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Hydrolysis of an Exogenous ^{125}I-Labelled Protein by Rat Yolk Sacs

EVIDENCE FOR INTRACELLULAR DEGRADATION WITHIN LYSOSOMES

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When added to the serum-free medium in which 17.5-day rat yolk sacs were incubated, formaldehyde-denatured ^{125}I-labelled bovine serum albumin was rapidly degraded. More than 80% of the radiolabelled digestion products appearing in the incubation medium consisted of [^{125}I]iodo-L-tyrosine; larger digestion products were found only in association with the yolk-sac tissue. In the early stages of an incubation, low-molecular-weight digestion products began to appear in the incubation medium only after they could be detected within the tissue, and progressive association of trichloroacetic acid-insoluble radioactivity with the tissue preceded both these events. None of the observed proteolysis could be attributed to proteinases released into the incubation medium. Tissue-associated acid-insoluble radioactivity showed a lysosomal distribution on subcellular fractionation, and cell-free homogenates of yolk sacs degraded albumin only at acid pH values. Progressively decreasing the rate of pinosome formation (either by progressively lowering the incubation temperature or by the use of increasing concentrations of the metabolic inhibitor rotenone) caused a corresponding decrease in the rate of degradation of albumin. These findings indicate that, *in vitro*, formaldehyde-denatured ^{125}I-labelled bovine serum albumin is digested by rat yolk sacs exclusively intracellularly, within lysosomes.

Many reports indicate that on adding radioiodinated proteins to the medium in which mammalian cells are cultured, low-molecular-weight digestion products accumulate progressively in the culture medium (see e.g. Ehrenreich & Cohn, 1967; Gabathuler & Ryser, 1969; Kirsch *et al.*, 1972; Williams *et al.*, 1975*b*; Goldstein *et al.*, 1975; Terris & Steiner, 1975; Stein *et al.*, 1976; Pratten *et al.*, 1978; Nilsson & Berg, 1977; Tolleshaug *et al.*, 1977). In the majority of these studies, the digestion of the exogenous protein has been assumed to occur intralysosomally. Although intralysosomal digestion of proteins is undoubtedly of major importance, a possible contribution from extracellular proteolysis cannot be dismissed. Indeed, Tökes & Sorgente (1976) and Tokes & Csipke (1977) claim to have observed the extracellular digestion of ^{125}I-labelled casein (bound to large latex beads) in several cell types, namely T-lymphocytes, macrophages, bovine aorta endothelial cells, normal and transformed quail fibroblasts and normal and carcinogen-treated liver epithelial cells. Moreover, digestion of ^{125}I-labelled peptide hormones by extracellular membrane-bound peptidases has been suggested by a number of authors: insulin (Freychet *et al.*, 1972) and possibly glucagon (Pohl *et al.*, 1972) by liver plasma membranes; corticotropin by crude preparations of adrenal-cortex membranes (Saez *et al.*, 1975); calcitonin (Marx *et al.*, 1973), glucagon and possibly insulin (Kerr & Kenny, 1974) by kidney plasma membranes. The microvilli of the rat yolk sac may, like those of the kidney proximal tubule (see Kenny, 1977), carry significant quantities of neutral endopeptidases on their outer surface, and such enzymes could possibly make a significant contribution to the overall catabolic activity of the rat yolk sac. Moore *et al.* (1977) briefly outline certain circumstantial evidence that suggests that lysosomes are involved in the digestion of proteins when incubated with 17.5-day rat yolk sacs, but there is no conclusive evidence to show that ^{125}I-labelled proteins are digested exclusively intracellularly by this tissue.

Formaldehyde-denatured ^{125}I-labelled bovine serum albumin has been used by several workers (Moore *et al.*, 1974, 1977; Roberts *et al.*, 1976; Pratten *et al.*, 1978; Duncan & Lloyd, 1978; Ibbotson & Williams, 1979) in quantitative studies of pinocytosis by the rat yolk sac using the methods originally described by Williams *et al.* (1975*a*,*b*). In all the above-mentioned studies the quantity of ^{125}I-labelled albumin captured by pinocytosis at a given time was calculated by summing the quantity of substrate accumulated in the yolk-sac tissue and the quantity of substrate-digestion products appearing in the incubation medium during the same incubation period. Calculation of the rate of pinocytic capture of

the digestible protein in this manner requires the albumin to be digested exclusively intracellularly, but, to date, conclusive evidence of this has been lacking.

Materials and Methods

Sodium [^{125}I]iodide (preparation IMS.4) and ^{125}I-labelled polyvinylpyrrolidone (preparation IM.33P) were products of The Radiochemical Centre, Amersham, Bucks., U.K. Tissue-culture medium 199 and heat-inactivated calf serum (products TC 20 and CS 07) were from Wellcome Reagents, Beckenham, Kent, U.K. Bovine serum albumin (preparation 0124t) was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. L-Tyrosine, glycyl-L-tyrosine and rotenone were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

All other chemicals were of analytical grade.

Preparation of ^{125}I-labelled compounds

Formaldehyde-treated ^{125}I-labelled bovine serum albumin was prepared by the method described by Moore *et al.* (1977); L-tyrosine and glycyl-L-tyrosine were both labelled with ^{125}I and separated from free ^{125}I on columns of Sephadex G-25 as described by Williams *et al.* (1971). These products were stored at −20°C.

Analysis of low-molecular-weight ^{125}I-labelled species

Radioactive species present in the incubation media (centrifuged for 5000*g*-min) were examined both on columns (35cm × 1.7cm) of Sephadex G-25 and on similar columns of the copper complex of Sephadex G-25, by using the methods described by Williams *et al.* (1971).

Yolk-sac-associated radioactivity was analysed in the same manner after homogenizing the tissue (three yolk sacs in 5ml of water) in a hand-operated Potter–Elvehjem-type glass/glass homogenizer.

Assay of proteolytic activity in a cell-free yolk-sac homogenate

A cell-free homogenate of 17.5-day yolk-sac tissue was prepared by homogenizing yolk sacs (ten in 10ml of water) in the above manner, diluting the homogenate with 20ml of water and centrifuging (1500*g*-min) to remove tissue debris. To portions of the centrifuged homogenate (50μl; containing approx. 150μg of protein) were added 130μl of an appropriate buffer solution and 20μl of a solution of formaldehyde-denatured ^{125}I-labelled albumin (0.5mg/ml). (Over the range pH 3.0–6.0, 0.1M-sodium acetate/acetic acid was used as buffer, but over the range pH 6.5–9.0 this was replaced by 0.1M-Tris/HCl.)

After incubating the mixture for 1h at 37°C the reaction was stopped by the addition of 0.5ml of aq. 20% (v/v) calf serum, immediately followed by 0.5ml of aq. 20% (w/v) trichloroacetic acid. After centrifugation, the acid-soluble radioactivity was measured. Blanks, at each pH, contained water in place of yolk-sac homogenate.

Rate of pinocytic uptake of ^{125}I-labelled polyvinylpyrrolidone

The rate of uptake of ^{125}I-labelled polyvinylpyrrolidone, from serum-free medium 199, by 17.5-day yolk sacs was determined by using the method described by Ibbotson & Williams (1979).

Rate of digestion and rate of uptake of a ^{125}I-labelled protein

The rates of uptake and digestion were determined either by the method of Ibbotson & Williams (1979), or by a modified version of this method that enabled data to be obtained by using a single yolk sac, incubated in a 50ml flask as in the original method (Williams *et al.*, 1975*b*), but in 19.0ml of serum-free medium 199. To the flask was added 1.0ml of medium containing ^{125}I-labelled albumin (20μg) the flask was re-gassed (O_2/CO_2, 19:1), sealed and incubated at 37°C. Portions of medium (0.5ml) were removed at regular intervals over an 0.5–3h period, the flask being re-gassed after each sampling. At the end of the incubation period a final portion of medium was removed and the tissue washed, then assayed for radioactivity and protein content as described by Williams *et al.* (1975*b*). Acid-soluble radioactivity associated with the yolk-sac tissue was assayed after adding 0.1ml of calf serum and 1.0ml of 20% trichloroacetic acid to a 1.0ml portion of the alkaline yolk-sac solution (Williams *et al.*, 1975*b*).

To each 0.5ml sample of medium was added 0.5ml of 20% calf serum, then the sample was assayed for both total and trichloroacetic acid-soluble radioactivity.

T_n, the quantity of acid-soluble radioactivity (c.p.m.) appearing in the incubation medium, by the time of the n^{th} sampling, was calculated by using the expression:

$$T_n = V_{i(i=n)} \cdot 2C_{i(i=n)} + \sum_{i=1}^{i=n-1} C_i$$

where V_i is the volume (ml) of the incubation medium remaining in the flask at the time of the i^{th} sampling and C_i is the acid-soluble radioactivity (c.p.m.) contained in the i^{th} sample (volume 0.5ml). Each value of C_i was corrected for: (*a*) background radioactivity, (*b*) acid-soluble radioactivity initially present in the ^{125}I-labelled protein preparation, (*c*) altered counting geometry (see Ibbotson & Williams, 1979)

and (*d*) loss of acid-soluble radioactivity occluded in the protein precipitate. By plotting T_n against sampling time, the rate of hydrolysis of the protein was obtained.

If the above hydrolysis occurs entirely intracellularly after pinocytic capture of the protein, then Q, the total quantity of ^{125}I-labelled protein captured by unit quantity of yolk-sac tissue (ng of ^{125}I-labelled protein per mg of yolk-sac protein) by the end of the overall incubation period, can be calculated from the expression:

$$Q = \frac{S}{P} \cdot \frac{(T+Y)}{M}$$

where T is the total amount of acid-soluble radioactivity (c.p.m.) appearing in the incubation medium during the overall incubation period and Y is the quantity of radioactivity (c.p.m.) associated with the yolk-sac tissue at the end of the same period. S is the initial concentration of substrate in the incubation medium (ng/μl), M is the mean quantity of acid-insoluble radioactivity in the medium over the incubation period (c.p.m./μl) and P is the protein content of the yolk sac (mg). The mean rate of putative uptake of ^{125}I-labelled protein over the incubation period is Q/t, where t is the duration of the incubation period (h).

Hydrolysis of ^{125}I-labelled albumin by conditioned incubation medium

Medium in which yolk sacs had previously been incubated for 3h was centrifuged (1500*g*-min) to remove any detached yolk-sac cells, then ^{125}I-labelled albumin (1μg/ml) was added and the medium re-incubated at 37°C for a further 2h before determining the amount of acid-soluble radioactivity present.

Distribution of radioactivity in subcellular fractions

Yolk sacs were incubated in serum-free medium 199 containing ^{125}I-labelled albumin (5μg/ml) for 2h, then removed and washed for 2min in three changes of ice-cold NaCl (1%, w/v), then homogenized and the distribution of acid-soluble and -insoluble radioactivity in subcellular fractions determined by the methods used by Williams *et al.* (1971).

Results

Production and characterization of the hydrolysis products of ^{125}I-labelled albumin

In agreement with the findings of Moore *et al.* (1977) and Ibbotson & Williams (1979), on incubating yolk sacs with formaldehyde-denatured ^{125}I-labelled bovine serum albumin the quantity of acid-soluble hydrolysis products in the incubation medium increased progressively with time over a 6h incubation period. Analysis of the radioactivity in the medium at 0, 1, 2, 3 and 6h indicated that the majority of the acid-soluble products eluted from Sephadex G-25 in the same position as [^{125}I]iodo-L-tyrosine, but there was also a minor peak corresponding to ^{125}I (Fig. 1*a*). In common with the major peak, the minor peak increased in size as the incubation period increased, but this minor peak never accounted for more than 5% of the total acid-soluble radioactivity in the medium. Where comparisons were made, the degree of hydrolysis calculated from the rise in acid-soluble products in the medium closely corresponded to that calculated from the relative amounts of radioactivity in the void volume and other fractions obtained from chromatography on Sephadex G-25.

Further analysis of the non-iodide acid-soluble radioactivity present in the medium at 3h, by using the copper complex of Sephadex G-25 (Fazakerley & Best, 1965), confirmed that 89% consisted of [^{125}I]iodo-L-tyrosine. In contrast, there was no detectable production of acid-soluble radioactivity on incubating formaldehyde-denatured ^{125}I-labelled bovine serum albumin (1μg/ml of medium) for 2h at 37°C, either in fresh medium 199 or in conditioned medium in which yolk sacs had previously been incubated for 3h; rates of hydrolysis were in both cases less than 0.1%/h.

Yolk sacs that had been incubated with ^{125}I-labelled albumin (2h), washed in fresh medium, then re-incubated in medium containing no ^{125}I-labelled albumin, liberated hydrolysis products that eluted almost entirely in the position of [^{125}I]iodo-L-tyrosine (Fig. 1*c*), and there was no evidence for the presence of large radiolabelled polypeptides. However, in homogenates of yolk sacs previously incubated with ^{125}I-labelled albumin, approx. 60% of the tissue-associated radioactivity was acid-insoluble and the majority eluted in the same position as ^{125}I-labelled albumin, but there was also evidence of the presence of polypeptides eluting between the void volume and ^{125}I (Fig. 1*b*).

Initial events during an incubation

A study of the initial events, on incubating ^{125}I-labelled albumin with yolk sacs by using the method of Ibbotson & Williams (1979), showed that acid-soluble hydrolysis products first appeared in the incubation medium in detectable amounts only after a period of 12–15min, but, by 25min, their rate of production became constant (4.4ng/min per mg of yolk-sac protein). However, acid-soluble digestion products could be detected in the tissue before 10min, and detectable amounts of acid-insoluble radioactivity accumulated in the tissue immediately on adding the labelled protein to the incubation medium (Fig. 2). Moreover, the maximum rate of accumulation of acid-insoluble radioactivity by the tissue in

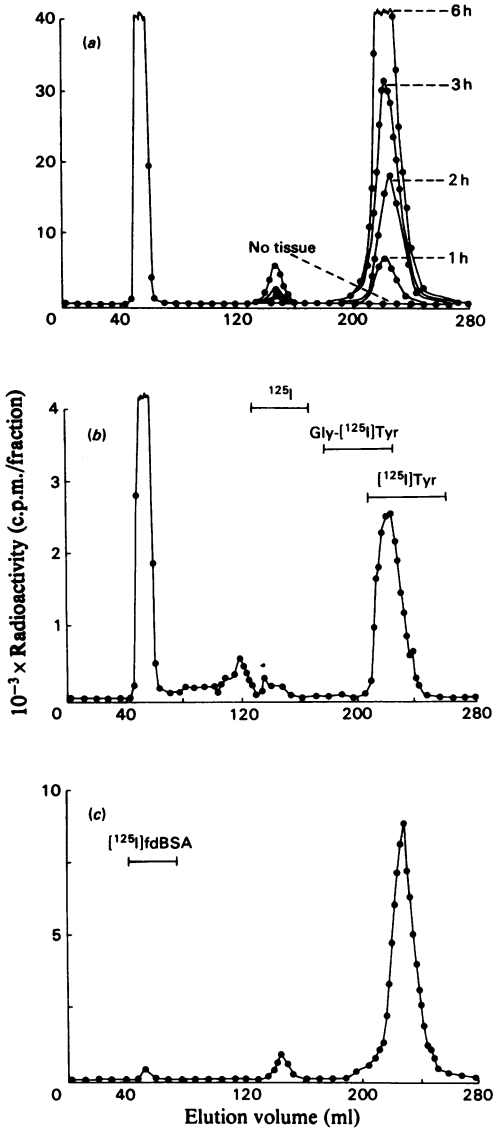

Fig. 1. *Sephadex G-25 chromatography of formaldehyde-denatured ^{125}I-labelled bovine serum albumin and its hydrolysis products*

Experiments were performed as described in the Materials and Methods section. The elution positions of formaldehyde-denatured ^{125}I-labelled bovine serum albumin ([^{125}I]fdBSA), ^{125}I, [^{125}I]iodo-L-tyrosine and glycyl[^{125}I]iodo-L-tyrosine are indicated by the horizontal bars. (*a*) Medium containing ^{125}I-labelled albumin after incubation in the absence of yolk-sac tissue for 2h or with a single yolk sac for 1, 2, 3 or 6h. (*b*) ^{125}I-labelled species associated with the yolk-sac tissue after incubation with ^{125}I-labelled albumin for 2h. (*c*) ^{125}I-labelled species released on reincubating (2h) yolk sacs that previously had been incubated with ^{125}I-labelled albumin.

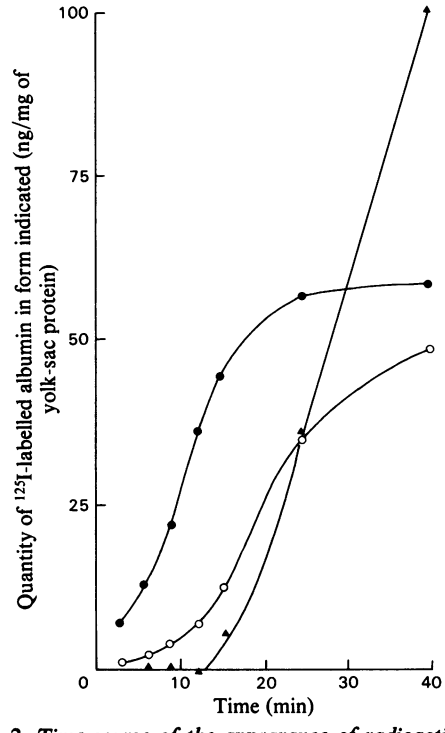

Fig. 2. *Time-course of the appearance of radioactivity in the yolk-sac tissue and acid-soluble radioactivity in the incubation medium when yolk sacs were incubated with ^{125}I-labelled albumin*

Yolk sacs were incubated separately in serum-free medium 199 (20ml) containing ^{125}I-labelled albumin (1 μg/ml) as described by Ibbotson & Williams (1979); ●, acid-insoluble, and ○, acid-soluble, radioactivity associated with the tissue; ▲, acid-soluble radioactivity in the incubation medium.

the initial 15min period (4.3ng/min per mg of yolk-sac protein), was almost identical with the constant rate of appearance of the acid-soluble products in the incubation medium beyond the initial 20min. By 1.0–1.5h, although the acid-soluble products in the medium continued to increase steadily with time, the quantities of acid-soluble and -insoluble radioactivity associated with the yolk-sac tissue became constant (results not shown); this finding is in agreement with those of Moore *et al.* (1977) and Ibbotson & Williams (1979).

Calculation of the concentration of acid-soluble products in the tissue (assuming the specific gravity of the tissue to be 1.0 and that its whole volume was available to acid-soluble products), and comparison of this estimated value with the concentration of acid-soluble products in the medium at 40min, showed the tissue concentration to exceed that in the medium by a factor of approx. 200.

Subcellular distribution of radioactivity and acid-proteinase activity

When a homogenate prepared from yolk sacs that had accumulated ^{125}I-labelled albumin was resolved into five particulate fractions and a final supernatant by differential centrifugation, the distributions of acid-proteinase activity, acid-insoluble and -soluble radioactivity were virtually identical with those described by Williams *et al.* (1971). The acid-insoluble radioactivity was mostly particle-associated and its distribution was very similar to that of acid-proteinase activity. The majority of the acid-soluble radioactivity was found in the final supernatant, but the portion that was associated with the particulate fractions showed a similar distribution to acid-proteinase activity.

Hydrolysis of ^{125}I-labelled albumin by a cell-free homogenate

A cell-free homogenate of yolk-sac tissue, when buffered to an acid pH, rapidly hydrolysed formaldehyde-treated ^{125}I-labelled albumin and showed a very sharp optimum at pH4.0 (Fig. 3). At a pH of 6.5 and above there was no detectable proteinase activity, suggesting that such homogenates contain little or no enzymes capable of degrading ^{125}I-labelled albumin at the pH of the incubation medium (pH7.1).

Effects of rotenone and incubation temperature on the rate of hydrolysis of labelled albumin by intact yolk sacs

Yolk sacs, incubated at 37°C in serum-free medium 199 with formaldehyde-denatured ^{125}I-labelled bovine serum albumin, caused a linear increase with time in the quantity of low-molecular-weight digestion products present in the medium in the interval beyond the initial lag period. The mean value of the

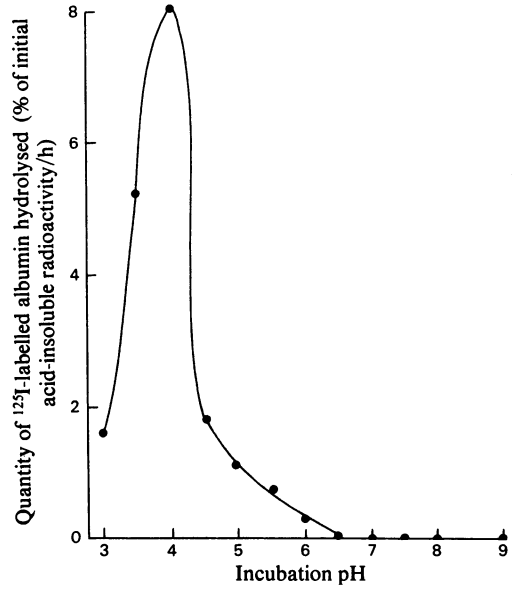

Fig. 3. *Effect of pH on the rate of production of acid-solubles from ^{125}I-labelled albumin by a cell-free extract of 17.5-day yolk sacs*
Incubations were performed as described in the Materials and Methods section. Each point represents the mean value for four individual determinations each using a single preparation of cell-free yolk-sac homogenate.

Table 1. *Effects of incubation temperature on the rate of uptake of ^{125}I-labelled polyvinylpyrrolidone and on both the rate of digestion and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin by 17.5-day yolk sacs*
Each value of the rate of uptake of ^{125}I-labelled polyvinylpyrrolidone ([^{125}I]PVP) was derived from a plot of uptake against time obtained by using six to eight yolk sacs taken from a single animal and incubated for up to 3h in serum-free medium 199 as described by Ibbotson & Williams (1979), but at various temperatures. Each value of the rate of digestion of ^{125}I-labelled albumin ([^{125}I]albumin) was derived from data, obtained from two yolk sacs incubated separately (see the Materials and Methods section), by determining the gradient of the acid-soluble-products plot over the interval 0.5–3.0h. Individual values of the putative rate of uptake were obtained from the sum of the quantity of acid-soluble radioactivity released into the medium over 3.0h and the amount of radioactivity associated with the tissue at 3.0h (see the Materials and Methods section). Mean values of the rates (±S.D.) are expressed as a percentage of the corresponding rate at 37°C for the numbers of experiments indicated.

Incubation temperature (°C)	No. of expts.	Relative rates: Uptake of [^{125}I]PVP	Digestion of [^{125}I]albumin	Putative uptake of [^{125}I]albumin
37	4	100.0± 5.9	100.0±14.4	100.0±11.8
34	4	88.9± 9.2	72.7±17.2	78.0±19.5
30	3	51.4± 8.9	36.3± 1.8	42.6± 3.3
25	3	15.6±10.1	11.7± 1.7	20.7±11.0
15	5	0.7± 5.8	0.1± 0.7	3.0± 1.0

rate of uptake of ^{125}I-labelled albumin, expressed as an Endocytic Index [i.e. the volume of medium whose contained substrate is captured/h by unit quantity of yolk-sac tissue (Williams *et al.*, 1975*b*)] was 345.4±49.7 (S.D.) μl/h per mg of yolk-sac protein. In corresponding experiments the non-digestible substrate, ^{125}I-labelled polyvinylpyrrolidone, was accumulated linearly with time by the tissue and showed a mean Endocytic Index of 3.6±0.2 (S.D.) μl/h per mg of yolk-sac protein. Both values are in close agreement with those reported by Ibbotson & Williams (1979).

Progressively lowering the incubation temperature from 37°C did not affect the linearity of the individual plots, either for the pinocytic uptake of the synthetic polymer or for digestion of the labelled albumin over a 3h period, but the gradients of the plots (i.e. the rate of uptake or the rate of digestion) were progressively decreased in a parallel manner (Table 1), so that at 15°C both processes were effectively fully inhibited.

A similar pattern of results was obtained for incubations performed at 37°C but in the presence of increasing concentrations of the metabolic inhibitor rotenone (Table 2). At the highest concentration of rotenone used (10μM), 85–90% inhibition of both processes was observed.

Discussion

When cells or tissues are incubated with radiolabelled proteins, most investigators uncritically attribute any observed proteolysis to lysosomal enzymes acting on the protein after its endocytic capture. Although, in such studies, subcellular-fractionation procedures can give evidence of the protein having entered the lysosomal compartment, and so suggest that at least some proteolysis occurs at this site, it is important in quantitative studies of endocytosis of proteins to establish whether this is the sole site of proteolysis in a particular tissue, since any extracellular degradation will lead to an overestimate of the rate of endocytic capture calculated by compounding the rate of accumulation of a protein by the tissue and the rate of appearance of digestion products in the incubation medium (Williams *et al.*, 1975*b*). It is relatively easy to identify and quantify any proteolysis caused by enzymes either present in serum components of the medium or liberated by the tissue during incubation, and, in the present investigation, such hydrolysis within the medium itself was of no significance. It is, however, more difficult to distinguish intralysosomal proteolysis from proteolysis caused by neutral peptidases, attached to the extracellular surface of the plasma membrane.

The finding that the subcellular distribution of acid-soluble and -insoluble species derived from formaldehyde-denatured ^{125}I-labelled bovine serum albumin, in yolk sacs that had been incubated with this protein, was essentially the same as previously reported (Williams *et al.*, 1971; Goetze *et al.*, 1976) suggested that at least some of the proteolysis occurred within lysosomes. This suggestion was further supported by the finding that almost all the acid-soluble radioactivity present in the medium was in the form of [^{125}I]iodo-L-tyrosine, a result compatible with the known permeability properties of the lysosomal membrane (Reijngoud & Tager, 1977). The data reported in Fig. 2 show that uptake of ^{125}I-labelled albumin by the tissue occurs before acid-soluble radioactivity can be detected in the tissue and that only after this time can such radioactivity be detected in the incubation medium. This sequence of events is that expected from pinocytic capture and intralysosomal hydrolysis. Moreover, although acid-soluble ^{125}I-labelled digestion products accumulated progressively in the incubation medium, there was no progressive accumulation of such species in the tissue beyond 1.0h of incubation; this strongly suggests that the direction of flow of digestion products is from the tissue out into the medium. A conservative

Table 2. *Effects of increasing concentrations of rotenone on the rate of uptake of ^{125}I-labelled polyvinylpyrrolidone ([^{125}I]PVP) and on both the rate of digestion and the putative rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin ([^{125}I]albumin)*

Day-17.5 yolk sacs were incubated at 37°C in serum-free medium 199 and data were gathered in the manner described in the legend to Table 1, but the medium contained various concentrations of rotenone, an inhibitor of mitochondrial function. Mean values of the rates (±S.D.), for the numbers of experiments indicated, are expressed as a percentage of the corresponding rate at 37°C in the absence of rotenone.

		Relative rates		
[Rotenone] (M)	No. of expts.	Uptake of [^{125}I]PVP	Digestion of [^{125}I]albumin	Putative uptake of [^{125}I]albumin
0	4	100.0±9.4	100.0±12.2	100.0±13.8
10^{-11}	4	99.0±7.7	96.3± 9.4	96.6±10.1
10^{-9}	4	66.2±2.0	82.8±12.7	85.6± 9.1
10^{-7}	4	25.4±9.4	40.5± 6.9	43.3±10.3
10^{-5}	4	13.1±3.6	13.0± 6.2	16.6± 7.0

estimate of the intracellular concentration of digestion products (based on the entire intracellular space being available to the ^{125}I-labelled acid-soluble products) indicated that, after 40min of incubation, the intracellular concentration was approx. 200-fold greater than that in the medium. In a tissue that is incapable of accumulating digestion products from the medium this must indicate that such products are formed, at least partially, within cells.

If a significant fraction of the overall digestion occurs at the surface of the tissue, through the action of neutral peptidases on the external surface of the plasma membrane, it would be expected that not only would the end-products of digestion be found in the medium, but also large peptides. The method of analysis used was incapable of detecting very large peptides, since these would elute at the void volume of Sephadex G-25 together with intact albumin. However, the absence of smaller peptides from the medium in which yolk sacs were incubated with ^{125}I-labelled albumin (Fig. 1*a*) and their restriction to the yolk-sac tissue (Fig. 1*b*) suggests that extracellular hydrolysis by membrane-bound enzymes is unlikely. This conclusion was supported by the absence from cell-free homogenates of yolk-sac tissue of any detectable proteolytic capacity at a pH of 6.5 or more (Fig. 3); this finding is very similar to that reported by Goldstein *et al.* (1975) for cell-free extracts of normal fibroblasts incubated with ^{125}I-labelled low-density lipoprotein. It therefore appears that ^{125}I-labelled albumin is only degraded by enzymes that are maximally active at a pH of 4.0, hence it is degraded only within the lysosomal system after endocytic uptake.

If this conclusion is correct, it should follow that the rate of digestion cannot possibly exceed the rate of endocytic capture of the protein. Direct evidence of this was sought by making use of two characteristics of the kinetics of the digestion of albumin. First, in the initial 10–15min after adding ^{125}I-labelled albumin to the incubated yolk sacs, no acid-soluble radioactivity appears within the medium, hence the rate of accumulation over the initial 15min period is a true measure of the rate of endocytic uptake. Second, beyond the initial 1.0h of incubation, the amount of radioactivity associated with the yolk-sac tissue remains constant (Moore *et al.*, 1977; Ibbotson & Williams, 1979); hence, once such a steady state has been reached, the rate of production of acid-soluble products within the medium will be equal to the rate of endocytic capture, provided digestion is entirely within lysosomes. (Should digestion of protein also occur outside the cells, then the rate of generation of acid-soluble radioactivity in the period beyond 1h would be expected to exceed the rate of endocytic capture in the initial 10–15min lag period.) The data reported in Fig. 2 indicate that the rate of endocytic capture of ^{125}I-labelled albumin in the initial 15min (4.3ng/min per mg of yolk-sac protein) is identical with the rate of production of acid-soluble radioactivity once a steady state has been reached (4.4ng/min per mg of yolk-sac protein); hence there is no evidence of the involvement of non-lysosomal proteolysis from these experiments with intact tissue.

Duncan & Lloyd (1978) reported that the endocytic capture of ^{125}I-labelled polyvinylpyrrolidone from medium 199 containing 10% (v/v) calf serum was strongly temperature-dependent and was also inhibited by a number of metabolic inhibitors. Tables 1 and 2 show that in equivalent experiments, in serum-free medium, progressively decreasing the incubation temperature or progressively increasing the rotenone concentration progressively decreased the relative rates of pinocytosis (as determined by the rate of capture of ^{125}I-labelled polyvinylpyrrolidone) and the relative rates of digestion of ^{125}I-labelled albumin in a closely parallel manner. Such parallel effects would be expected only when pinocytosis is the rate-limiting step in both events.

It therefore appears that, for formaldehyde-treated ^{125}I-labelled bovine serum albumin at least, yolk-sac-associated proteolysis occurs entirely within the lysosomal system; hence the estimates of rates of pinocytosis of this protein reported by Williams *et al.* (1975*b*), Moore *et al.* (1977), Ibbotson & Williams (1979) and others are accurate. Furthermore, since certain weak bases have been reported to inhibit tissue-associated proteolysis by interfering with lysosomal function (de Duve *et al.*, 1974), the effects of these agents on the proteolysis of ^{125}I-labelled albumin by yolk-sac tissue can now be attributed with confidence to effects on the lysosomal system alone (G. Livesey & K. E. Williams, unpublished work).

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References

de Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. & Van Hoof, F. (1974) *Biochem. Pharmacol.* **23**, 2495–2531

Duncan, R. & Lloyd, J. B. (1978) *Biochim. Biophys. Acta* **544**, 647–655

Ehrenreich, B. A. & Cohn, Z. A. (1967) *J. Exp. Med.* **126**, 941–958

Fazakerley, S. & Best, D. R. (1965) *Anal. Biochem.* **12**, 290–295

Freychet, P., Kahn, R., Roth, J. & Neville, D. M. (1972) *J. Biol. Chem.* **247**, 3953–3961

Gabathuler, M.-P. & Ryser, H. J.-P. (1969) *Proc. R. Soc. London Ser. B* **173**, 95–98

Goetze, T., Franke, H., Hühn, W., Tönjes, R., Schlag, B. & Goetze, E. (1976) *Cytobiologie* **12**, 274–286

Goldstein, J. L., Brunschede, G. Y. & Brown, M. S. (1975) *J. Biol. Chem.* **250**, 7854–7862

Ibbotson, G. E. & Williams, K. E. (1979) *Biochem. J.* **178**, 785–792

Kenny, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 393–444, North-Holland, Amsterdam, New York and Oxford

Kerr, M. A. & Kenny, A. J. (1974) *Biochem. J.* **137**, 477–488

Kirsch, R. E., Frith, L. O. & Saunders, S. J. (1972) *Biochim. Biophys. Acta* **279**, 87–91

Marx, S. J., Woodward, C., Aurbach, G. D., Glossman, H. & Keutmann, H. T. (1973) *J. Biol. Chem.* **248**, 4797–4802

Moore, A. T., Williams, K. E. & Lloyd, J. B. (1974) *Biochem. Soc. Trans.* **2**, 648–650

Moore, A. T., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **164**, 607–616

Nilsson, M. & Berg, T. (1977) *Biochim. Biophys. Acta* **497**, 171–182

Pohl, S. L., Krans, H. M. J., Birnbaumer, L. & Rodbell, M. (1972) *J. Biol. Chem.* **247**, 2295–2301

Pratten, M. K., Duncan, R. & Lloyd, J. B. (1978) *Biochim. Biophys. Acta* **540**, 455–462

Reijngoud, D.-J. & Tager, J. M. (1977) *Biochim. Biophys. Acta* **472**, 419–449

Roberts, A. V. S., Nicholls, S. E., Griffiths, P. A., Williams, K. E. & Lloyd, J. B. (1976) *Biochem. J.* **160**, 621–629

Saez, J. M., Dazord, A., Morera, A. M. & Bataille, P. (1975) *J. Biol. Chem.* **250**, 1683–1689

Stein, O., Weinstein, D. B., Stein, Y. & Steinberg, D. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 14–18

Terris, S. & Steiner, D. F. (1975) *J. Biol. Chem.* **250**, 8389–8398

Tökes, Z. A. & Csipke, C. (1977) *J. Cell. Biol.* **75**, 221*a*

Tökes, Z. A. & Sorgente, N. (1976) *Biochem. Biophys. Res. Commun.* **73**, 965–971

Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **499**, 73–84

Williams, K. E., Lloyd, J. B., Davies, M. & Beck, F. (1971) *Biochem. J.* **125**, 303–308

Williams, K. E., Kidston, E. M., Beck, F. & Lloyd, J. B. (1975*a*) *J. Cell Biol.* **64**, 113–122

Williams, K. E., Kidston, E. M., Beck, F. & Lloyd, J. B. (1975*b*) *J. Cell Biol.* **64**, 123–134