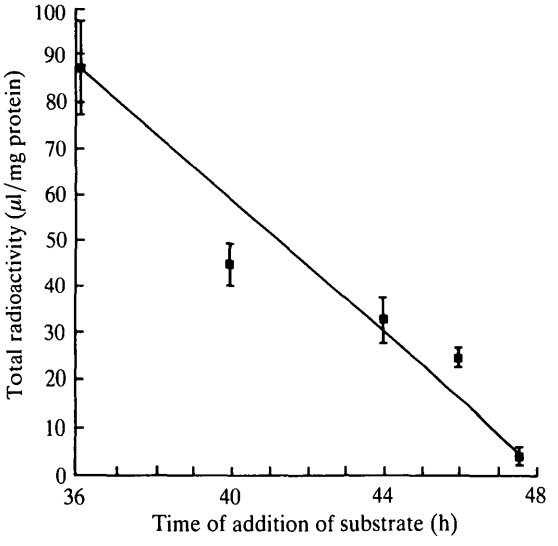

Fig. 3. Total radioactivity taken up by yolk sacs after incubation of conceptuses in the presence of [^{3}H]leucine-labelled haemoglobin. Each point represents the mean (±S.D.) of at least six determinations. As in Fig. 1, uptake values are expressed as clearances.

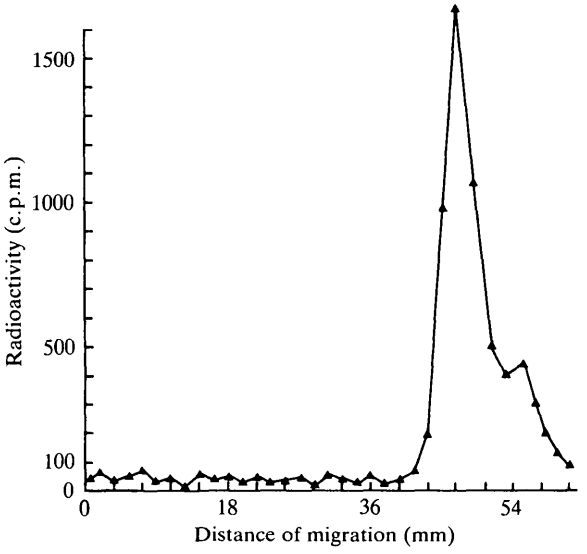

Fig. 4. Profile of protein-associated radioactivity after SDS polyacrylamide gel electrophoresis of [^{3}H]leucine-labelled haemoglobin.

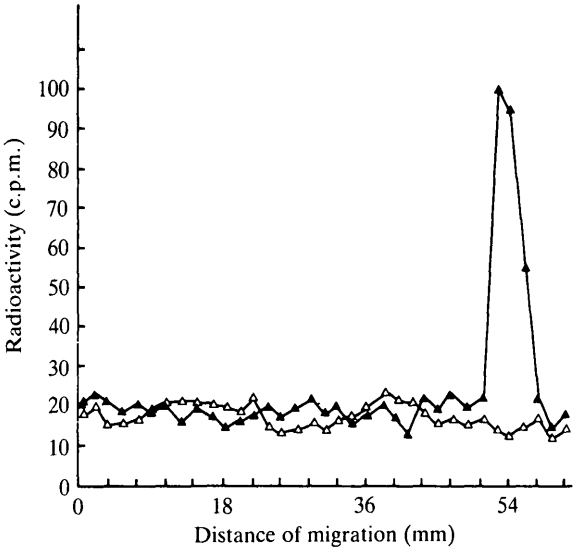

Fig. 5. Profile of protein-associated radioactivity after SDS polyacrylamide gel electrophoresis of yolk-sac proteins (▲) and embryo proteins (△) from conceptuses that had been incubated in the presence of [^{3}H]leucine-labelled haemoglobin for the final hour of a 48 h culture period.

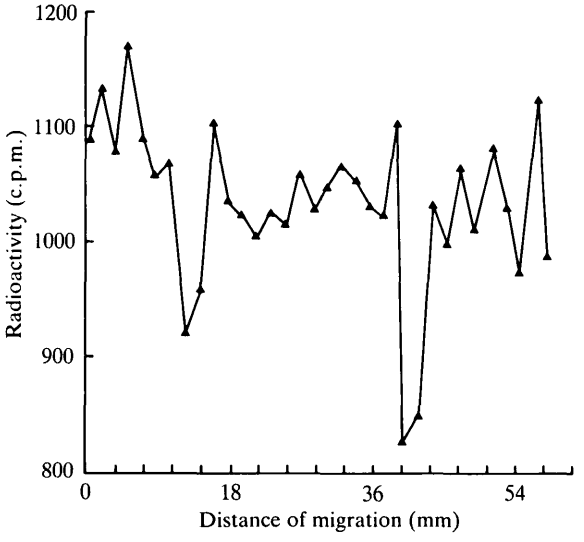

Fig. 6. Profile of protein-associated radioactivity after SDS polyacylamide gel electrophoresis of yolk-sac proteins from conceptuses that had been incubated in the presence of [^{3}H]leucine-labelled haemoglobin for the final 12 h of a 48 h culture period.

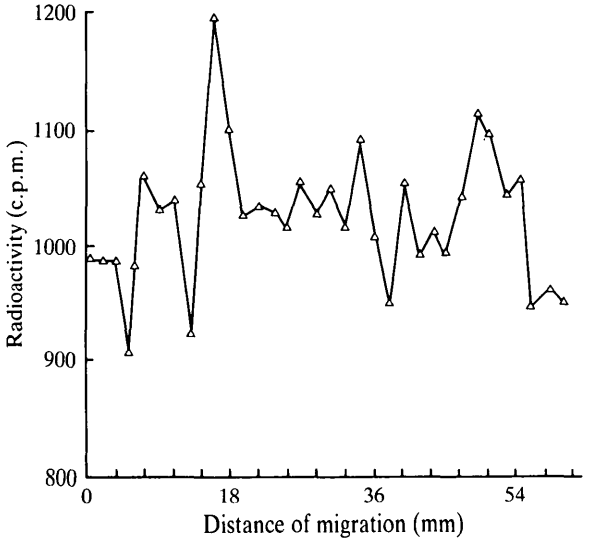

Fig. 7. Profile of protein-associated radioactivity after SDS polyacrylamide gel electrophoresis of embryo proteins from conceptuses that had been incubated in the presence of [^{3}H]leucine-labelled haemoglobin for the final 12 h of a 48 h culture period.

haemoglobin are all retained within the tissues. On the assumption, shown below to be correct, that the yolk sac is the only site of uptake of haemoglobin by the early rat conceptus, and that radioactivity associated with both the yolk sac and the embryo is derived from pinocytosis of radiolabelled haemoglobin by the yolk sac, the total clearance of radioactivity by the yolk sac may be calculated (Fig. 3).

Electrophoretic separation of conceptus proteins

The profile of radioactivity associated with yolk-sac proteins after a 1 h exposure to radiolabelled haemoglobin (Fig. 5) showed a single peak corresponding to radiolabelled haemoglobin itself (see Fig. 4). Embryonic proteins under the same incubation conditions showed no radioactivity (Fig. 5). After incubation of conceptuses in the presence of [^{3}H]leucine-labelled haemoglobin for the final 12 h of culture, both yolk sac and embryo displayed a wide spectrum of radiolabelled proteins (Figs 6 and 7).

DISCUSSION

When conceptuses were cultured in medium whose only radiolabelled component was [^{3}H]leucine-labelled haemoglobin, radioactivity accumulated in both yolk sac and embryo. The time course of this accumulation, and the changing percentage of radioactivity in macromolecular form, are similar to the data previously obtained (Freeman *et al.* 1981) using unfractionated ^{3}H-labelled rat serum proteins as the source of radiolabel in the culture medium. The interpretation of these patterns of uptake is discussed fully by Freeman *et al.* (1981) and in the Introduction above, and no further comment is needed. The present results do however identify haemoglobin as a protein that can be captured by the visceral yolk-sac epithelial cells, and comparison of Fig. 3 with Fig. 9 of Freeman *et al.* (1981) shows that its rate of capture is three times the average value for ^{3}H-labelled serum proteins. No significance can be attached to this factor, since it was not possible for Freeman *et al.* (1981) to identify which radiolabelled protein(s) was captured from the mixture presented. Furthermore the present experiments used 50 % serum as the culture medium; those of Freeman *et al.* (1981) used 100 %.

The use of ^{3}H-labelled haemoglobin in the present experiments was to demonstrate conclusively that the radiolabelled protein found in the embryo after incubation of conceptuses with a ^{3}H-labelled protein was not the result of direct uptake from the medium by the embryo, but arose from *de novo* synthesis from radiolabelled amino acids released from the yolk-sac lysosomes. The presence (Fig. 7) of a large number of radiolabelled proteins in the embryo after 12 h incubation of the conceptus in a medium containing a single radiolabelled protein, haemoglobin, proves this point unambiguously. The presence, after the same incubation period, of many radiolabelled proteins in the yolk sac itself

(Fig. 6) shows additionally that protein synthesis in the growing yolk sac also uses amino acids generated from proteins pinocytically ingested by its epithelial cells. Haemoglobin was the only radiolabelled protein found in the yolk sac after a very brief (1 h) exposure to culture medium containing [^{3}H]leucine-labelled haemoglobin, and no radiolabelled protein was found in the embryo (Fig. 5). These findings further establish the role of the visceral yolk sac as the site of pinocytic ingestion and consequently the source of amino acids for the needs of the embryo.

Finally, it should be stressed that no suggestion is made here that haemoglobin is *in vivo* an important source of protein nutrition for the early rat conceptus. Haemoglobin was chosen for experimental convenience, and it is likely that other radiolabelled polypeptides, whether homologous or heterologous, would have given similar results. *In vivo* the yolk-sac epithelial cells are probably omnivorous with respect to plasma proteins, and the most abundant species such as albumin are likely to be quantitatively the most significant sources of amino acids for protein synthesis in the embryo (and yolk sac).

We thank the Medical Research Council for a grant in support of this work, and Professor F. Beck for many useful discussions.

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(*Accepted 14 October 1982*)