Rates of pinocytic capture of simple proteins by rat yolk sacs incubated *in vitro*

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The rates of pinocytic uptake of a number of small ^{125}I -labelled simple proteins (insulin, ribonuclease A and lysozyme) by rat yolk sacs incubated *in vitro* were determined both before and after treating these proteins with reagents that are known to increase the rate of capture of ^{125}I -labelled bovine serum albumin. Uptake of the untreated forms of all three proteins was extremely rapid, indicating that adsorptive pinocytosis is the principal mechanism by which yolk-sac cells capture these simple proteins, but these rates showed no simple correlation with molecular charge. In contrast with albumin, the rates of uptake of the treated proteins were either unchanged or lower than that of the corresponding untreated protein preparation; polymeric forms of ^{125}I -labelled lysozyme larger than dimers were ingested at rates significantly lower than that of the monomer.

Previous studies of the rates of pinocytosis of radiolabelled substrates by rat yolk sacs incubated *in vitro* have demonstrated that certain substrates {e.g. ^{125}I -labelled polyvinylpyrrolidone (1-vinylpyrrolidin-2-one polymers) and $[\text{U-}^{14}\text{C}]\text{sucrose}$ } are captured at similar low rates as a result of their being ingested only in the fluid phase (Williams *et al.*, 1975a; Roberts *et al.*, 1977; Ibbotson & Williams, 1979). In contrast, chemically-treated, but non-aggregated, preparations of ^{125}I -labelled bovine serum albumin have been shown to be ingested at higher rates as a result of their adsorbing to the plasma-membrane surface of the pinocytic cells of yolk-sac tissue (Williams *et al.*, 1975b; Moore *et al.*, 1977; Ibbotson & Williams, 1979). Since serum albumin is exceptional in that it avidly binds a wide variety of molecules, including fatty-acid derivatives (Spector, 1975), it is possible that the adsorption of serum albumin to the plasma membrane is a further manifestation of this capacity and that adsorptive pinocytosis is not generally shown by simple proteins.

It was therefore of interest to determine the rates of ingestion of ^{125}I -labelled preparations of other simple proteins (insulin, lysozyme and ribonuclease A) and to re-determine the rates of ingestion after subjecting each protein to those chemical treatments that caused ^{125}I -labelled albumin to become much more extensively adsorbed to the plasma membrane of yolk-sac tissue (Moore *et al.*, 1977). By using a series of small well-characterized simple proteins and by subjecting each protein to the same

chemical treatments it was hoped to identify a structural feature of these simple protein molecules that is important in determining their ability to adsorb to the plasma membrane of the epithelial cells of the yolk-sac tissue.

Materials and methods

Preparation and treatment of ^{125}I -labelled proteins

Bovine insulin (25 i.u./mg), bovine pancreatic ribonuclease A (type XA) and hen egg-white lysozyme (grade 1), all products of Sigma (London) Chemical Co., London S.W.6, U.K., were iodinated by the same procedure as used for the iodination of bovine serum albumin (for details see Moore *et al.*, 1977). The maximum extent of iodination was approx. 1 iodine atom/500 molecules of protein.

After dialysis, portions of a single preparation of each ^{125}I -labelled protein were either titrated to pH 2.5 with acetic acid and incubated at 37°C for 2 h, or treated with urea or alkaline formaldehyde by the methods described by Moore *et al.* (1977) for treating serum albumin. Each preparation was then exhaustively dialysed against 1% (w/v) NaCl and the protein concentration was determined [by comparing A_{280} with that of a standard solution (0–1 mg/ml) of the native form of the protein] before storing the solution at –20°C.

Determination of the presence of aggregates by gel filtration

Portions (5–25 µg) of each of the above prep-

arations were applied to a Sephadex G-75 column (1.7 cm $\times$ 80 cm) and eluted with a suitable buffer solution at a flow rate of 24 ml/h. Insulin and ribonuclease preparations were eluted with 0.2 M NaH_2PO_4 buffer (pH 6.5) but, to obtain acceptable recoveries of lysozyme preparations from the column, this buffer had to be replaced by 0.05 M sodium acetate (pH 6.5) containing 1% (w/v) NaCl (Hampe, 1972).

Uptake of ^{125}I -labelled proteins by 17.5-day rat yolk sacs incubated in vitro

Rat yolk sacs were incubated in medium 199 containing 10% (v/v) calf serum by the method described in detail by Williams *et al.* (1975a,b) and modified as described by Moore *et al.* (1977). Each ^{125}I -labelled protein preparation was also incubated for 5.0 h in medium alone to establish its degree of hydrolysis in the absence of yolk-sac tissue.

Each rate of uptake was expressed as an Endocytic Index as defined by Williams *et al.* (1975a,b).

Assays of radioactivity

After measuring the total amount of radioactivity in a sample of medium (1.0 ml), the fraction of the ^{125}I -labelled protein that was hydrolysed was determined by precipitating macromolecular species with phosphotungstate and trichloroacetic acid by the method employed by Moore *et al.* (1977) to precipitate glycoproteins. The trichloroacetic acid-soluble radioactivity was measured and the observed value (c.p.m.) was normalized for changes in both sample volume (Ibbotson & Williams, 1979) and loss of acid-soluble material occluded within the pellet of precipitated protein (Livesey & Williams, 1979).

Determination of the rate of release of labelled substrate from yolk sacs

The progressive release of radioactivity into substrate-free medium from washed yolk sacs that had previously ingested a ^{125}I -labelled protein for 2 h *in vitro* was determined by the method of Williams *et al.* (1975a). The time course for the release of trichloroacetic acid-soluble radioactivity was also determined by assaying each medium sample again, after precipitating the macromolecular species by the above method.

Nature of the digestion products derived from ^{125}I -labelled proteins when incubated with yolk sacs

For each of the ^{125}I -labelled proteins, the radioactive species released into the incubation medium from the yolk-sac tissue, both during uptake studies and during 2 h re-incubations of the type described above, were examined on columns of Sephadex G-25 (using the same buffers as used for Sephadex G-75

studies) and on columns of its copper-complex. Details of these methods are given in Williams *et al.* (1971).

Results

The progressive uptake and digestion of ^{125}I -labelled lysozyme (frozen, -20°C) with time is shown in Fig. 1; the other ^{125}I -labelled protein preparations each showed a similar pattern. The quantity of radioactivity associated with the tissue rose initially but became virtually constant after 1–2 h of incubation, by which time trichloroacetic acid-soluble radioactivity was released into the medium at a constant rate. Such findings indicate that pinocytic capture of labelled protein is the rate-limiting step in the overall process of pinocytic capture and intracellular hydrolysis. [This is

Fig. 1. Uptake and digestion of ^{125}I -labelled lysozyme by 17.5-day yolk sacs

Uptake and digestion of ^{125}I -labelled lysozyme started when this protein ($25.0\text{ }\mu\text{g}$) was added to the 10.0 ml of medium 199 containing 10% (v/v) calf serum, in which each yolk sac was incubated. Each point represents a value obtained from a single yolk sac; ○, radioactivity associated with the tissue; ■, trichloroacetic acid-soluble radioactivity released into the medium; ●, uptake of ^{125}I -labelled lysozyme by the tissue (calculated by summing the quantity of tissue-associated radioactivity and the amount of acid-soluble radioactivity released into the incubation medium).

the pattern previously established for the digestion of ^{125}I -labelled albumin preparations by yolk sacs (Williams *et al.*, 1975b; Moore *et al.*, 1977).] Fig. 2 shows the results of a number of experiments in which washed yolk sacs containing ^{125}I -labelled lysozyme (not treated) were re-incubated in substrate-free medium after accumulating this labelled protein *in vitro* over a 2 h period. Within 2 h of re-incubation, 99.5% of the radioactivity initially associated with the tissue was released back into the medium, 90–95% of it in an acid-soluble form. Similar results were obtained on re-incubating yolk sacs that had accumulated each of the other ^{125}I -labelled protein preparations.

Examination, on Sephadex G-25 columns, of the trichloroacetic acid-soluble radioactivity released into the medium when yolk sacs were either incubated in the continuous presence of ^{125}I -labelled lysozyme (frozen, -20°C) or re-incubated in substrate-free medium after accumulating this protein *in vitro* (as described above), showed the majority of

Fig. 2. Release of radioactivity from yolk sacs previously loaded with ^{125}I -labelled lysozyme

Yolk sacs were incubated for 2 h in medium containing untreated ^{125}I -labelled lysozyme ($10\text{ }\mu\text{g/ml}$), washed three times for 2 min in substrate-free medium before being re-incubated singly for a further 2 h in 10 ml of substrate-free medium. The time course of release of radioactivity is shown for three determinations (mean $\pm$ s.d.). ●, Total radioactivity; ○, trichloroacetic acid-soluble radioactivity; ▲, acid-insoluble radioactivity.

the acid-soluble products to elute in the position corresponding to iodo-L-tyrosine. The equivalent results with the other ^{125}I -labelled protein preparations were virtually identical and these results are all essentially the same as recently reported in detail (Livesey & Williams, 1979) for equivalent experiments with formaldehyde-denatured ^{125}I -labelled bovine serum albumin as substrate. Moreover, further examination of the acid-soluble radioactivity on columns of the copper-complex of Sephadex G-25 showed that, for all the protein preparations studied, 85.6–91.4% of the acid-soluble material generated eluted from the columns in the same position as ^{125}I iodo-L-tyrosine and only 8.6–14.4% eluted in the same position as the dipeptide marker, glycyl[^{125}I iodo-L-tyrosine. These results indicate that, as judged by the fate of ^{125}I iodo-L-tyrosine residues, all these proteins are hydrolysed to the level of amino acids with little or no attendant release of oligopeptides. Such findings are compatible with the observed hydrolysis occurring exclusively within lysosomes (see Livesey & Williams, 1979, for more detailed arguments in support of this conclusion).

Although the results reported above showed only marginal differences between the different ^{125}I -labelled proteins, the rates of uptake of the individual protein preparations, when expressed as Endocytic Indices (Williams *et al.*, 1975a,b), showed some striking differences (Table 1). The Endocytic Indices of all the protein preparations were much greater than that of ^{125}I -labelled polyvinylpyrrolidone, which, from the results of 35 separate experiments, showed a mean ($\pm$ s.d.) value of $1.71 \pm 0.33\text{ }\mu\text{l/mg}$ of yolk-sac protein per h, a value almost identical with that previously reported (Williams *et al.*, 1975a). However, none of the native or formaldehyde-treated preparations of the ^{127}I -iodinated (non-radioactive) proteins had any effect on the rate of uptake of ^{125}I -labelled polyvinylpyrrolidone when added to the incubation medium in tracer quantities ($1\text{--}2.5\text{ }\mu\text{g/ml}$ of medium) indicating that none of these proteins has the ability to modulate the rate of pinosome formation (Table 2).

When examined on Sephadex G-75, all of the insulin and ribonuclease preparations eluted as single peaks in the same position as the monomeric form of the corresponding native protein, indicating that aggregation was not the cause of the modified rates of uptake of the formaldehyde-treated forms of these two proteins. However, Sephadex G-75 chromatography showed lysozyme to be markedly heterogeneous after treatment with either alkaline formaldehyde or with bicarbonate buffer (pH 10) alone (Fig. 3). No monomeric lysozyme remained after treatment with alkaline formaldehyde. One component of the formaldehyde-treated material eluted in the same position as α -chymotrypsin (mol.wt. 24000), and hence is presumably a dimer (Galen-

Table 1. Rates of uptake of various ^{125}I -labelled protein preparations by 17.5-day yolk sacs incubated in medium containing calf serum

Rates of uptake are expressed as Endocytic Indices (see Williams *et al.*, 1975a,b); units are $\mu\text{l}/\text{mg}$ of yolk-sac protein per h. Each value of the Endocytic Index was derived from a plot of uptake against time using data from experiments in which 5–11 yolk sacs, taken from a single animal, were incubated separately for up to 3.0 h in medium 199 containing 10% (v/v) calf serum and tracer quantities (1–2.5 $\mu\text{g}/\text{ml}$) of one of the ^{125}I -labelled protein preparations that had been treated as indicated before exhaustive dialysis. Where appropriate, results are expressed as means $\pm$ s.d. for the numbers of experiments shown in parentheses. Data marked * are taken from Moore *et al.* (1977).

Protein	Endocytic Index ($\mu\text{l}/\text{mg}$ of yolk-sac protein per h) of the ^{125}I -labelled protein preparation treated as indicated				
	Frozen (-20°C)	Acetic acid (pH 2.5)	Urea (4 M)	Formaldehyde (pH 10)	Buffer (pH 10)
Insulin	150.3 $\pm$ 24.3 (6)	155.7 $\pm$ 8.7 (4)	147.3 $\pm$ 15.8 (5)	44.8 $\pm$ 13.4 (4)	123.9 $\pm$ 13.5 (3)
Lysozyme	129.9 $\pm$ 16.6 (6)	134.2 $\pm$ 3.9 (3)	133.0 (2)	87.3 $\pm$ 12.4 (3)	76.6 $\pm$ 14.3 (3)
Ribonuclease	146.6 $\pm$ 42.0 (8)	120.5 (2)	139.3 (1)	57.6 $\pm$ 7.8 (4)	121.1 $\pm$ 11.8 (3)
Bovine serum albumin	—	—	—	71.3 $\pm$ 6.4 (4)	—
	8.9 $\pm$ 2.0 (5)*	14.5 $\pm$ 3.1 (4)*	73.3 $\pm$ 6.7 (2)*	65.0 $\pm$ 11.0 (4)*	8.1* (1)

Table 2. Effects of ^{127}I -iodinated (non-radioactive) preparations of proteins on the rate of uptake of ^{125}I -labelled polyvinylpyrrolidone by 17.5-day yolk sacs incubated in medium containing calf serum

Each experiment was performed in a manner essentially the same as that described in the legend to Table 1, but the incubation medium contained both ^{125}I -labelled polyvinylpyrrolidone (2.0 $\mu\text{g}/\text{ml}$) and tracer quantities (1–2.5 $\mu\text{g}/\text{ml}$) of one of the ^{127}I -iodinated (non-radioactive) protein preparations. The rate of uptake of ^{125}I -labelled polyvinylpyrrolidone, calculated as described by Williams *et al.* (1975a), is reported as the mean value of the Endocytic Index ($\pm$ s.d.) from three separate experiments in each case.

Endocytic Index ($\mu\text{l}/\text{mg}$ of yolk-sac protein per h) of ^{125}I -labelled polyvinylpyrrolidone in the presence of the protein preparation indicated

Protein	Frozen (-20°C)	Formaldehyde (pH 10)
Insulin	1.62 $\pm$ 0.36	1.61 $\pm$ 0.22
Lysozyme	1.58 $\pm$ 0.35	1.98 $\pm$ 0.39
Ribonuclease	1.51 $\pm$ 0.19	1.50 $\pm$ 0.14

beck *et al.*, 1977), whereas another polymeric fraction eluted in the same position as albumin (mol.wt. 66 000), and hence possibly consists of tetramers. Monomeric and polymeric fractions, isolated from the heterogeneous preparations of ^{125}I -labelled lysozyme, were shown to be stable in solution by re-examination on Sephadex G-75. Determination of their individual rates of uptake indicated that the dimeric form of ^{125}I -labelled lysozyme is ingested at essentially the same rate as the monomer, but that the fraction containing higher polymers was ingested far less rapidly (Table 3).

Fig. 3. Sephadex G-75 chromatography of ^{125}I -labelled lysozyme preparations

Mixtures containing 5–25 μg of one of the ^{125}I -labelled lysozyme preparations and 10 mg of unlabelled lysozyme (internal marker) were analysed on Sephadex G-75. Radioactivity ($\bullet$) and A_{280} ($\square$) of the eluate were monitored. Horizontal bars indicate the positions of marker substrates; reduced lysozyme was prepared by the method of White (1967). (a) Untreated lysozyme (recovery of radioactivity 98%); (b) formaldehyde-treated lysozyme (recovered radioactivity 84%); (c) bicarbonate-treated lysozyme (recovered radioactivity 92%). Abbreviation used: BSA, bovine serum albumin.

Table 3. *Rates of uptake of different fractions of ^{125}I -labelled lysozyme preparations when incubated with 17.5-day yolk sacs in medium containing calf serum*

A preparation of ^{125}I -labelled lysozyme, shown to be heterogeneous on Sephadex G-75 (see Fig. 3) after exposure to either formaldehyde (pH 10) or bicarbonate buffer (pH 10) alone, was resolved into fractions on Sephadex G-75 using aq. 1% (w/v) NaCl as eluent. The rate of uptake of each fraction was separately investigated. Each species was present at a concentration of $1\text{ }\mu\text{g/ml}$ of medium in these experiments; other details are as described in the legend to Table 1. Results are means $\pm$ S.D. for the numbers of experiments shown in parentheses.

Fraction of the ^{125}I -labelled lysozyme preparation investigated	Endocytic Index (μg /mg of yolk-sac protein per h)
Untreated monomer	136.0 ± 6.8 (4)
Bicarbonate-treated monomer	118.5 ± 8.3 (3)
Formaldehyde-induced dimer	124.9 ± 16.6 (3)
Bicarbonate-induced aggregates	32.3 ± 5.7 (3)
Formaldehyde-induced aggregates	58.3 ± 4.9 (3)

Discussion

Exposure of ^{125}I -labelled bovine serum albumin to a number of denaturing agents increases the rate of pinocytic uptake of this protein by rat yolk sacs incubated *in vitro* (Moore *et al.*, 1974, 1977) and a preliminary study showed these increases to correlate well with the observed decrease in the α -helical content of the protein molecule as estimated by o.r.d. measurements (Lloyd *et al.*, 1975). However, although albumin has frequently been used as a model soluble protein it is known to show complex behaviour. It readily forms dimers (Franglen, 1974), is thought to exhibit microheterogeneity (since the mean content of sulphhydryl groups is less than one per albumin monomer; Noel & Hunter, 1972), expanded conformations exist below pH 3.5–4.5 (Franglen, 1974) and intramolecular re-shuffling of disulphide bonds occurs through sulphhydryl-catalysed disulphide-exchange reactions (Foster, 1960; Sogami *et al.*, 1969). Moreover, albumin is an exceptional protein in that it binds certain anionic metabolites, in particular fatty acids (Spector, 1975), so avidly that it is almost impossible to completely remove them without also denaturing the protein molecule; also, variation in the fatty acid content may well affect the tertiary structure of the native molecule (Noel & Hunter, 1972; Verbruggen *et al.*, 1968). Thus, although the differences in the rates of pinocytic capture of the various albumin preparations are very marked (Table 1), identification of the

molecular feature(s) responsible for the enhanced rates of uptake of this simple protein, by adsorptive pinocytosis, is a formidable task.

For the various preparations of the smaller, more fully characterized, simple proteins (insulin, ribonuclease A and lysozyme) investigated in the current study, the general features of the uptake kinetics in the rat yolk-sac system are essentially the same as reported for albumin (Williams *et al.*, 1975b; Moore *et al.*, 1977). Once captured pinocytically, these proteins are released almost exclusively as acid-soluble digestion products (mostly [^{125}I]iodo-L-tyrosine), indicating that exocytosis occurs at a negligible rate, and thus the rate of pinocytosis can be calculated by the method of Williams *et al.* (1975b). Moreover, since tracer quantities of the ^{127}I -iodinated (non-radioactive) proteins do not alter the rate of pinosome formation, as determined by the rate of capture of ^{125}I -labelled polyvinylpyrrolidone, the native forms of all these small, simple proteins must adsorb much more extensively to the plasma membrane of the epithelial cells of the yolk-sac tissue than does albumin. Thus albumin preparations are by no means exceptional in their capacity to bind to the plasma membrane of these cells. Furthermore, although the simple proteins investigated in the present study are far smaller than albumin, they are ingested at much higher rates. Such findings fail to support the contention of Ryser (1967) that higher-molecular-weight proteins are cleared selectively; the rates of degradation of serum proteins (Dice & Goldberg, 1976) likewise fail to support this contention. Similarly, in the current study, the observation that alkaline formaldehyde- and bicarbonate-induced polymers of lysozyme (possibly tetramers) were ingested more slowly than lysozyme monomers or dimers (Table 3) conflicts with Ryser's suggestion and contrasts with the findings of Kooistra *et al.* (1979a) that polymeric forms of both ribonuclease A and lysozyme are cleared more rapidly *in vivo* by rat liver sinusoidal cells. Rat yolk sacs appear to be capable of only pinocytosis, but other cells that are capable of both pinocytosis and phagocytosis may well capture aggregated proteins by the latter route rather than by adsorptive pinocytosis; this will complicate the interpretation of clearance patterns of materials of colloidal dimensions.

In contrast with bovine serum albumin, the smaller simple proteins showed unaltered rates of uptake after exposure to acetic acid or urea. These findings suggest that, unlike albumin, they suffer no permanent conformational change on exposure to these agents. Moreover, in contrast with albumin, formaldehyde treatment caused the uptake rates of the smaller proteins to fall markedly rather than to increase (Table 1). This last observation conflicts with the suggestion (Moore *et al.*, 1977) that

formaldehyde may increase albumin binding through destroying the ϵ -amino groups of lysine residues, thus increasing the negative charge on the molecule, a suggestion compatible with the observation (Dice & Goldberg, 1976) that, *in vivo*, the more acidic proteins of rat serum are degraded more rapidly than the less acidic ones.

The decreased rates of uptake of the formaldehyde-treated proteins cannot be attributed to these proteins possessing cytotoxic properties since, in tracer concentrations, they were without effect on the rate of pinosome formation (Table 2). Hence there appears to be no obvious correlation between either the size or the charge of the protein preparations reported in Table 1 and their ability to adsorb to the plasma membrane of yolk-sac epithelial cells. However, ribonuclease and lysozyme are basic proteins and the surface of the insulin monomer is predominantly hydrophobic in character (Chothia & Janin, 1975). It is thus possible to explain the pattern of uptake rates reported in Table 1 in terms of both basic and hydrophobic characteristics if some simple proteins adsorb to regions of the plasma membrane that are negatively charged, whereas others adsorb to hydrophobic regions. For example, native bovine serum albumin, which is negatively charged at physiological pH, would be able to interact neither with the negatively charged nor with the hydrophobic binding sites and hence would be expected to be ingested only slowly. But if denaturation causes hydrophobic groups normally buried within the compact native conformation of the albumin molecule (Spector, 1975) to be brought to the surface, uptake could be greatly enhanced through hydrophobic interactions with the plasma membrane. Likewise, insulin will enter almost exclusively by adsorbing to hydrophobic sites, but lysozyme and ribonuclease will enter by adsorbing to negatively charged sites in the manner suggested by Kooistra *et al.* (1979b).

Such explanations of the observed behaviour in terms of multiple classes of binding site on the surface of yolk-sac epithelial cells require evidence for the existence of more than one type of site to be advanced. Experiments to support this view are the subject of other publications (Livesey, 1979; Kooistra & Williams, 1981).

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