Digestion of an Exogenous Protein by Rat Yolk-Sac Cultured *in vitro*

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Yolk-sacs were removed from 17.5-day pregnant rats injected 2-5 h previously with ^{125}I -labelled bovine serum albumin. The specific activities of acid phosphatase and acid proteinase, and the specific radioactivities (trichloroacetic acid-insoluble and trichloroacetic acid-soluble) were measured in subcellular fractions prepared by homogenization and differential centrifugation. The conversion of acid-insoluble into acid-soluble radioactivity within cultured tissue was followed and the nature of the liberated products was investigated by gel chromatography. The results are consistent with the protein entering lysosomes and being digested there. The radiolabel was released chiefly as free iodotyrosine.

The embryos of certain mammals, including all rodents and lagomorphs, are surrounded by an inverted yolk-sac for a large part of gestation. Following Goldman's (1909) demonstration of the intense phagocytic capacity of rat yolk-sac towards azo dyes, numerous studies have demonstrated the ability of its epithelial cells to accumulate a wide variety of colloidal and macromolecular materials in membrane-bound vacuoles (Al-Abass & Schultz, 1966; Lambson, 1966; Schultz, Reger & Schultz, 1966; Krzyzowska-Gruca & Schiebler, 1967; Beck & Lloyd, 1968; Lloyd, Beck, Griffiths & Parry, 1968). The biological significance of this phagocytic activity is not known, although roles in embryonic nutrition (Beck & Lloyd, 1968), embryonic protection (Wilson, Beaudoin & Free, 1959) and, in certain species, the prenatal transmission of passive immunity (Brambell, 1970) have been proposed. Each of these hypotheses assumes that the yolk-sac can digest and not merely ingest macromolecules. There is circumstantial evidence in a number of studies that the yolk-sac can digest certain proteins after their endocytosis (Beck, Parry & Lloyd, 1970; Brambell, 1970) but conclusive proof of this has been lacking. In the present experiments, yolk-sacs were isolated from pregnant rats that had been injected intravenously with ^{125}I -labelled bovine serum albumin. The intracellular location of the ingested protein was investigated by centrifugal fractionation. Digestion of the protein within the yolk-sac was followed in organ cul-

ture and the products analysed by chromatography on Sephadex. A preliminary report of some of the work has been made (Williams, Lloyd & Beck, 1969).

MATERIALS AND METHODS

Chemicals. [^{125}I]Iodide (preparation IMS. 4, 2 mCi in 0.5 ml) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and bovine serum albumin (type II), L-tyrosine, 3-iodo-L-tyrosine, and 3,5-di-iodo-L-tyrosine were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Glycyl-L-tyrosine was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other chemicals were of analytical grade.

Preparation of acid-denatured ^{125}I -labelled albumin. The method was modified from that of Bocci (1969). Bovine serum albumin (5 mg) was dissolved in 5.0 ml of 0.05 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 8.0, cooled to 0°C and sodium [^{125}I]iodide (2 mCi) added. After stirring for 2 min, chloramine-T (1 mg in 5.0 ml of water) was added and the mixture stirred for a further 8 min, when reaction was stopped by the addition of sodium metabisulphite (1 mg in 5.0 ml of water). Sodium iodide (50-100 mg) was added and the pH adjusted to 3.5 with acetic acid. The solution was maintained at 37°C for 1 h and then freed from inorganic iodide by dialysis (48 h at 4°C) in Visking tubing against three changes of 1% (w/v) NaCl. In the resulting solution, which contained 0.25-0.40 mg of protein/ml, only 0.5-2.0% of the total radioactivity remained soluble when precipitated by trichloroacetic acid in the presence of carrier albumin. The preparation was deep-frozen in 2 ml lots in sterile bottles. Storage for up to 8 weeks did not lead to any detectable increase in the acid-soluble radioactivity.

Preparation of ^{125}I -labelled 3-iodo-L-tyrosine and ^{125}I -labelled glycyl-3-iodo-L-tyrosine. L-Tyrosine and glycyl-L-tyrosine were labelled with [^{125}I]iodide by the method described above for the iodination of albumin, and the products were freed from iodide by chromatography on Sephadex G-25 (see below). Both compounds were shown to remain soluble when precipitation of protein

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was performed by the addition of carrier protein and trichloroacetic acid.

Determination of protein. This was carried out by the method described by Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as reference protein.

Enzyme assays. Acid phosphatase was assayed at pH 5.0 by using *p*-nitrophenylphosphate (10 mM) as substrate (Torriani, 1960) and pre-incubating the fractions for 10 min to inactivate microsomal phosphatases as described by Neil & Horner (1964). Acid proteinase was assayed at pH 3.8 by using haemoglobin (2%, w/v) as substrate, the released tyrosine being determined by the method of Anson (1937).

Assays for radioactivity. Samples (1 ml) were treated with aq. 20% (w/v) trichloroacetic acid (0.5 ml) and centrifuged at low speed to sediment the precipitated protein. The total radioactivity was then measured in a Nuclear-Chicago γ -spectrometer. Tubes were then centrifuged at 2000g for 20 min, the clear supernatant was decanted and its radioactivity measured. This value is referred to below as the acid-soluble radioactivity and the difference between this value and the total radioactivity is designated the acid-insoluble radioactivity.

Centrifugal fractionation of rat visceral yolk-sac. Wistar rats (200–250 g), from an inbred laboratory colony, were brother-sister mated overnight. If sperm were detected in the vagina the following morning, pregnancy was timed from midnight of the night of mating. At 17.5 days a solution of ^{125}I -labelled albumin (2.5 ml/kg body wt.) was injected into the femoral vein and conceptuses removed under ether anaesthesia 3.5 h later. (Preliminary experiments had shown that the specific trichloroacetic acid-insoluble radioactivity in the yolk-sac reached a peak 2–5 h after injection.) Immediately after removal from the animal the conceptuses were placed in cold 0.25 M-sucrose (adjusted to pH 7.4 with NaHCO_3) and the whole yolk-sacs dissected free. The yolk-sacs (12) were homogenized in 0.25 M-sucrose by using a Potter-Elvehjem-type Teflon-glass homogenizer. The homogenate was then diluted to 20 ml and fractionated by differential centrifugation. The fractionation scheme used (Lloyd & Beck, 1969) yields five particulate fractions, isolated with successively higher centrifugal forces, and a final supernatant (S). The five fractions are designated first nuclear (N_1 , isolated at 1500g-min), second nuclear (N_2 , 10000g-min), heavy mitochondrial (M, 33000g-min), light mitochondrial (L, 326000g-min) and microsomal (P, 300000g-min). Each pellet was resuspended in 0.25 M-sucrose and all fractions were assayed for protein, acid phosphatase, acid proteinase, total radioactivity and acid-insoluble radioactivity.

Organ culture of rat visceral yolk-sac. Pregnant rats were injected with ^{125}I -labelled albumin as described above. Conceptuses were removed 2–3 h after injection, placed in cold medium TC 199 (preparation TC 20; Wellcome Reagents Limited, Beckenham, Kent, U.K.) and the proximal part of each yolk-sac was dissected free and cut into two portions of approximately equal size, as described by Beck *et al.* (1970). One portion was laid flat, villous surface uppermost, on a tantalum gauze raft for culture by the technique of Beck *et al.* (1970). When three such explants had been placed on one raft, the cold medium was removed and the raft quickly rinsed with

warm (37°C) medium TC 199 previously gassed with air+CO₂ (95:5). A further 5 ml of the same medium was then added and the culture vessel placed in a desiccator containing air+CO₂ (95:5) and maintained at 37°C. The second (control) portions of the three yolk-sacs were held in cold medium TC 199 until incubation of the corresponding explanted portions had begun. Controls were then rinsed in fresh medium TC 199 and deep frozen.

At 0.5 h intervals the desiccator was opened, a sample (1.0 ml) of medium removed from the culture dish and immediately replaced by an equal volume of warm, fresh medium from a dish held in the same desiccator. The sample of medium was divided into two 0.5 ml portions and to each was added 0.5 ml of carrier protein solution (4 mg of bovine serum albumin/ml) before assay for total and acid-insoluble radioactivity.

After 3.5 h incubation the explants were removed from the raft, washed for 2 min in 1% NaCl at 4°C and deep-frozen until assay. After thawing they were homogenized in water (2 ml) for 6 min by using a Virtis homogenizer at top speed. The homogenate was diluted to 5.0 ml with water and portions (1.0 ml) were assayed for total and acid-soluble radioactivity. Further portions of the homogenate were assayed for protein.

Examination of radioactive material released from yolk-sacs into culture medium. (a) Separation on Sephadex G-25. A method described by Mougey & Mason (1963) was slightly modified. Sephadex G-25, hydrated under sodium acetate (0.02 M) was packed into a column (40 cm $\times$ 1.8 cm diam.). Marker compounds (^{125}I iodide, ^{125}I -labelled albumin, L-tyrosine, ^{125}I iodo-L-tyrosine, 3-iodo-L-tyrosine, 3,5-di-iodo-L-tyrosine and glycyl ^{125}I -iodo-L-tyrosine) were applied to the column in water or 0.05 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 7.0. Culture media were centrifuged at 2000g for 20 min before application. Sodium acetate (0.02 M) was used as eluent (flow rate 20 ml/h) and 2 ml fractions were collected and assayed for either radioactivity or the colour developed with the procedure described for protein determination by Lowry *et al.* (1951). In the latter assay it was shown that tyrosine, iodotyrosine and di-iodotyrosine gave colour yields (at 540 nm) proportional to concentration. Recovery (%) of radioactive compounds was calculated from the residual activity on the column gel.

(b) Separation on a copper complex of Sephadex G-25. In the method of Fazakerley & Best (1965) copper hydroxide is precipitated in Sephadex G-25 beads, so that amino acids chelate with copper ions and their elution is retarded relative to dipeptides, giving a substantial separation of the two. The amount of CuSO_4 used in preparing the complex was decreased to 70% of that used in the original method, since this was found to improve resolution. The column was eluted with 0.05 M-sodium tetraborate-sodium hydroxide buffer, pH 11.0, for the first 20 ml fractions and thereafter with HCl (0.2 M). Compounds in the eluate were detected by their radioactivity and recoveries were calculated as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the distribution of acid phosphatase, acid proteinase and of acid-soluble and acid-insoluble radioactivity in the various centrifugal

Fig. 1. Distribution of (a) acid phosphatase, (b) acid proteinase, (c) acid-soluble radioactivity and (d) acid-insoluble radioactivity in subcellular fractions of yolk-sac removed from pregnant rats injected intravenously with ^{125}I -labelled bovine serum albumin. Graphical presentation as described by de Duve *et al.* (1955).

fractions of yolk-sac obtained from rats injected with labelled protein. The results are presented in the manner described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The distribution of the two lysosomal enzymes is similar to that reported for 13-day (Schultz, 1969) and 15-day (Lloyd *et al.* 1968; Lloyd & Beck, 1969) rat yolk-sac, and is consistent with the heterogeneity of lysosome size in this tissue that is indicated by morphological studies (see Lloyd *et al.* 1968, for discussion). The close parallel between the profiles for acid-insoluble radioactivity and acid proteinase suggests strongly that the two are associated with the same subcellular organelle, namely the lyso-

some. Acid-soluble radioactivity, which represents the digestion products of labelled protein, is found predominantly in the S fraction, with amounts in the particulate fractions that correlate with the acid proteinase activity and acid-insoluble radioactivity. This is consistent with digestion taking place in lysosomes and the low-molecular-weight products diffusing into the cytosol through the lysosomal membrane.

The radioactivity released into the medium from explants containing ^{125}I -labelled albumin was calculated by the expression:

$$T_n = 5C_{i(i-n)} + \sum_{i=1}^{i=n-1} C_i$$

where T_n is the radioactivity (c.p.m.) released up to the time of the n th sampling, C_i the c.p.m./ml of medium of the i th sample, corrected for background. Values for specific radioactivity in the medium are expressed as c.p.m./mg of protein in the cultured yolk-sac tissue. Fig. 2 depicts the progressive release of radioactivity from a typical explant (expt. 2 of Table 1). Most of the radioactivity is acid-soluble, and the rate of its release much decreased after 3.5 h culture. Table 1 shows the results of three separate experiments and compares specific radioactivities after culture for 3.5 h with those in uncultured controls. Approximately two-thirds of the radioactivity in the control yolk-sacs was acid-insoluble, i.e. macromolecular, whereas after culture approximately two-thirds of the total radioactivity in the whole system was acid-soluble. Calculation of the precise extent to which acid-insoluble radioactivity is converted into acid-soluble radioactivity is complicated by discrepancies in total specific radioactivities as between cultured systems and corresponding controls. Table 1 (column 7) shows that the ratio of these two values is greater than 1. The reason for this is not known, but the result would be explained if, as indicated by earlier work (Beck *et al.* 1970), explants lose protein by autophagic cytoplasmic reduction during culture. To make a calculation possible, values for specific radioactivities in the control explants have been multiplied by a factor that makes the total specific radioactivities in the two series identical. The adjusted values are given in *italics* in Table 1, and are used to calculate the percentage of the initially insoluble radioactivity rendered soluble during culture. This value (last column of Table 1) is approx. 50%.

Fig. 3 shows the elution of post-culture medium from Sephadex G-25; 92% of the activity applied to the column appeared in the eluate. The elution positions of marker compounds in the same system are also shown. Recoveries of 3[125 I]-iodo-L-tyrosine and glycyl[125 I]iodotyrosine were 96.9% and 98.3% respectively. Three peaks are seen with culture medium: two minor peaks in positions corresponding to 125 I-labelled albumin and iodide, and the major component which appears in a

Fig. 2. Progressive release of radioactivity into medium on culturing rat yolk-sac that had ingested 125 I-labelled bovine serum albumin *in vivo*. ■, Total radioactivity; ○, radioactivity soluble in trichloroacetic acid.

Table 1. *Change in nature and distribution of radioactivity on culturing yolk-sacs containing ingested 125 I-labelled bovine serum albumin*

Values are expressed as specific radioactivities (c.p.m./mg of yolk-sac protein). The values in *italics* have been adjusted to allow for the change in total specific radioactivity during culture (see the text for details).

Expt. no.	Nature of radioactivity	Specific radioactivity				Cultured/control sp. radioactivity ratio	% Hydrolysis of insoluble activity in control
		Culture system (after 3.5 h)			Control (deep-frozen) tissue		
		Medium	Tissue	Whole system			
1	Soluble	743	78	821	313 (374)	1.20	53.5
	Insoluble	217	172	389	700 (836)		
	Total	960	250	1210	1013 (1210)		
2	Soluble	685	110	795	330 (454)	1.38	45.4
	Insoluble	130	280	410	545 (751)		
	Total	815	390	1205	875 (1205)		
3	Soluble	490	63	553	260 (263)	1.01	58.4
	Insoluble	80	127	207	490 (497)		
	Total	570	190	760	750 (760)

Fig. 3. Elution of post-culture medium from Sephadex G-25. Elution positions of marker compounds in the same system are indicated. Fractions indicated by shaded area were pooled for subsequent fractionation on the copper complex of Sephadex G-25.

Fig. 4. Elution from the copper complex of Sephadex G-25 of pooled fractions 73-93 from the separation shown in Fig. 3. Elution positions of marker compounds in the same system are indicated.

position where both iodotyrosine and glycylidotyrosine elute. Fractions 73-93, representing this major peak, were pooled and concentrated by freeze-drying before application to a column of the copper complex of Sephadex G-25. The copper complex of Sephadex G-25 resolved glycyl[125 I]-

iodo-L-tyrosine and [125 I]iodo-L-tyrosine into two well-separated peaks in fractions 30-50 and 90-120 respectively. Corresponding recoveries were 98.9% and 99.2%. Fig. 4 shows the elution pattern of the fractions from the culture medium that eluted from Sephadex G-25 in fractions 73-93. The

recovery was 98.4% of the radioactivity applied to the column, 86% of the recovered radioactivity appearing in the position corresponding to [125 I]-iodotyrosine. The remaining 14%, which was eluted in the position corresponding to glycyl-iodotyrosine, is presumably a mixture of small peptides containing iodotyrosine; these would be expected to elute with the glycyl dipeptide.

The results show that 125 I-labelled albumin is subject to digestion after uptake *in vivo* into the rat visceral yolk-sac. [125 I]iodotyrosine appeared to be the major labelled product, indicating that the tyrosyl residues, at least, were largely hydrolysed to free amino acids. Similar results have been obtained in analogous studies with cultured mouse macrophages (Ehrenreich & Cohn, 1967, 1968; Unanue & Askonas, 1968) and Sarcoma S180 cells (Gabathuler & Ryser, 1969). The site of digestion of exogenous proteins in yolk-sac may confidently be supposed to be the lysosomes, both because other foreign proteins, such as horseradish peroxidase, have been shown to congregate there (Beck & Lloyd, 1968) and because of the results of differential centrifugation presented here. Coffey & de Duve (1968) have demonstrated the ability of the hydrolases from rat liver lysosomes to digest acid-denatured bovine serum albumin. The origin of the small amount of iodide found in post-culture media is uncertain. It may originate in iodide present in the 125 I-labelled albumin preparation or may be produced by deiodination of labelled protein in some tissue of the rat before death. Subsequent experiments with yolk-sac cultured in medium containing 125 I-labelled albumin suggest that the yolk-sac tissue cannot itself remove iodide from this substrate (K. E. Williams, unpublished work).

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