The fate of homologous ^{125}I -labelled immunoglobulin G within rat visceral yolk sacs incubated *in vitro*

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1. When ^{125}I -labelled rat IgG (immunoglobulin G) is incubated *in vitro* with visceral yolk sacs from 17.5-day-pregnant rats, the protein is readily degraded. The major radioactive digestion product that accumulates in the medium is [^{125}I]iodo-L-tyrosine. 2. When rotenone (10 μM) is also present in the incubation medium, the rate of digestion of IgG is inhibited to the same extent as the rate of pinocytosis of ^{125}I -labelled polyvinylpyrrolidone. Proteolysis is likewise inhibited when either NH_4Cl (30 mM) or leupeptin (30 $\mu\text{g}/\text{ml}$) is present in the medium. The above findings strongly suggest that the observed proteolysis occurs within lysosomes. 3. Normally, yolk sacs that have been exposed *in vitro* to radiolabelled substrates release radioactivity slowly when the tissue is re-incubated, unless the substrate can be degraded within lysosomes and released in the form of low-molecular-weight hydrolysis products. However, in such experiments ^{125}I -labelled rat IgG shows quite exceptional behaviour in being rapidly released in an apparently intact form (as well as being degraded). 4. If an agent that inhibits pinocytosis (e.g. rotenone or 2,4-dinitrophenol) is present in the incubation medium during exposure of the tissue to ^{125}I -labelled rat IgG, it abolishes release of macromolecular radioactivity on re-incubation of the tissue. 5. Enhanced tissue accumulation of ^{125}I -labelled rat IgG, induced by the presence of leupeptin in the medium during the uptake phase, resulted in no concomitant increase in the amount of ^{125}I -labelled IgG released in macromolecular form on re-incubation of the tissue. 6. These findings indicate that the observed rapid release of ^{125}I -labelled IgG is unlikely to represent release from lysosomes and is more compatible with release from a separate class of vesicle that does not fuse with lysosomes.

Brambell (1966) postulated a mechanism, for the transport of homologous IgG across the epithelial cells of the yolk sac, that depends on those molecules that are transmitted intact adsorbing to a limited number of highly specific receptors on the inner face of pinocytic vesicles to protect the IgG from proteolytic attack when the vesicles fuse with lysosomes. Any IgG free in the lumen of such vesicles would be unprotected, hence liable to degradation on encountering lysosomal enzymes. The validity of this mechanism has been challenged more recently, and an alternative mechanism (Wild, 1975), advanced largely on the basis of ultrastructural findings, postulates that such transport involves a separate class of endocytic vesicles (coated micro-pinocytic vesicles). It is suggested that

these vesicles carry receptors specific for homologous IgG, but do not fuse with lysosomes and hence can selectively transport IgG molecules across yolk-sac cells without any possible attendant proteolysis.

Studies *in vitro*, using intact yolk sacs maintained in organ culture, offer a possible means of obtaining biochemical evidence to help to evaluate the status of these rival hypotheses, but to date only preliminary evidence from this source has been reported (Williams & Ibbotson, 1979). An advantage of studies *in vitro* using isolated yolk sacs is that (unlike studies *in vivo*) the catabolism of proteins, including IgG, can be investigated quantitatively (Williams, 1981). The data reported in the present paper substantiate the claims of the preliminary report (Williams & Ibbotson, 1979) that, in comparison with all other proteins studied to date, homologous IgG is treated

Abbreviation used: IgG, immunoglobulin G.

in an entirely atypical manner by the rat yolk sac in being the only protein that is released in quantity in an apparently undegraded form after becoming associated with the tissue. Moreover, the results of additional experiments, in particular those with leupeptin, an agent that has been shown to inhibit strongly the digestion of pinocytically captured protein in yolk-sac tissue (Knowles *et al.*, 1981), show that agents that inhibit proteolysis do not increase the amount of ^{125}I -labelled IgG released from the tissue. This suggests that the released IgG does not originate from secondary lysosomes, as required by the Brambell (1966) mechanism.

Essentially the same methodology as reported here has revealed a similar pattern of data (Weisbecker, 1982) when equivalent experiments were conducted with yolk sacs from the rabbit, a species in which yolk-sac transport is the only route of transmission of passive immunity.

Materials and methods

Preparation of ^{125}I -labelled proteins

Formaldehyde-denatured ^{125}I -labelled bovine serum albumin was prepared by the method of Moore *et al.* (1977), and rat γ -globulins (Fraction II; Miles Laboratories, Slough, Bucks., U.K.) were labelled by the same chloramine-T method, but, except for one small quantity, this material was not treated with formaldehyde. The concentration of the stock solution (approx. 1 mg/ml) of each protein preparation was estimated from the A_{280} before such solutions were stored at -20°C .

Uptake of ^{125}I -labelled proteins by yolk sacs

In some experiments the net rate of uptake of each ^{125}I -labelled protein by 17.5-day rat yolk sacs incubated in medium 199 containing heat-inactivated calf serum (10%, v/v) was determined by separately incubating single yolk sacs for up to 6 h in the presence of the labelled protein (1 $\mu\text{g}/\text{ml}$ of medium) and then summing the quantity of radioactivity associated with the tissue and the quantity of digestion products released back into the incubation medium by a given time, by using the method of Moore *et al.* (1977). In other experiments the net rate of uptake of each of these ^{125}I -labelled proteins from serum-free medium 199 was determined over a 5 h period by the modified method described by Ibbotson & Williams (1979). The concentration of ^{125}I -labelled protein was again 1 $\mu\text{g}/\text{ml}$ of medium.

Release of ^{125}I radioactivity from yolk sacs

By using essentially the same method as described by Williams *et al.* (1975a), 17.5-day yolk sacs were first incubated in serum-free medium 199 for 2 h with ^{125}I -labelled protein (5 $\mu\text{g}/\text{ml}$ of medium), then washed three times for 2 min in fresh medium 199 at

37°C before being re-incubated in serum-free medium (10.0 ml). Samples of medium were removed at regular intervals during the 3 h re-incubation period and assayed for both total radioactivity and trichloroacetic acid-soluble radioactivity by using the methods described for the assay of ^{125}I -labelled albumin and its hydrolysis products (see Ibbotson & Williams, 1979). The time course of the release of each type of radioactivity was determined as described by Williams *et al.* (1975a). Release was expressed either as a percentage of the radioactivity associated with the tissue at the start of the re-incubation or as ng of ^{125}I -labelled protein released from unit quantity of yolk-sac tissue by a given time. In some experiments, a metabolic inhibitor (2,4-dinitrophenol or NH_4Cl) was present in either the uptake or the release medium. In similar experiments, the effects of the cathepsin inhibitor leupeptin were established.

Nature of the acid-soluble hydrolysis products of ^{125}I -labelled protein

The acid-soluble ^{125}I -labelled degradation products released into the incubation medium were examined on columns of Sephadex G-25 by the method described in detail by Williams *et al.* (1971).

Nature of the acid-insoluble radioactivity released from yolk sacs previously exposed to ^{125}I -labelled rat IgG

Gel chromatography. Medium 199 (5.0 ml) containing radioactivity that had been released over a period of 3 h (from washed yolk sacs that had previously been incubated with ^{125}I -labelled rat IgG) was concentrated to a volume of 1–2 ml, then mixed with approx. 8 mg of native rat IgG. The mixture, containing approx. 4×10^4 c.p.m., was centrifuged (40 000 g-min), to remove tissue debris, then applied to a Sephadex G-200 column (75 cm $\times$ 1.5 cm) and co-chromatographed by using 0.05 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.5) containing 0.08 M- NaCl as eluent, at a flow rate of 12 ml/h.

Fractions (1 ml) were collected and monitored both for A_{280} and for their content of radioactivity.

Immunodiffusion. Further samples of the concentrated medium were examined by immunodiffusion against rabbit antiserum raised against rat IgG.

Results

Incubations either in medium 199 alone or in medium 199 containing heat-inactivated calf serum (10%, v/v) showed ^{125}I -labelled rat IgG to be degraded when incubated in the presence of yolk-sac tissue. No hydrolysis occurred in the absence of tissue, and the formation of trichloroacetic acid-soluble radioactivity ceased when the yolk sac was

Table 1. *Effects of NH_4Cl and of rotenone on the relative rates of uptake of ^{125}I -labelled polyvinylpyrrolidone and on the relative rates of degradation of ^{125}I -labelled rat IgG and of formaldehyde-treated bovine serum albumin*

Rates of uptake of ^{125}I -labelled polyvinylpyrrolidone (PVP), calculated as described in the text, were compared with the rates of formation of acid-soluble radioactive digestion products from ^{125}I -labelled rat IgG and from formaldehyde-treated ^{125}I -labelled bovine serum albumin (BSA) when the substrates ($1\text{ }\mu\text{g/ml}$ of medium) were incubated in serum-free medium 199 in the presence of yolk sacs. Data are mean values ($\pm$ S.D.) for three determinations and are normalized for tissue weight and expressed relatively, by taking the corresponding control rate in agent-free medium to be 100%.

Agent in the incubation medium (concn.)	Relative rate of uptake of ^{125}I -labelled PVP (% of control)	Relative rate of degradation of ^{125}I -labelled protein (% of control)	
		^{125}I -labelled BSA	^{125}I -labelled rat IgG
NH_4Cl (5 mM)	30.4 ± 10.8	40.2 ± 9.5	18.6 ± 11.4
NH_4Cl (20 mM)	15.9 ± 2.9	<1.0	<1.0
Rotenone ($10\text{ }\mu\text{M}$)	13.1 ± 3.6	13.0 ± 6.2	7.4 ± 2.1

Table 2. *Rates of uptake of ^{125}I -labelled polyvinylpyrrolidone and of different batches of ^{125}I -labelled rat IgG by 17.5-day rat yolk sacs incubated in vitro*

Each value of the net rate of uptake was obtained from a separate experiment in which yolk sacs (seven to ten) from a 17.5-day-pregnant rat were incubated in the presence of ^{125}I -labelled polyvinylpyrrolidone (PVP) or rat IgG ($1\text{ }\mu\text{g/ml}$ of medium) in either medium 199 alone or medium 199 containing 10% (v/v) of heat-inactivated calf serum (see text). Each result is expressed in the form of an Endocytic Index (see the text). Mean values, $\pm$ S.D., are reported for the numbers of experiments shown in parentheses.

^{125}I -labelled substrate	Endocytic Index ($\mu\text{l/mg}$ of yolk-sac protein per h)	
	Medium 199 + 10% calf serum	Serum-free medium 199
PVP	1.7 ± 0.30 (55)	2.5 ± 1.0 (11)
Rat IgG (batch I)	9.1 ± 4.7 (3)	64 ± 10 (3)
Rat IgG (batch II)	13.4 ± 2.0 (4)	97 ± 17 (3)
Rat IgG (batch III)	12.1 ± 2.2 (9)	67 ± 17 (10)

removed from the incubation flask. Chromatography of centrifuged post-incubation medium (i.e. medium in which yolk sacs had been incubated) on Sephadex G-25 showed the acid-soluble radioactivity to consist almost entirely of [^{125}I]iodo-L-tyrosine, a finding similar to that reported for equivalent experiments with formaldehyde-denatured ^{125}I -labelled bovine serum albumin as substrate (Livesey & Williams, 1979). Chromatography of post-incubation medium on Sephadex G-200 showed no evidence of the presence of large radiolabelled peptides. Such findings (results not reported in detail) are compatible with the degradation occurring within lysosomes (Livesey & Williams, 1979).

Further evidence for the acid-soluble radioactivity arising from intralysosomal proteolysis is given in Table 1. These data indicate that NH_4Cl

and rotenone, agents that have previously been shown to inhibit pinocytosis in the rat yolk sac (Livesey & Williams, 1979; Livesey *et al.*, 1980), each similarly affect the hydrolysis of formaldehyde-denatured ^{125}I -labelled albumin and of ^{125}I -labelled rat IgG. [There is now considerable evidence for the hydrolysis of the former protein occurring entirely within the lysosomal system of the rat yolk sac (Livesey & Williams, 1979).] At the higher concentration of NH_4Cl , the much lower relative rates of degradation ($<1\%$) of the two ^{125}I -labelled proteins, compared with the relative rate of uptake of ^{125}I -labelled polyvinylpyrrolidone (16%) reported in Table 1, are readily explained. At the higher concentration (20 mM) the lysosomotropic agent NH_4Cl causes an intralysosomal accumulation of undigested protein so that the rate of proteolysis is less than the rate of pinocytic capture (Livesey *et al.*, 1980). In contrast, rotenone has no effect on the digestion of proteins within lysosomes (Livesey, 1978). It exerts its effect by simply decreasing the rate of pinocytosis, which is conveniently determined by measuring the rate of uptake of the non-degradable fluid-phase marker, ^{125}I -labelled polyvinylpyrrolidone (Williams *et al.*, 1975a).

The quantity of acid-soluble digestion products released into the incubation medium during an uptake experiment rises linearly with time (results not shown in detail). This permits the net rate of capture of ^{125}I -labelled rat IgG to be expressed as an Endocytic Index [a clearance index defined by Williams *et al.* (1975a) as the number of μl of medium whose contained substrate is captured/mg of yolk-sac protein per h]. The summarized results (Table 2) show the Endocytic Index of ^{125}I -labelled rat IgG to be fairly reproducible for a given batch of the material, but there appears to be some inter-batch variation. In all experiments, the Endocytic Index of the ^{125}I -labelled rat IgG is considerably greater than that of ^{125}I -labelled polyvinylpyrrolidone, the difference being particularly marked for those data that relate to incubations in serum-free

Fig. 1. Time-course for the release of radioactivity from yolk sacs that have previously ingested radiolabelled albumin or IgG

Yolk sacs were incubated in serum-free medium 199 containing either formaldehyde-treated ^{125}I -labelled bovine serum albumin or ^{125}I -labelled rat IgG, then rinsed and re-incubated in fresh medium 199 (see the text for details). For each protein, the quantity of radioactivity released is expressed as a percentage of the total radioactivity associated with the tissue at the start of the re-incubation period. Each point represents the mean amount of radioactivity released ($\pm$ s.d.) at times up to 3.0 h in three separate experiments; $\bullet$, acid-soluble and $\blacksquare$, acid-insoluble radioactivity derived from ^{125}I -labelled albumin; $\circ$, acid-soluble and $\square$, acid-insoluble radioactivity derived from ^{125}I -labelled rat IgG.

medium 199. This indicates that most of the ^{125}I -labelled IgG that is being degraded by the yolk sac is captured by adsorptive pinocytosis in the manner described in detail for ^{125}I -labelled serum albumin and other simple proteins (Williams *et al.*, 1975b; Livesey & Williams, 1979, 1981).

The rate of release of acid-insoluble radioactivity from yolk sacs that had previously ingested ^{125}I -labelled albumin (Fig. 1) was similar to that of ^{125}I -labelled polyvinylpyrrolidone. Over the 3 h re-incubation period, $64 \pm 2\%$ of the total radioactivity associated with the tissue was released; most of this radioactivity was acid-soluble. However, the results of equivalent experiments with yolk sacs that had ingested ^{125}I -labelled rat IgG differed markedly from the above results with ^{125}I -labelled albumin. Approx. 80% of the tissue-associated radioactivity was released over 3 h, but the amounts of acid-soluble and -insoluble radioactivity released at any given time were almost equal (Fig. 1). The release pattern remained unchanged in experiments in which the re-incubation medium contained native IgG ($10\mu\text{g/ml}$). However, formaldehyde treatment of the ^{125}I -labelled IgG caused the release pattern (results not shown in detail) to become almost identical with that of formaldehyde-treated ^{125}I -labelled albumin, as shown in Fig. 1.

When different concentrations of the metabolic inhibitor 2,4-dinitrophenol were added to serum-free uptake medium, the Endocytic Index of ^{125}I -labelled polyvinylpyrrolidone decreased (Table 3). There was a similar decrease in the quantity of radioactivity (derived from ^{125}I -labelled rat IgG) that was released into the re-incubation medium after exposure of the tissue to the labelled protein in the presence of increasing amounts of 2,4-dinitrophenol (Table 3). The relative amounts of acid-soluble and -insoluble radioactivity released into the inhibitor-free re-incubation medium remained approximately equal. [The lower percentage of acid-insoluble material in the

Table 3. Effects of 2,4-dinitrophenol on the rate of uptake of ^{125}I -labelled polyvinylpyrrolidone by yolk sacs and on the release of radioactivity from yolk sacs previously exposed to ^{125}I -labelled rat IgG in the presence of this compound

The rates of uptake of ^{125}I -labelled polyvinylpyrrolidone (PVP) by yolk sacs incubated in serum-free medium 199 containing different concentrations of 2,4-dinitrophenol (2,4-DNP) were determined as described in the text. Other yolk sacs were first incubated for 2 h in serum-free medium 199 containing both 2,4-DNP and ^{125}I -labelled rat IgG ($10\mu\text{g/ml}$), then washed and re-incubated in fresh serum-free medium 199 containing no additives. The quantities of acid-soluble and -insoluble radioactivity released over a 3 h period were measured as in Fig. 1, but only the net quantities released by 3 h are shown. Mean values ($\pm$ s.d.) are reported for the numbers of experiments in parentheses.

Concn. of 2,4-DNP in medium during uptake of radiotracer ($\mu\text{g/ml}$)	Rate of uptake of ^{125}I -labelled PVP ($\mu\text{l/mg}$ of yolk- sac protein per h)	Amount of radioactivity released by 3 h from yolk sacs exposed to ^{125}I - labelled rat IgG in medium containing 2,4-DNP (ng/mg of yolk-sac protein)		
		Total	Soluble	Insoluble
0	2.69 ± 0.29 (3)	889 (1)	619 (1)	270 (1)
5	1.47 ± 0.61 (3)	623 ± 126 (4)	337 ± 76 (4)	286 ± 50 (4)
10	0.17 ± 0.08 (3)	64 ± 14 (6)	27 ± 8 (6)	37 ± 6 (6)
25	0.10 ± 0.14 (3)	66 ± 40 (6)	36 ± 26 (6)	30 ± 16 (6)

Fig. 2. Effect of NH_4Cl on the tissue association of ^{125}I -labelled rat IgG and on the release of radioactivity from tissues that have ingested ^{125}I -labelled IgG

Yolk sacs were incubated essentially as described in the legend to Fig. 1, but with NH_4Cl (30 mM) present in either the uptake or the release medium (see the text for details). The quantity of ^{125}I -labelled IgG released in a given form by 3.0 h is reported, together with the quantity present in the tissue at the start of the re-incubation period. □, Total radioactivity associated with the tissue at the start of the 3.0 h re-incubation; ■, total radioactivity released into the re-incubation medium by 3.0 h; ▤, acid-insoluble radioactivity released; ▨, acid-soluble radioactivity released.

released radioactivity compared with that reported in Fig. 1 probably results from a higher concentration of tracer protein (10 $\mu\text{g}/\text{ml}$ of medium) being used (to maintain measurable amounts of radioactivity in spite of a decreased rate of uptake) rather than the usual 5 $\mu\text{g}/\text{ml}$ as used in the experiments reported in Fig. 1. Williams & Ibbotson (1979) reported that the relative amount of acid-insoluble material in the released radioactivity fell as the concentration of ^{125}I -labelled rat IgG in the uptake medium increased.]

A similar series of experiments investigated the effects of a single concentration of NH_4Cl (30 mM)

on the association of ^{125}I -labelled rat IgG with the tissue and on the release of radioactivity into the re-incubation medium. The Endocytic Index of the ^{125}I -labelled rat IgG measured over a 6.5 h period in the presence of NH_4Cl (30 mM) was less than 1% of the control value (no NH_4Cl). Other results (Fig. 2) show that the presence of NH_4Cl (30 mM) in the uptake medium strongly inhibits tissue association of radioactivity. The results ('Release only' and 'Control', Fig. 2) also show that, when uptake occurs in the absence of inhibitor, release of acid-insoluble material is not measurably affected by the inhibitor being present only during the re-incubation stage. However, there is a fall, relative to the control, in the total quantity of radioactivity released by 3 h. The fall results from a decrease in the extent of release of acid-soluble radioactivity. This observation is compatible with the finding that NH_4^+ ions rapidly penetrate yolk sacs during incubation and, at such high concentrations, cause almost total inhibition of the proteolysis of ^{125}I -labelled albumin (Livesey *et al.*, 1980). [Again the ^{125}I -labelled rat IgG concentration in the uptake medium is 10, not 5 $\mu\text{g}/\text{ml}$, so the relative amount of acid-insoluble radioactivity released in the control experiment is lower than that reported in Fig. 1.]

The presence of the cathepsin inhibitor leupeptin (30 $\mu\text{g}/\text{ml}$) in the serum-free uptake medium did not alter the rate of uptake of the ^{125}I -labelled rat IgG (the Endocytic Index remained unchanged at $65 \pm 10 \mu\text{l}/\text{mg}$ of yolk-sac protein per h; $n=3$), but the rate of proteolysis of the tissue-associated protein fell to approx. 50% of the rate of capture. These findings are almost identical with those reported for equivalent experiments with formaldehyde-denatured ^{125}I -labelled albumin as substrate (Knowles *et al.*, 1981). Moreover, when yolk sacs were re-incubated after simultaneous exposure to ^{125}I -labelled rat IgG and leupeptin (30 $\mu\text{g}/\text{ml}$), the quantity of radioactivity that became associated with the tissue before re-incubation was started was much greater than in the matched controls, but there was no detectable increase in the amount of acid-insoluble radioactivity released during the 3 h re-incubation (Fig. 3).

Finally, examination of the radioactivity released into the re-incubation medium by a yolk sac that had previously taken up ^{125}I -labelled rat IgG showed that the radioactivity that was eluted at the void volume of a Sephadex G-25 column ran as a single symmetrical peak (that was co-eluted with unlabelled rat IgG) when applied to a Sephadex G-200 column. Immunodiffusion studies in which this released acid-insoluble radioactivity was run against antisera raised to rat IgG resulted in precipitin lines that were indistinguishable from those generated by the ^{125}I -labelled rat IgG preparation used in the related uptake experiment.

Fig. 3. Effect of leupeptin on the tissue association of ^{125}I -labelled rat IgG and on the release of radioactivity from tissues that have ingested ^{125}I -labelled IgG. Yolk sacs were incubated as described in the legend to Fig. 2, but with leupeptin (30 $\mu\text{g}/\text{ml}$) in place of NH_4Cl . Symbols are as in Fig. 2.

Discussion

The 17.5-day rat visceral yolk sac when incubated *in vitro* has been shown to accumulate slowly and progressively a number of non-digestible radiotracers, e.g. ^{125}I -labelled polyvinylpyrrolidone, [^{14}C]-sucrose and certain batches of colloidal [^{198}Au]gold (Williams *et al.*, 1975a; Roberts *et al.*, 1977). In contrast, extensive studies with ^{125}I -labelled proteins (Williams *et al.*, 1975b; Moore *et al.*, 1977; Livesey, 1978; Livesey & Williams, 1981) have shown that such proteins are not progressively accumulated by the tissue, but, within 2 h of the start of incubation, the quantity of radioactivity associated with the tissue becomes constant as a steady state is reached in which uptake becomes balanced by intralysosomal proteolysis accompanied by release back into the

incubation medium of acid-soluble degradation products. The rate of uptake of each protein, when expressed in the form of an Endocytic Index (see the Results section for definition), is usually markedly greater than that of ^{125}I -labelled polyvinylpyrrolidone, indicating that proteins are normally captured by adsorptive pinocytosis. In the current study, ^{125}I -labelled IgG too is taken up at a much higher net rate than ^{125}I -labelled polyvinylpyrrolidone (Table 2). Moreover, the acid-soluble radioactivity produced consists mainly of [^{125}I]iodo-L-tyrosine (as found with other ^{125}I -labelled proteins), and, in the presence of metabolic inhibitors, the rate of generation of acid-soluble radioactivity is decreased in proportion to the decrease in the rate of pinocytosis of ^{125}I -labelled polyvinylpyrrolidone (Table 1). Such findings indicate that a portion of the ^{125}I -labelled rat IgG taken up by the yolk-sac tissue enters by adsorptive pinocytosis and passes into the lysosomal system, where it undergoes complete proteolysis.

After pinocytic uptake, the rates of release of non-degradable radiotracers from rat yolk sacs are usually very low (Williams *et al.*, 1975a; Roberts *et al.*, 1977), the rate of release of the fluid-phase marker ^{125}I -labelled polyvinylpyrrolidone being no more than 1–2%/h. Provided that acid-insoluble radioactivity is monitored, the same is also true for ^{125}I -labelled proteins other than rat IgG (Fig. 1; Livesey, 1978; Livesey & Williams, 1981). Only ^{125}I -labelled rat IgG is rapidly released by the tissue in an acid-insoluble form (Fig. 1). However, this pattern of behaviour is destroyed if the ^{125}I -labelled rat IgG is exposed to formaldehyde after iodination (results not shown in detail). These findings are in sharp contrast with those of Besterman *et al.* (1981), who analysed in detail the biphasic release of the fluid-phase marker [^{14}C]sucrose from macrophages and from fibroblasts and suggested that the rapid initial phase of release [$t_{\frac{1}{2}}$ (half-life) < 10 min] could arise from pinosomes rapidly recycling to and fusing with the plasma membrane. [It is unlikely that transmembrane permeation is the explanation of these results with fibroblasts, since Dean & Jessup (1982) have shown inulin and dextran to exhibit similar kinetics.] Likewise, Adams *et al.* (1982) reported that pulse exposure of Chinese-hamster ovary cells to horseradish peroxidase results in the release of 30–50% of the tissue-associated enzyme within 10–20 min of re-incubation.

The interpretation of the initial phase of release in all experiments of the type described in the preceding paragraph is inherently problematic, because it is technically difficult to differentiate release of extracellular tracer (adsorbed to or occluded at the tissue surface) from rapid exocytosis of internalized tracer. However, it seems unlikely that the intact ^{125}I -labelled rat IgG released in such experiments

originates by slow desorption from the tissue surface rather than by release of material that has been captured by pinocytosis, since the release pattern is unchanged when native IgG was present in the re-incubation medium and the release is inhibited by decreasing the temperature of the re-incubation medium (Williams & Ibbotson, 1979). Moreover, the presence in the uptake medium of either 2,4-dinitrophenol (Table 3) or NH_4Cl (Fig. 2), agents whose ability to inhibit pinocytosis in the yolk sac is well documented (Duncan & Lloyd, 1978; Livesey *et al.*, 1980), causes the amount of ^{125}I -labelled rat IgG that becomes associated with the tissue and the amount of ^{125}I -labelled polyvinylpyrrolidone that is captured to be decreased by a similar percentage. A noteworthy observation (Fig. 2) is that, although the presence of NH_4Cl (30 mM) in the re-incubation medium decreases the extent of release of acid-soluble radioactivity, a finding that is expected from the high speed of penetration of NH_4^+ ions into yolk-sac tissue and their ability to inhibit intralysosomal proteolysis (Livesey *et al.*, 1980), the extent of release of acid-insoluble radioactivity remains unchanged relative to the control. NH_4^+ ions have been shown both to decrease the rate of pinocytosis of ^{125}I -labelled polyvinylpyrrolidone in rat yolk sacs and to decrease reversibly the [ATP]/[ADP] ratio in the tissue (Livesey *et al.*, 1980). Thus the release process may not be as energy-dependent as uptake. This suggestion merits further experimental investigation, since Dean & Jessup (1982) also report that exocytosis is only marginally affected by NH_4Cl concentrations in the 30–50 mM range.

The findings with leupeptin, a potent inhibitor of cysteine proteinases, including cathepsins B, H and L (Barrett, 1980), are particularly interesting from the standpoint of testing Brambell's (1966) original mechanism of passage of IgG across the yolk sac. When leupeptin (30 $\mu\text{g}/\text{ml}$) is present in the medium, uptake remains unaffected (cf. effect of NH_4Cl), but the rate of proteolysis is decreased by approx. 50%, and there is a marked increase in the amount of radioactivity that becomes associated with the tissue during the uptake phase (Fig. 3). However, the quantity of acid-insoluble radioactivity released on re-incubation remains the same. This observation suggests that protecting the homologous IgG from degradation within secondary lysosomes does not lead to an enhanced rate of release. It appears that macromolecules, other than IgG, that enter the yolk sac by pinocytosis effectively enter an intracellular cul-de-sac from which they can escape only by being degraded to fragments small enough to diffuse across the lysosomal membrane. If the molecules are either inherently incapable of being degraded by lysosomal enzymes (e.g. ^{125}I -labelled polyvinylpyrrolidone or colloidal [^{198}Au]gold) or a sufficient

degree of inhibition of lysosomal cathepsins can be achieved to prevent degradation of ^{125}I -labelled proteins, they simply accumulate in the vacuolar system [cf. findings of Besterman *et al.* (1981), Adams *et al.* (1982) and Dean & Jessup (1982)]. It thus appears that a single class of pinocytic vesicle destined to fuse with lysosomes is inadequate to explain the observations in the yolk-sac system. Either a second type of vesicle must be involved in the transport process, as proposed by Wild (1975), or a portion of the vesicle membrane carrying receptors specific for IgG must bud off from the endosomes before they fuse with lysosomes. The latter is effectively a variation on Wild's (1975) mechanism, with selection occurring within the vacuolar system rather than at the plasma-membrane surface.

This preliminary study is encouraging in that it indicates that such kinetic studies *in vitro* with intact yolk-sac tissue can shed more light on the dynamics of degradation of IgG by the yolk sac and can help to resolve the question of the mechanism of passage of IgG across the tissue. Further experiments are required to establish whether evidence for a separate class of vesicle that contains only homologous IgG can be obtained by subcellular fractionation of yolk sacs. Other experiments could be designed to establish whether the observed release of IgG is vectorial.

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