The role of the visceral yolk sac
in mediating protein utilization by rat embryos
cultured in vitro

By STUART J. FREEMAN,¹ FELIX BECK²
AND JOHN B. LLOYD¹,³

From the Universities of Keele and Leicester

SUMMARY

Conceptuses from 9-5-day pregnant rats have been cultured for 48 h in heat-inactivated homologous serum. Embryonic development was normal. The protein contents of embryos and visceral yolk sacs after different periods of culture were recorded.

When ¹²⁵I-labelled polyvinylpyrrolidone or [³H]dextran were added to the culture serum, radioactivity was accumulated by the yolk sac, but only background levels were detected in the embryo itself. The amount of radioactivity found in the yolk sac varied with the length of the interval before harvesting during which ¹²⁵I-labelled PVP or [³H]dextran was present.

When formaldehyde-denatured ¹²⁵I-labelled bovine serum albumin was added to the culture serum, little radioactivity accumulated in the yolk sac and only background levels were found in the embryo. Trichloroacetic acid-soluble radioactivity steadily appeared in the culture serum, however.

When conceptuses were cultured in glucose- and vitamin-supplemented dialysed serum from rats injected 2 h previously with [³H]leucine, radioactivity was found in both embryos and yolk sacs. The amount of radioactivity in these tissues increased with duration of exposure to ³H-labelled serum proteins. After short exposures little of the yolk sac and embryonic radioactivity was acid-insoluble, but this proportion increased with duration of exposure.

These results are interpreted as follows. Intact macromolecules cannot enter the cells of the embryo itself, but are captured by pinocytosis into the cells of the visceral yolk-sac endoderm. Indigestible macromolecules such as ¹²⁵I-labelled polyvinylpyrrolidone and [³H]dextran accumulate within the yolk-sac lysosomes, but proteins are digested there by the lysosomal enzymes. The radiolabelled digestion product of ¹²⁵I-labelled bovine albumin is [¹²⁵I]iodotyrosine, which cells cannot utilize and so is excreted into the culture serum. The labelled digestion product of the ³H-labelled rat serum proteins is [³H]leucine, which is used for protein synthesis in both embryo and yolk sac.

The experiments provide direct evidence for the long-suspected role of the yolk sac in mediating embryonic nutrition in the period of development prior to the establishment of a functional chorioallantoic placenta.

¹ Authors' address: Department of Biological Sciences, University of Keele, Keele, Staffordshire, ST5 5BG, England.
² Author's address: Department of Anatomy, University of Leicester, Leicester LE1 7RH, England.
³ To whom correspondence should be addressed.
INTRODUCTION

Prior to the establishment of a chorioallantoic placenta the mammalian embryo must derive its nutrition from substances present in its immediate environment. In rodents the marked pinocytic properties of the visceral yolk sac endoderm have led to the suggestion that these cells perform a nutritional role by capturing macromolecules, transferring these to the lysosomes where they are digested, and passing the digestion products to the developing embryo (see Williams et al. 1976). The stage of development at which this mechanism is likely to be of greatest importance lies between gastrulation and the onset of chorioallantoic placental function and is the period when the embryo is maximally sensitive to teratogenic insult. It has been proposed that some teratogens, particularly trypan blue, act by inhibiting pinocytosis or intra-lysosomal catabolism in the yolk sac (Williams et al. 1976).

Several authors (Beck, Lloyd & Griffiths, 1967; New & Brent, 1972; Payne & Deuchar, 1972; Goetze et al. 1975; Sharma & Peel, 1979) have adduced circumstantial evidence for the yolk sac having a nutritional role, but direct evidence has hitherto been lacking. We here report experiments using the rat embryo cultured in vitro for 48 h from 9-5 days of gestation that show that radio-labelled amino acid present in protein in the culture medium is incorporated into embryonic protein. Further experiments, using the non-digestible macromolecules 125I-labelled polyvinylpyrrolidone and [3H]dextran and the digestible but non-utilizable 125I-labelled bovine serum albumin, show that uptake and digestion by the yolk sac are necessary for this process.

Brief reports of some of this work have been published elsewhere (Freeman & Lloyd, 1980, 1981).

MATERIALS AND METHODS

Preparation of rat serum in which proteins have been labelled with [3H]leucine

Male rats weighing approximately 250 g received an intraperitoneal injection of L-[4,5-3H]leucine (Radiochemical Centre, Amersham, Bucks, U.K., preparation TRK510) contained within 10 ml of 0-9% saline, at a dosage of 40μCi/100 g body wt. After 2 h, at which time incorporation of radioactivity into serum proteins is maximal (Schreiber, Boutwell, Potter & Morris, 1966), the rat was anaesthetized with ether. Blood was withdrawn from the dorsal aorta and serum prepared by the method of New, Coppola & Cockroft (1976). To remove free [3H]leucine the serum was dialysed at 4°C for 4 days against four changes of a balanced salt solution (Cockroft, 1979). The dialysed serum contained approximately 1% of its total radioactivity in a form soluble in 6-7% (w/v) trichloroacetic acid (TCA). The dialysed serum was kept at −20°C until use, whereupon it was heat-denatured at 56° for 30 min, centrifuged at 2000 g for 5 min to remove any particulate matter, and to it was added (per ml)
Yolk sac mediates protein nutrition in rat embryos in vitro

10 µl of a vitamin concentrate (Flow Laboratories, Irvine, Scotland, U.K.; preparation 16-014-49) and 1.5 mg D-glucose.

Preparation of an aqueous solution of formaldehyde-denatured $^{125}$I-labelled bovine serum albumin

The method of Moore, Williams & Lloyd (1977) was used to prepare a solution containing 0.55 mg protein/ml. Radioactivity soluble in 6-7% (w/v) TCA accounted for approximately 1% of the total radioactivity and no increase in this level was observed following storage for several months at -20°C.

Culture of 9-5-day rat egg cylinders

Rat egg cylinders, explanted at 9-5 days of gestation, were cultured in 100% homologous serum for 48 h. Details of preparation of the culture medium, explanation and conditions of culture were as described by New, Coppola & Terry (1973) and New et al. (1976). 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).

Studies on the uptake of radiolabelled substrates by embryo and yolk sac

(i) $^{125}$I-labelled polyvinylpyrrolidone as substrate. Rat conceptuses were cultured as described above, and $^{125}$I-labelled polyvinylpyrrolidone (PVP) (Radiochemical Centre, preparation IM 33P) added to the culture medium at a particular interval before harvesting. In different experiments 1.4, 2.8, or 5.6 µg $^{125}$I-labelled PVP/ml (final concentration) were added at different times from 24 h to 1 h before harvesting. At harvesting, conceptuses were washed in Hanks medium (Flow Laboratories, Irvine), yolk sacs and embryos dissected apart and washed separately in a further three changes of Hanks medium. Each tissue was then solubilized by adding 0.5 ml of 0.25 M-NaOH and incubating for 1 h at 37°C. This solution was neutralized by adding 0.5 ml of 0.25 M-HNO₃ and the volume brought to 1.1 ml by adding 0.1 ml of water. Protein assay (Lowry, Rosebrough, Farr & Randall, 1951) was performed on duplicate 0.05 ml portions, using bovine serum albumin (Sigma (London) Chemical Company, Poole, Dorset) as reference protein. The remaining 1.0 ml was assayed for radioactivity using a Packard auto-gamma scintillation spectrometer 5136 (Packard Instruments Ltd., Caversham, Berks, U.K.). The observed count, corrected for background, was multiplied by 1.1 to give a value for the radioactivity of the whole tissue. Duplicate 1.0 ml samples of each culture medium were assayed for radioactivity and values corrected for background.

The level of uptake of radiolabel into yolk sac and embryo was expressed as a clearance, as described by Williams, Kidston, Beck & Lloyd (1975a).
Results are presented as microlitres of medium whose contained substrate was captured per milligram of tissue protein.

(ii) $[^{3}H]$Dextran as substrate. Following the same basic procedure as in (i), $[^{3}H]$dextran (Radiochemical Centre; preparation TRA.382) was used as substrate at a concentration of 2, 5 or 10 $\mu$g/ml culture medium. Tissues were solubilized, neutralized and diluted as in (i) above and protein assay performed on duplicate 0.05 ml fractions. Of the remaining 1.0 ml, 0.5 ml was added to approximately 4.5 ml of Lumagel scintillation fluid (Lumac Systems, Basle, Switzerland) and assayed for radioactivity in a Packard 2425 liquid scintillation spectrometer. Duplicate 0.5 ml samples of the media were counted similarly. All observed counts were corrected for background and quenching, and tissue counts were multiplied by 2.2 to give a value for the radioactivity of the whole tissue. Uptake of radioactivity by tissues was, as in (i), expressed as a clearance.

(iii) Formaldehyde-denatured $^{125}$I-labelled bovine serum albumin as substrate. Formaldehyde-denatured $^{125}$I-labelled bovine serum albumin (BSA) at a final concentration of 26.8, 33.5 or 40.2 $\mu$g/ml was added to the culture medium at some time-point between 12 and 0.5 h before harvesting. At harvesting, conceptuses were solubilized, neutralized and diluted as in (i) above. It was demonstrated that solubilization of tissues at 37 °C in 0.25 M NaOH for 1 h did not lead to a significant increase in the proportion of TCA-soluble radioactivity.

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; Wellcome Reagents Ltd, Beckenham, Kent) was added. The solution was then centrifuged for 20 min at 2000 g, the supernatant (1.5 ml) decanted into a fresh tube and its radioactivity re-measured – the TCA-soluble count. Owing to the increase in volume for 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 yield a more accurate value. This correction factor was determined empirically by TCA-precipitation of medium, or tissue solutions, to which $[^{125}$I]iodotyrosine had been added.

Duplicate 1.0 ml fractions of culture medium were assayed for total and TCA-soluble radioactivity in the same way, but without carrier protein. Corrections were made as above.

(iv) $[^{3}H]$Leucine-labelled serum proteins as substrate. 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 h before harvesting. At harvesting, conceptuses were washed as before and tissues solubilized, neutralized and diluted. The 1.0 ml of tissue solution for radioactivity counting was divided into two 0.5 ml fractions, one of which was added to approximately 4.5 ml of Lumagel scintillant and assayed for total radioactivity. To the remaining 0.5 ml was added 0.25 ml of 20% TCA and 0.05 ml of carrier protein. After centrifugation at 2000 g for 20 min, the super-
Yolk sac mediates protein nutrition in rat embryos in vitro

Fig. 1. Increase in protein content of yolk sac (●), and embryo (○) of 9.5-day rat conceptuses cultured in vitro. Numbers of conceptuses (in parentheses) and standard deviations (S.D.) are shown.

Fig. 2. Radioactivity associated with yolk sac (●), and embryo (○) after incubation of rat conceptuses in the presence of $^{125}$I-Labelled PVP. $^{125}$I-Labelled PVP (2.8 µg/ml) was added at different intervals from the start of the culture period. All cultures were terminated at 48 h. Numbers of conceptuses (in parentheses) and S.D. are shown.

natant (0.75 ml) was added to approximately 4.5 ml of Lumagel scintillant and counted to give the TCA-soluble radioactivity. This figure was adjusted, as in (iii), by multiplication by an empirical correction factor. Appropriate corrections were made for background and quenching.

Duplicate 0.5 ml samples of culture medium were similarly assayed for total and TCA-soluble radioactivity, with the omission of carrier protein. Uptake values for total radioactivity were calculated and the percentage of TCA-soluble material of this worked out.

RESULTS

Development of conceptuses in culture

Conceptuses grew and developed progressively throughout the 48 h culture period. Viability was at least 90% and conceptuses that failed to satisfy the criteria for normal development (described above) were discarded.

Figure 1 shows the protein content of yolk sacs and embryos from 24 to 48 h of culture.

Uptake of $^{125}$I-labelled PVP and $[^{3}H]$dextran by embryo and yolk sacs

Figure 2 shows how the amount of radioactivity found in the embryo and yolk sac at 48 h varied with the time at which $^{125}$I-labelled PVP was added to the culture serum. It may be seen that no radioactivity enters the embryo. In contrast the yolk sacs contained significant amounts of radioactivity; the
Fig. 3. Radioactivity associated with yolk sac (●), and embryo (○) after incubation of rat conceptuses in the presence of [3H]dextran. [3H]Dextran (5-0 μg/ml) was added at different intervals from the start of the culture period. All cultures were terminated at 48 h. Numbers of conceptuses (in parentheses) and S.D. are shown.

Fig. 4. Radioactivity associated with yolk sac (●), and embryo (○) after incubation of rat conceptuses in the presence of formaldehyde-denatured 125I-labelled BSA. Formaldehyde-denatured 125I-labelled BSA (5.5 μg/ml) was added at different intervals from the start of the culture period. All cultures were terminated at 48 h. Numbers of conceptuses (in parentheses) and S.D. are shown.

Fig. 5. Percentage tissue radioactivity soluble in TCA after incubation of rat conceptuses in the presence of formaldehyde-denatured 125I-labelled BSA. Numbers of conceptuses (in parentheses) and S.D. are shown. (●) Yolk sac, (○) embryo.

Fig. 6. Total radioactivity taken up by yolk sac after incubation of rat conceptuses in the presence of formaldehyde-denatured 125I-labelled BSA. Numbers of conceptuses (in parentheses) and S.D. are shown.
amount found was roughly proportional to the duration of exposure to $^{125}$I-labelled PVP, except that little difference was seen between tissues incubated with radiolabel from 24 h and those in which radiolabel was added at 30 h.

Essentially identical data were obtained at each of three different concentrations of $^{125}$I-labelled PVP in the culture medium.

Figure 3 shows the results obtained using $[^3]$Hdextran as substrate. Uptake into the yolk sac was qualitatively and quantitatively similar to that of $^{125}$I-labelled PVP. Some radioactivity, but not progressive accumulation, was found in the embryo, and this probably represents either some slight degree of digestion of dextran to glucose or the presence of some contaminating $[^3]$H-glucose in the $[^3]$Hdextran sample.

Since the amount of $^{125}$I-labelled PVP (or of $[^3]$Hdextran) captured by the yolk sac between 24 and 30 h of culture was quantitatively insignificant by the time of harvesting, subsequent experiments used conceptuses exposed to radiolabelled substrate for a maximum period of 12 h before harvesting.

*Experiments using formaldehyde-denatured $^{125}$I-labelled BSA as substrate*

The amount of radioactivity present in yolk sacs and embryos at harvesting is shown in Fig. 4. In the yolk sac the level of radioactivity was independent of duration of exposure to $^{125}$I-labelled BSA, except where this duration was below 2 h. Essentially the same was observed for radioactivity in the embryo, though at a very much lower level. The percentage of yolk sac and embryo radioactivity that was TCA-soluble is shown in Fig. 5. Evidently, the small amount of radioactivity reaching the embryo is all in a form soluble in TCA. The TCA-soluble radioactivity of the yolk sac, expressed as a percent of total yolk sac radioactivity, increases with increasing time of exposure to substrate, attaining a maximum value of 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 (which did not appear when $^{125}$I-labelled BSA was incubated in serum but without conceptuses) must have been released from the tissues following digestion of radiolabelled protein, and must properly be included in any calculation of uptake. The pattern of uptake of radioactivity, including that which is retained within yolk-sac tissue and that released back into the medium, is shown in Fig. 6. Uptake is linear over the 12 h, and at a much higher rate than is observed for $^{125}$I-labelled PVP or $[^3]$Hdextran.

*Experiments using $[^3]$Hleucine-labelled serum proteins as substrates*

As found by Cockroft (1979), conceptuses cultured in glucose- and vitaminsupplemented dialysed rat serum developed normally, as judged by the chosen criteria (see above).

The uptake of radioactivity into yolk sac and embryo is shown in Fig. 7.
In the yolk sac, the level of radioactivity increases in proportion to the duration of exposure to radiolabelled protein. There is, furthermore, an appreciable amount of radioactivity reaching the embryonic tissue; the amount increases linearly with time of exposure to substrate and reaches a level slightly in excess of that in the yolk sac. The percentage of tissue radioactivity that is TCA-soluble is shown in Fig. 8. After short periods of exposure to substrate, the yolk sac contains a high percentage of its radioactivity in TCA-soluble form. On longer exposures this percentage declines steadily, apparently tending towards a minimum value of 30%.

A qualitatively similar pattern is observed in the embryo, where the percentage of TCA-soluble radioactivity is higher than in the yolk sac at short exposure times and declines more rapidly with longer exposures, to a steady level of about 20%, lower than that seen in yolk sac.

The acid-soluble radioactivity of the culture medium did not increase during the incubation period. This must indicate that any digestion products of $^3$H-labelled proteins must be retained within the tissues. The pattern of uptake of radioactivity by the conceptus, calculated from values for yolk sac and embryo radioactivity and for the total radioactivity of the culture medium, are shown in Fig. 9.

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 macromolecular substrates. Therefore the uptake of $^{125}$I-
Yolk sac mediates protein nutrition in rat embryos in vitro

Fig. 9. Total radioactivity taken up by yolk sac after culture of rat conceptuses in serum whose proteins were [3H]leucine-labelled. Numbers of conceptuses (in parentheses) and S.D. are shown.

Labelled PVP was studied following exactly the same procedure described in (i) of the Materials and Methods, but using glucose- and vitamin-supplemented dialysed serum as a culture medium. At a concentration of 2-3 µg 125I-labelled PVP/ml, the pattern of uptake of radioactivity did not differ from that observed when whole serum was used as culture medium (see Fig. 1).

Discussion

125I-Labelled PVP is a macromolecule that cannot be digested by lysosomal enzymes. Experiments using 17-5-day rat yolk sac incubated in vitro (Williams et al. 1975a; Duncan & Lloyd, 1978) have demonstrated uptake of this substrate by pinocytosis and its progressive accumulation within the tissue. In the present experiments the accumulation of 125I-labelled PVP by the yolk sac demonstrates the pinocytic capacity of the 9-5- to 11-5-day rat yolk sac. The inability of 125I-labelled PVP to penetrate into the embryo indicates that embryonic cells do not capture the macromolecule directly and also that indigestible macromolecules captured by the yolk sac are immobilized within this tissue's lysosomes and are not transferred into the embryo.

The amount of radioactivity present in the yolk sac at harvesting depended on the length of time the conceptus had been exposed to 125I-labelled PVP in the culture medium. A linear relationship between amount captured and duration of exposure would not be expected, owing to the rapid growth of the tissue during the incubation period. Fig. 2 shows that relatively little of the radioactivity present at harvesting derives from uptake in the early phase of exposure: this is consistent with the relatively small amount of tissue present at this stage (Fig. 1).

In the 17-5-day rat yolk-sac uptake of 125I-labelled PVP (and of [14C]sucrose)
is by fluid-phase non-adsorptive pinocytosis (Williams et al. 1975a; Roberts, Williams & Lloyd, 1977). Since the rates of uptake of [3H]dextran and 125I-labelled PVP in the present experiments are quantitatively similar, when expressed as clearances (Figs. 2, 3), it is likely that adsorption to plasma membrane does not occur with either substrate in the 9.5- to 11.5-day yolk sac. This conclusion is reinforced by the observation that clearance of 125I-labelled PVP and [3H]-dextran appeared to be independent of substrate concentration.

When conceptuses were cultured in the presence of formaldehyde-denatured 125I-labelled BSA, there was little accumulation of radioactivity by the conceptus (Fig. 4), but a release of TCA-soluble radioactivity into the culture serum whose extent depended on duration of exposure to substrate. With the exception of very short exposures, the amount of radioactivity in the tissues did not depend on the duration of exposure. This pattern is reminiscent of the results obtained using 17.5-day rat yolk sac (Williams et al. 1975b; Moore et al. 1977) and indicates pinocytic uptake followed by intralysosomal proteolysis.

The radioactivity found in the yolk sac after incubations with 125I-labelled BSA was partly TCA-insoluble, indicating that the yolk sac is the likely site of pinocytosis and digestion. In contrast the extremely small amount of radioactivity in embryonic tissue was all TCA-soluble, suggesting it derives from the products of digestion within yolk sac lysosomes. [125I]Iodotyrosine cannot be used for protein synthesis and so cannot be incorporated into newly formed protein in either yolk sac or embryo.

If it is assumed that the yolk sac alone is responsible for the digestion of 125I-labelled BSA, it is possible to calculate the clearance of this substrate (Fig. 6) and compare it directly with the graphs for 125I-labelled PVP (Fig. 2) and [3H]dextran (Fig. 3). Apparently 125I-labelled BSA is captured some four times more rapidly, presumably because its pinocytosis is aided by membrane adsorption (Williams et al. 1975b; Moore et al. 1977).

When conceptuses were cultured in the presence of 3H-labelled serum proteins, the amount of radioactivity found in the yolk sac increased with length of exposure to 3H-labelled proteins. For very short exposures almost all the radioactivity was TCA-soluble, but the proportion that was protein-bound increased to 65% with exposures up to 12 h. Taken together with the failure of the yolk sac to accumulate intact 125I-labelled BSA, these data indicate that 3H-labelled proteins accumulating in the yolk sac derive from de novo synthesis from amino acids released from the lysosomes following pinocytic uptake and lysosomal degradation of 3H-labelled serum proteins.

The results from incubations with 125I-labelled PVP, [3H]dextran and formaldehyde-denatured 125I-labelled BSA all indicate that intact macromolecules are incapable of reaching the developing embryo from the culture serum. It is therefore particularly striking that conceptuses cultured in the presence of 3H-labelled serum proteins incorporate radiolabel into embryonic protein. The amount of radioactivity and the proportion that was TCA-insoluble increased
Yolk sac mediates protein nutrition in rat embryos in vitro

with duration of exposure. These data are consistent with the embryo drawing for its amino acid needs on the products of yolk-sac lysosomal digestion. The alternative explanation that serum proteins can be incorporated intact either directly or by a route through the yolk sac that avoids lysosomal action is much less plausible and is inconsistent with the decreasing proportion of TCA-soluble radioactivity in the embryo with increasing duration of exposure to ³H-labelled serum proteins.

If it is assumed that all the ³H-radioactivity found in both yolk sac and embryo derives from labelled protein pinocytosed by the yolk sac, the rate of clearance of ³H-labelled serum proteins may be calculated (Fig. 9) and compared with those calculated for the radiolabelled substrates used in earlier experiments. The rate is approximately twice that for ¹²⁵I-labelled PVP and [³H]dextran and one half that for formaldehyde-denatured ¹⁸⁵I-labelled BSA.

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S. J. Freeman, F. Beck and J. B. Lloyd


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