

# QUANTITATIVE STUDIES OF PINOCYTOSIS

## II. Kinetics of Protein Uptake and Digestion by Rat Yolk Sac Cultured In Vitro

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### ABSTRACT

Pinocytic uptake of  $^{125}\text{I}$ -labeled bovine serum albumin by 17.5-day rat visceral yolk sac cultured in vitro has been examined. Uptake was followed by intracellular digestion and, after an initial period, the content of radioactivity in the tissue itself remained constant during the incubation. Radiolabel was returned to the culture medium predominantly as [ $^{125}\text{I}$ ]iodotyrosine; exocytosis of undigested protein did not occur.

The rate of uptake of labeled protein, which was constant within an experiment and reproducible between experiments, was much higher than that of a nondigestible macromolecule,  $^{125}\text{I}$ -labeled polyvinylpyrrolidone. The higher rate of uptake was a consequence of the protein entering the cells chiefly by adsorption to the plasma membrane being internalized;  $^{125}\text{I}$ -labeled albumin did not stimulate, nor did  $^{125}\text{I}$ -labeled polyvinylpyrrolidone inhibit pinocytosis. Different preparations of  $^{125}\text{I}$ -labeled albumin had characteristically different rates of uptake, probably reflecting differences in affinity for plasma membrane receptors. The physiological significance of the findings is discussed.

The pinocytosis of proteins by mammalian cells was described in 1968 (1) as an underdeveloped area of research. Although in the intervening 6 yr the subject has attracted some attention, chiefly because of increasing interest in the mechanisms of turnover of plasma proteins, this judgment is still valid. The present paper is concerned with kinetic aspects of protein uptake by epithelial cells and of the subsequent intracellular digestion of the protein.

Studies of the uptake of proteins by animal cells have been conducted in a number of experimental systems. Cell fractionation of tissues from rats injected intravenously with horseradish peroxidase

(2), yeast invertase (2), or  $^{131}\text{I}$ -labeled albumin and transferrin (3) has indicated that these proteins congregate in particulate cell fractions with sedimentation characteristics similar to those of lysosomes. These biochemical findings have been confirmed and extended by the use of histochemical methods and proteins that are either fluorescent labeled (4), or possess enzymic activity (5). Ingested exogenous proteins are seen first in peripheral vacuoles that do not react positively for lysosomal enzymes and later in deeper vacuoles that do so react (6).

The evidence that exogenous proteins enter cells by pinocytosis is strong but indirect. First, there is

a good correlation between the ability of different types of cell to take up protein and the presence of morphological features suggestive of pinocytosis. Second, entry by endocytosis seems likely in view of the proteins being localized within the vacuolar system of the cytoplasm. Third, the size of protein molecules makes it unlikely that they can pass freely across biological membranes.

After uptake, exogenous proteins gradually disappear from tissues at rates characteristic of each protein (2, 7). That this is chiefly a result of protein digestion rather than of exocytosis of intact protein was at first inferred from the knowledge that lysosomes contained several proteinases and peptidases, but in 1967 Ehrenreich and Cohn (8) provided direct evidence of digestion when they showed that macrophages exposed to  $^{125}\text{I}$ -labeled human serum albumin ingested the protein and released the radioactivity as iodotyrosine. Similar observations have since been made with sarcoma S-180 cells (9). Moreover, Mego and colleagues (10) have shown that digestion of an exogenous protein takes place in osmotically active subcellular particles prepared from rat liver by differential centrifugation. Several authors (11–13) have recently provided evidence that amino acids and some dipeptides can pass through the lysosome membrane, but that larger molecules, such as intact proteins and their partial degradation products, cannot escape. Thus it is taken as a general rule that proteins and other exogenous macromolecules are confined to the vacuolar system of heterophagosomes and heterolysosomes until they are digested by lysosomal enzymes to molecules small enough to pass through the lysosome membrane into the cytosol. It should be noted, however, that in some special cases (14) exogenous proteins can apparently escape intact from the vacuolar system and reach other subcellular sites; the mechanisms involved are, however, not understood.

The preceding paper (15) reports an organ culture system that yields quantitative data on the uptake of  $^{125}\text{I}$ -labeled polyvinylpyrrolidone, a macromolecule that is not susceptible to attack by lysosomal hydrolases and which therefore progressively accumulates in the cells. In the present paper, the same system has been used to study the uptake and degradation of  $^{125}\text{I}$ -labeled bovine serum albumin. A number of additional questions are posed when a protein is used as substrate. Can a steady state be achieved where the rate of accumulation by endocytosis is balanced by the

rates of digestion and of release of digestion products from the cells? If so, endocytosis is the rate-determining step and thus the step most likely to be subject to metabolic control mechanisms. Is protein ingested at the same rate as is  $^{125}\text{I}$ -labeled polyvinylpyrrolidone? If not, is this because one substrate stimulates endocytosis (in the sense of increasing the rate of internalization of membrane and external liquid) or because one substrate is internalized to a greater extent than the other by adsorption on the plasma membrane? How is it possible to determine whether adsorption to the plasma membrane is important in the pinocytosis of protein, when experimentally it is impossible to distinguish directly between truly adsorbed protein and that contained in extracellular solution occluded at the tissue surface? Such questions are frequently raised in discussions of pinocytosis (16, 17), but satisfactory answers have awaited more conclusive experimentation.

## MATERIALS AND METHODS

### *Chemicals*

$^{125}\text{I}$ -Labeled polyvinylpyrrolidone of average molecular weight 30,000–40,000 ( $^{125}\text{I}$ PVP; preparation IM 33P) and  $^{125}\text{I}$ iodide (preparation IMS4, 2 mCi in 0.5 ml) were purchased from the Radiochemical Centre, Amersham, Bucks., U. K. Polyvinylpyrrolidone (PVP) of average molecular weight 40,000 (PVP-40, lot 41C-1460) and bovine serum albumin (type II) were obtained from Sigma (London) Chemical Co. Ltd. All other chemicals were of analytical grade.

### *Preparation of Acid-Treated $^{125}\text{I}$ -Labeled Bovine Serum Albumin*

The method used was that of Williams et al. (18). The product was stored at  $-15^{\circ}\text{C}$  without preservative as 5.0-ml lots in sterile bottles. Storage for up to 7 mo led to no detectable increase in the percentage of the radioactivity that failed to precipitate on addition of trichloroacetic acid (to 6.7% wt/vol) in the presence of carrier protein. During the course of the work, six batches of  $^{125}\text{I}$ BSA were prepared, ostensibly by identical methods.

### *Preparation of Acid-Treated $^{127}\text{I}$ -Labeled Bovine Serum Albumin*

With the same iodination procedure as employed in preparing the radiolabeled albumin, but with the same number of gram atoms of  $^{127}\text{I}$ iodide in place of the  $^{125}\text{I}$ iodide, the protein was iodinated to the same extent as its radioactive analog. Since the  $^{125}\text{I}$ iodide preparation possessed a degree of isotopic purity close to 100%,

0.08  $\mu\text{g}$  KI was substituted for each microCurie of [ $^{125}\text{I}$ ]iodide.

### *Preparation of $^{125}\text{I}$ -Labeled 3-Iodo-*L*-Tyrosine and $^{125}\text{I}$ -Labeled Glycyl-3-Iodo-*L*-Tyrosine*

These compounds were prepared and purified by the methods described by Williams et al. (18).

### *Assay of $^{125}\text{I}$ -Radioactivity*

Radioactivity was measured in a Packard gamma spectrometer (Packard Instrument Ltd., Caversham, Berks., U. K.), samples being presented to the machine in disposable 3-ml plastic tubes. Where possible, a standard sample volume of 1.0 ml was employed, but when assay procedures dictated that volumes in excess of 1.0 ml had to be counted (see below), the observed count was multiplied by an empirical correction factor to give the count that would have been given if the same amount of radioactivity had been presented in a volume of 1.0 ml. This procedure removed any volume-dependence of the counting efficiency.

### *Organ Culture of 17.5-Day Rat Visceral Yolk Sac*

The method used has been described in the preceding paper (15). In experiments in which the uptake and digestion of  $^{125}\text{I}$ -labeled bovine serum albumin were studied, culture vessels were returned to the water bath after removal of the yolk sac tissue and kept there until the end of the experiment, when all the media were deep frozen until assayed. This procedure was adopted to allow for possible proteolytic activity associated with the culture medium.

### *Examination of the Hydrolysis Products of $^{125}\text{I}$ -Labeled Albumin*

Preliminary experiments showed that  $^{125}\text{I}$ -labeled albumin was taken up by cultured yolk sac and that the products of catabolism were released back into the culture medium. A detailed examination of the hydrolysis products was made, using the Sephadex chromatography methods of Mougey and Mason (19) and Fazakerley and Best (20) as modified by Williams et al. (18). Culture media were centrifuged at 2,000  $g$  for 20 min before applying up to 3.0 ml to the columns (40 cm  $\times$  1.8 cm diam)

### *Determination of the Extent of Hydrolysis of $^{125}\text{I}$ -Labeled Albumin*

Once the nature of the hydrolysis products had been established by column chromatography, a simpler and more rapid estimation of the degree of hydrolysis was

employed for kinetic studies. Samples (1.0 ml) of culture medium were first counted (total radioactivity), and then aqueous trichloroacetic acid (20% wt/vol, 0.5 ml) was added. After thorough mixing, the tubes were immediately centrifuged at 2,000  $g$  for 20 min, and the entire clear supernate (1.3 ml) was carefully decanted into a fresh tube and counted. The observed count, corrected for the change in counting geometry (see above), is referred to below as the TCA-soluble radioactivity of the culture medium, and the difference between this value and the total radioactivity is designated the TCA-insoluble radioactivity.

The corresponding values of the TCA-insoluble and TCA-soluble radioactivities within the cultured yolk sacs were also determined. Yolk sacs were washed in saline, homogenized in water, and diluted to 5.0 ml with water; then portions (1.0 ml) were treated as described above for the culture medium. Further portions (0.2 ml) were assayed for total protein, as described in the preceding paper (15).

To determine the TCA-soluble radioactivity released into the medium during an incubation, the measured value of the TCA-soluble radioactivity in the medium was corrected for the TCA-soluble radioactivity (approximately 1%) known to be introduced initially with the preparation of [ $^{125}\text{I}$ ]BSA. To make this possible, samples of the [ $^{125}\text{I}$ ]BSA preparation diluted in culture medium were assayed in at least quadruplicate to determine accurately the percentage of TCA-solubles in the preparation on the day of use. Since the total volume of the medium was 10.0 ml and the protein content of each yolk sac was known, the quantity of hydrolysis products released into the medium could be expressed as TCA-soluble counts per minute per milligram yolk sac protein.

### *Determination of the Rate of Exocytosis of [ $^{125}\text{I}$ ]BSA from Cultured Yolk Sac*

The possibility that radiolabeled albumin could be returned to the medium in the undigested form after internalization by the epithelial cells was investigated in the following manner. Yolk sacs were cultured for 3.0 h in culture medium containing [ $^{125}\text{I}$ ]BSA, it having been shown that the level of TCA-insoluble radioactivity within the tissue reaches a maximum value after an incubation period of approximately 2.0 h (see below). Extracellular radioalbumin was removed by transferring each yolk sac to fresh medium containing no radiolabel and incubating for 2-min periods at 37°C in a total of three changes of culture medium (10.0 ml). Finally, the yolk sac was transferred to a culture vessel containing fresh medium (10.0 ml), gassed, and incubated for a further period of 4.0 h. The medium was then centrifuged (2,000  $g$ , 30 min) to remove detached cells and applied to a column of Sephadex G-25, eluting with sodium acetate buffer (0.02M, pH 7.35) as described by Williams et al. (18).

## RESULTS

### Digestion Products of $^{125}\text{I}$ -Labeled Bovine Serum Albumin

Fig. 1 shows the elution pattern on Sephadex G-25 of centrifuged culture medium containing  $^{125}\text{I}$ BSA in which a yolk sac had been incubated for a period of 7.5 h. Three peaks are seen; the two corresponding to undegraded  $^{125}\text{I}$ BSA and to  $^{125}\text{I}$ iodide were also present when culture medium was chromatographed before incubation with a yolk sac. The third peak, which eluted in a position corresponding to iodotyrosine or an iodotyrosyl dipeptide, was not present in culture medium containing  $^{125}\text{I}$ BSA that had been incubated without yolk sac under standard culture conditions for up to 24 h. Furthermore, the size of the  $^{125}\text{I}$ iodide peak was not increased by incubation in either the presence or the absence of tissue. These experiments show that there is no detectable proteolytic activity associated with the culture medium itself and that neither the tissue nor the medium liberates iodide from iodotyrosyl residues. Increased levels of low molecular weight hydrolysis products could thus be safely ascribed to the proteolytic action of yolk sac alone. Moreover, the site of proteolysis was shown to be the tissue itself, since no further digestion took place when, after a 4-h incubation in the presence of a yolk sac, culture medium containing  $^{125}\text{I}$ BSA was incubated for a second 4-h period with the yolk sac removed. This experiment excludes the possibility that digestion of  $^{125}\text{I}$ BSA takes place in the culture medium by enzymes extruded by the yolk sac tissue.

Fig. 2 shows the elution pattern on the copper complex of Sephadex G-25 of the concentrated pooled fractions 71–75, inclusive, from the separation shown in Fig. 1. This system of chromatography separates amino acids from oligopeptides, and the results in Fig. 2 indicate that 95% of the hydrolysis products of  $^{125}\text{I}$ BSA released into the medium was  $^{125}\text{I}$ -labeled 3-iodo L-tyrosine and 5% was compounds that elute in the same position as glycyl-iodotyrosine, presumably a mixture of small peptides containing iodotyrosine.

It was shown that chromatographically pure samples of  $^{125}\text{I}$ iodotyrosine and  $^{125}\text{I}$ glycyl-iodotyrosine remained in solution when precipitation with trichloroacetic acid was attempted in the presence of 10% calf serum. Hence, in the subsequent studies the extent of hydrolysis was esti-

mated from the radioactivity remaining in the supernate after precipitation of  $^{125}\text{I}$ BSA with trichloroacetic acid (see above).

### Uptake and Digestion of $^{125}\text{I}$ -Labeled Bovine Serum Albumin

Fig. 3 shows the results of a typical experiment in which six yolk sacs from the same rat were cultured in medium containing  $3\ \mu\text{g}$  of  $^{125}\text{I}$ BSA (batch no. 6) per ml, incubations being terminated at intervals up to 6.5 h. The rate of release of TCA-soluble radioactivity into the culture medium and the quantities both of TCA-insoluble and

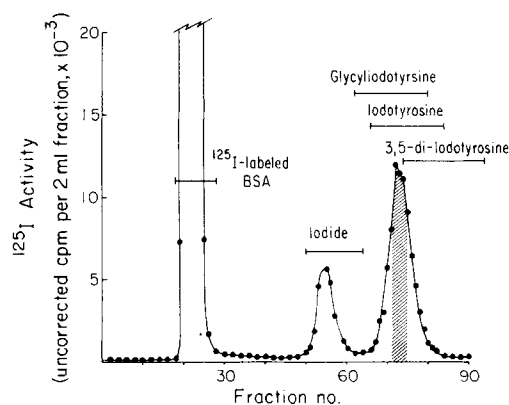


FIGURE 1 Elution from Sephadex G-25 of centrifuged postculture medium. Elution positions of marker compounds in the same system are indicated. Those fractions represented by the cross-hatched area were pooled, concentrated by freeze-drying, and reanalyzed (see Fig. 2).

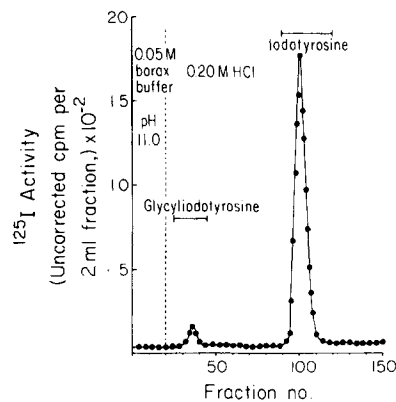


FIGURE 2 Elution from the copper complex of Sephadex G-25 of the pooled, concentrated fractions (71–75) from the separation shown in Fig. 1. Elution positions of marker compounds in the same system are indicated.

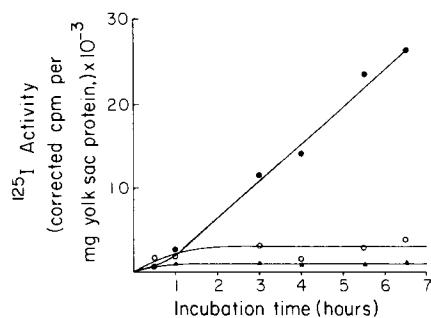


FIGURE 3 Levels of  $^{125}\text{I}$ -activity in tissue and culture medium on incubation of yolk sacs in the presence of  $^{125}\text{I}$ BSA (batch no. 6). Data at each time interval are from a single yolk sac. ●, TCA-soluble activity released into the culture medium; ○, TCA-insoluble activity in the yolk sac; ▲, TCA-soluble activity in the yolk sac.

TCA-soluble radioactivity in the yolk sac tissue each rose over the initial 2 h and then became constant. These observations are compatible with a steady state being reached beyond 1–2 h, when the rate of ingestion of  $^{125}\text{I}$ BSA is exactly balanced by the rate of release of its hydrolysis products into the culture medium.

Fig. 4 shows the elution pattern on Sephadex G-25 of centrifuged culture medium in which a washed yolk sac, laden with  $^{125}\text{I}$ BSA by prior incubation in medium containing  $^{125}\text{I}$ BSA, had been incubated for 4.0 h. Of the radioactivity released by the tissue into the culture medium, only 2.7% was in the macromolecular form. These results indicate that after  $^{125}\text{I}$ BSA has been ingested by the yolk sac, it is returned to the medium only as low molecular weight hydrolysis products. Because there is no exocytosis of  $^{125}\text{I}$ BSA, its uptake by a yolk sac in culture must be balanced by the combined effects of two processes, accumulation within the tissue and release of TCA-soluble hydrolysis products into the medium. Thus the rate of uptake of  $^{125}\text{I}$ BSA may be calculated from data such as those of Fig. 3. Since the total radioactivity found in the cultured tissue was approximately constant beyond 2 h of incubation (see above), the rate of appearance of TCA-soluble radioactivity in the medium between 2 and 8 h affords a good approximation to the rate of uptake of  $^{125}\text{I}$ BSA by the yolk sac. However, the rate of uptake is given more precisely by summing at each time interval the total radioactivity in the tissue and the TCA-soluble radioactivity in the medium. This latter method is more generally

applicable since it is not dependent on tissue levels of radioactivity being constant; changing tissue levels are met, for example, in experiments where compounds are added that modify either the uptake or the digestion of  $^{125}\text{I}$ albumin (21).

### Method of Calculating Uptake

In the preceding paper (15) the uptake of a nondigestible substrate,  $^{125}\text{I}$ -labeled polyvinylpyrrolidone ( $^{125}\text{I}$ PVP) was expressed as the volume of culture medium whose contained substrate had been captured by unit quantity of tissue. With the digestible substrate  $^{125}\text{I}$ BSA, the bulk of the ingested radiolabel is not retained within the yolk sac tissue but released back into the medium as TCA-soluble digestion products. In addition, because  $^{125}\text{I}$ BSA is found to be ingested at a much higher rate than is  $^{125}\text{I}$ PVP, a significant depletion of the TCA-insoluble radioactivity in the medium occurs during the incubation period. Both these factors must be allowed for when the uptake of  $^{125}\text{I}$ BSA is calculated. The appropriate expression is  $(Y + 10S)/M'P$ , where the numerator is the sum of the total radioactivity retained in the yolk sac tissue and the total quantity of TCA-soluble radioactivity released into the culture medium (10 ml) during the incubation period.  $Y$  is the total radioactivity in the yolk sac (counts per minute, corrected for background);  $S$  the TCA-soluble radioactivity in the culture medium at the end of an incubation period (counts per minute per milliliter medium, corrected for background and for the TCA-soluble radioactivity present at the beginning of the incubation);  $M'$  the mean TCA-insoluble radioactivity in the culture medium over the particular incubation period (counts per minute per milliliter medium, corrected for background); and  $P$  the protein content of the yolk sac (milli-

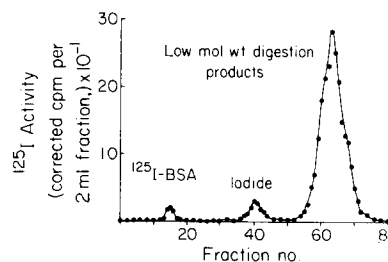


FIGURE 4 Elution from Sephadex G-25 of centrifuged culture medium, in which a yolk sac laden with  $^{125}\text{I}$ BSA had been cultured for 4 h.

grams). Uptake, so expressed, has the units of microliters per milligram tissue protein.

### Quantitative Data on the Uptake of $^{125}\text{I}$ -Labeled Bovine Serum Albumin

When the data of Fig. 3 are recalculated as explained above, the results are as shown in Fig. 5. It can be seen that uptake of  $^{125}\text{I}$ BSA is linear with time over the entire 8-h incubation period. In consequence, an endocytic index can be obtained from the slope of this plot. Table I shows the endocytic index for nine similar experiments in which the same batch (batch no. 6) of  $^{125}\text{I}$ BSA was used. Application of the same analysis of covariance as used in the preceding paper (15) to the data of Table I indicated that there was significantly more variability between experiments than within individual experiments. Therefore, in contrast to the data on  $^{125}\text{I}$ PVP, it was not legitimate to pool the uptake data for a batch of  $^{125}\text{I}$ BSA to give an overall plot from which mean values of the gradient and intercept could be determined along with associated confidence intervals for these parameters. Hence, only a mean endocytic index and its standard deviation can be derived from the data of Table I.

Similar experiments were carried out with five additional batches of  $^{125}\text{I}$ BSA and the results are shown in Table II. Each preparation of  $^{125}\text{I}$ BSA had its own characteristic and reproducible en-

docytic index. The endocytic indices of preparations of  $^{125}\text{I}$ BSA were independent of the concentration of  $^{125}\text{I}$ BSA in the culture medium (Fig. 6) within the range 1.5–52.0  $\mu\text{g}/\text{ml}$  and also independent of the age of the preparation (Fig. 7). In the experiments shown in Fig. 7, the concentration of  $^{125}\text{I}$ BSA added to the culture medium was increased as the sample became older, in order to provide sufficient radioactivity for accurate assays.

Fig. 8 shows the results of an experiment in which the rate of uptake of  $^{125}\text{I}$ PVP by yolk sac in culture was shown to be unaffected by the presence of 16.0 or 160  $\mu\text{g}$   $^{127}\text{I}$ -iodinated bovine serum albumin per milliliter culture medium. An analogous experiment (Fig. 9) showed that the uptake of  $^{125}\text{I}$ BSA was unchanged by the pres-

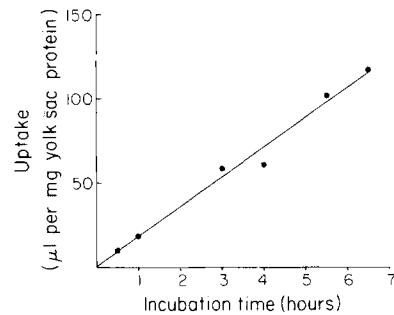


FIGURE 5 Uptake of  $^{125}\text{I}$ BSA by cultured yolk sac. The same data as shown in Fig. 3 recalculated in the manner explained in the text.

TABLE I  
Correlation Coefficient, Slope, and Intercept on the Ordinate Axis for the Regression Lines Through Each Set of Data From Nine Separate Experiments on the Uptake of  $^{125}\text{I}$ BSA (batch no. 6) by Rat Yolk Sac Cultured In Vitro

Experiment no.	Correlation coefficient	Slope	95% Confidence limits of slope	Intercept on ordinate axis
		$\mu/\text{h}/\text{mg protein}$	$\mu/\text{h}/\text{mg protein}$	$\mu/\text{mg protein}$
1	0.952	14.15	5.84–22.47	+0.95
2	0.991	17.74	14.37–21.12	+0.62
3	0.984	30.99	23.18–38.80	–6.85
4	0.976	17.29	11.93–22.65	+3.01
5	0.981	17.80	12.97–22.64	–2.79
6	0.997	24.96	22.22–27.70	+12.31
7	0.989	22.17	16.01–28.33	+20.68
8	0.987	21.20	16.31–26.09	+11.08
9	0.984	22.41	16.71–28.11	–10.08

95% confidence limits of the slopes are also indicated. The slope of the line is equal to the endocytic index, as defined in the text.

Endocytic index (mean) =  $20.97 \pm 5.00$  SD.

Intercept (mean) =  $3.21 \pm 9.85$  SD.

TABLE II  
Values of the Endocytic Index for Different Batches of [<sup>125</sup>I]BSA

Batch of [ <sup>125</sup> I]BSA	Experiments performed	Mean endocytic index	Standard deviation of endocytic index
no.	no.	μl/h/mg protein	μl/h/mg protein
1	4	18.65	±7.52
2	4	11.06	±0.94
3	6	31.98	±9.88
4	3	51.63	±5.13
5	8	80.85	±17.33
6	9	20.97	±5.00

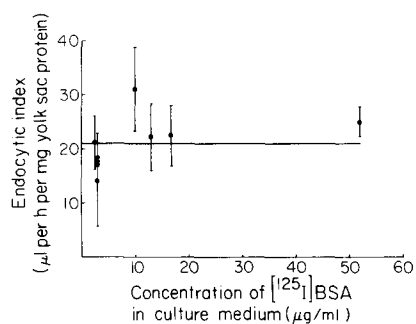


FIGURE 6 Endocytic index of [<sup>125</sup>I]BSA (batch no. 6) at different concentrations of [<sup>125</sup>I]BSA in the incubation medium. 95% confidence limits are shown.

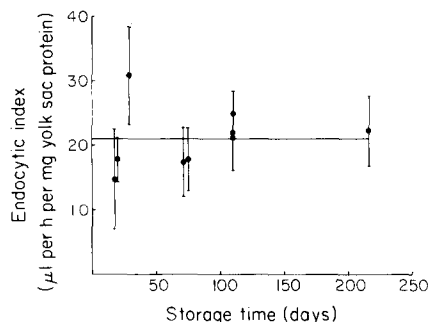


FIGURE 7 Effect of storage (-20°C) on the endocytic index of [<sup>125</sup>I]BSA (batch no. 6). 95% confidence limits are shown.

ence of 4.0 or 40 μg noniodinated PVP per milliliter. In the latter experiment, neither the level of radioactivity within the tissue nor the rate of release of TCA-soluble radioactivity was affected by the inclusion of PVP in the culture medium.

## DISCUSSION

The results described above show that when a yolk sac is incubated in medium containing [<sup>125</sup>I]BSA, the substrate is taken up by the tissue and digested within it. At least in the case of iodotyrosyl residues, digestion proceeds largely to the level of free amino acids which, together with a small percentage of oligopeptides, are released by the tissue. Only a very small amount (2.7%) of ingested protein is released back into the medium in macromolecular form, indicating that the combined effects of exocytosis, leakage from dead cells, and detachment of fragments of the cultured tissue are negligible. These findings agree well with those of a previous study (18) in which [<sup>125</sup>I]BSA was accumulated in the yolk sac in vivo after intravenous maternal administration, and catabolism followed in vitro on culturing of the laden yolk sacs by a raft technique.

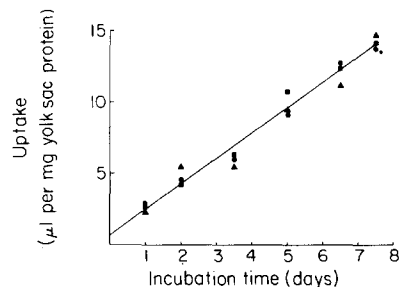


FIGURE 8 Uptake of [<sup>125</sup>I]PVP by yolk sacs in the absence and presence of [<sup>127</sup>I]BSA in the culture medium. ▲, control (no [<sup>127</sup>I]BSA); ■, [<sup>127</sup>I]BSA present (16 μg per ml of medium); ●, [<sup>127</sup>I]BSA present (160 μg/ml of medium).

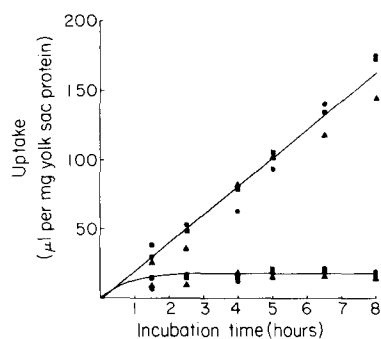


FIGURE 9 Uptake and digestion of [<sup>125</sup>I]BSA (batch no. 6) in the absence and presence of noniodinated PVP in the culture medium. ●, Control (no PVP); ▲, noniodinated PVP present (4 μg/ml of medium); ■, noniodinated PVP present (40 μg/ml of medium).

In the present experiments, uptake of [<sup>125</sup>I]BSA by yolk sac in this system proceeded at a constant rate, at least up to 8 h. Moreover, for any given batch of [<sup>125</sup>I]BSA, the rate of uptake was reproducible between experiments within quite narrow limits. In every experiment the levels of radioactivity in the tissue itself became constant after a short initial period, indicating an exact balance between the rate of uptake of protein by the tissue and the rate of digestion of protein within the tissue. A further implication of the constancy of the tissue radioactivity is that ingestion of [<sup>125</sup>I]BSA is the rate-determining step in the sequence of ingestion, digestion, and release.

As in the experiments using [<sup>125</sup>I]PVP reported in the preceding paper (15), the constancy of the rate of uptake within an experiment permits this rate to be described by a single numerical value, the endocytic index, a form of expression whose usefulness has been discussed in the previous paper (15). In these experiments, while the endocytic index of [<sup>125</sup>I]PVP was 1.71 μl/h/mg protein, the endocytic index of [<sup>125</sup>I]BSA ranged from 11.1 to 80.8 μl/h/mg protein according to the batch of [<sup>125</sup>I]BSA used. The remainder of this discussion is concerned with the question of how different substrates can show such markedly different endocytic indices.

Fig. 10 is a diagram of a pinocytic vesicle being formed by invagination of the plasma membrane. Solute molecules are shown as being internalized both in free solution and adsorbed on the plasma membrane. As stated by Jacques (17), the rate at which a solute enters a cell by endocytosis is given by the sum of two terms, the first representing

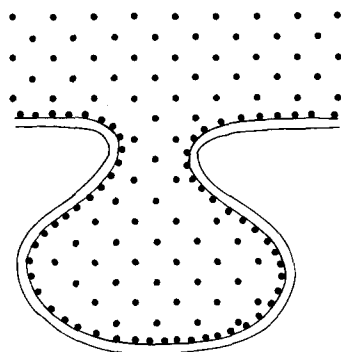


FIGURE 10 Diagram of the formation of a pinocytic vacuole to illustrate ingestion of solute molecules (●) both in free solution and adsorbed on the plasma membrane.

uptake in the liquid phase, the second uptake by adsorption on the membrane. Thus, assuming instantaneous and continuous adsorption equilibrium at the cell surface,

$$Q = Fc + \frac{SRc}{K + c}, \quad (1)$$

where  $Q$  is the rate of uptake of solute,  $c$  its concentration in the external liquid,  $K$  the dissociation constant of the substrate-surface complex, and  $R$  the maximum amount of substrate that can be adsorbed per unit area of cell surface.  $F$  and  $S$  are the rates of pinocytic internalization of external liquid and of cell surface, respectively; these two parameters are interrelated by the geometry of the pinocytic vesicles.

All the terms in equation (1) are potentially variable. For any given substrate, however,  $R$  and  $K$  will be constant, but if the substrate is itself an inducer or an inhibitor of pinocytosis, changes in  $c$  will cause changes in  $F$  and  $S$ .

If equation (1) is divided throughout by the solute concentration,  $c$ , an expression for the endocytic index, as defined above, is obtained:

$$\text{endocytic index} = \frac{Q}{c} = F + \frac{SR}{K + c}. \quad (2)$$

All our experiments have been performed in the presence of 10% calf serum. It is possible that if either [<sup>125</sup>I]PVP or [<sup>125</sup>I]BSA enters cells adsorbed to plasma membrane, it will be in competition with plasma proteins also entering the cells by the same route. Moreover the concentrations of [<sup>125</sup>I]PVP and [<sup>125</sup>I]BSA used are very low in comparison with the concentration of the serum proteins. It may be deduced that in the presence of a competing substrate  $I$ , at a concentration  $i$ , equation (2) becomes

$$\text{endocytic index} = F + \frac{SRM}{KM + Ki + Mc}, \quad (3)$$

where  $M$  is the dissociation constant of the surface-competitor complex. In the simplest case, where no adsorption of solute to membrane occurs, equation (3) simplifies to

$$\text{endocytic index} = F, \quad (4)$$

i.e. the endocytic index equals the rate of pinocytic capture of liquid.

Examination of equations (3) and (4) suggests



two alternative explanations for the observation that the endocytic index for [ $^{125}$ I]BSA was greater than that for [ $^{125}$ I]PVP. In the first explanation uptake of both substrates is entirely in the bulk liquid phase (equation (4) holds), but [ $^{125}$ I]BSA stimulates or [ $^{125}$ I]PVP inhibits vesicle formation, i.e.  $F$  is greater in experiments with [ $^{125}$ I]BSA than in experiments with [ $^{125}$ I]PVP. However, this cannot be so because the presence of [ $^{127}$ I]BSA did not increase the endocytic index of [ $^{125}$ I]PVP (Fig. 8), nor did PVP decrease the endocytic index of [ $^{125}$ I]BSA (Fig. 9). Moreover, electron micrographs of yolk sacs ingesting [ $^{125}$ I]BSA showed no evidence of increased pinocytic activity as compared to yolk sacs ingesting [ $^{125}$ I]PVP. Hence, we conclude that  $F$  and  $S$  are constants in all our experiments, and that the observed behavior cannot be accounted for by equation (4). In the second explanation, the rate of vesicle formation is unaffected by the substrate used ( $F$  and  $S$  are constant), and the endocytic index for [ $^{125}$ I]BSA is higher than that for [ $^{125}$ I]PVP because uptake by adsorption is greater in the case of [ $^{125}$ I]BSA, i.e.  $R$  is greater, or  $K$  lower, for [ $^{125}$ I]BSA than for [ $^{125}$ I]PVP.

As  $F$  is constant in our experiments, it follows that  $F$ , the rate of capture of liquid, is no greater than the endocytic index for [ $^{125}$ I]PVP, i.e. approximately  $1.7 \mu\text{l/h/mg}$  yolk sac protein (18). Indeed,  $F$  will be less than this value if any [ $^{125}$ I]PVP enters the cells adsorbed to the plasma membrane. The present experiments do not give any indication as to whether this is so. However, in the case of [ $^{125}$ I]BSA of endocytic index 11.1 (batch no. 2), no more than 15.4% ( $1.71/11.1$ ) of the uptake can be accounted for by endocytosis in the bulk liquid phase, indicating that at least 85% must enter adsorbed on membrane. For batch no. 5 [ $^{125}$ I]-BSA, at least 98% must enter by this route. The differences between the endocytic indices of the different batches of [ $^{125}$ I]BSA prepared (Table II) must reflect interbatch differences in the values of  $R$  or  $K$ , or even of both  $R$  and  $K$ . The simplest model conceptually would invoke a single type of "receptor" on the cell surface, with each individual receptor able to bind one molecule of substrate (i.e.  $R = \text{constant}$ ), different batches of albumin having different affinities (i.e. different  $K$ s) for these receptors. The present experiments do not afford any explanation of why each batch of [ $^{125}$ I]BSA behaved differently. Each batch was iodinated and acid treated by ostensibly the same procedure and

it must be assumed that small changes in the conformation of [ $^{125}$ I]BSA result in large changes in affinity for plasma membrane "receptors". It is certainly not unexpected that the rate of endocytic capture of a protein will be sensitive to small changes in its structure. Denatured proteins are removed from the circulation more rapidly than the undenatured species (22), and rat macrophages in vitro catabolize  $^{125}\text{I}$ -labeled rat albumin only if it is first heat denatured (23). In the case of glycoproteins, it is becoming clear (24) that removal of sialic acid residues increases susceptibility to uptake by the liver, presumably by pinocytosis. Further experiments are in progress to investigate this question.

From equation (3) it may be seen that the endocytic index for a solute can be independent of the concentration of solute in the external liquid. If entry is entirely in the bulk liquid phase, i.e. if  $R = 0$  (equation [4]), the endocytic index will be independent of concentration provided the rate of capture of liquid ( $F$ ) does not vary with change in substrate concentration. If entry is by both uptake of liquid and adsorption, the endocytic index will be independent of concentration only if two conditions both hold: neither  $F$  nor  $S$  must vary with changes of substrate concentration, and  $c$  must be insignificantly small in comparison either with  $K$  or with  $Ki/M$ . In our experiments, the endocytic indices of [ $^{125}$ I]PVP and [ $^{125}$ I]BSA were found to be independent of substrate concentration over a wide range. In the case of [ $^{125}$ I]PVP, entry could be entirely in the liquid phase, but in the case of [ $^{125}$ I]BSA this cannot be so. It is possible to conclude that the rates of vesicle formation ( $F$  and  $S$ ) are not affected by changes in concentration of [ $^{125}$ I]PVP or [ $^{125}$ I]BSA over the range studied. Unfortunately, and contrary to a statement made in a preliminary communication of this work (25), it is not possible to deduce anything about the value of  $K$  for [ $^{125}$ I]BSA. If calf serum were not needed for these cultures, independence of the endocytic index of [ $^{125}$ I]BSA of substrate concentration would have indicated that  $K$  was considerably higher than the highest concentration of [ $^{125}$ I]-BSA used. But in our experiments the concentration of [ $^{125}$ I]BSA used could be smaller than  $Ki/M$  ( $i$  is of course large), although larger than  $K$ .

The results described in this paper offer clear evidence that different macromolecules can be ingested pinocytically at different rates by an epithelial tissue such as the rat yolk sac without

attendant changes in the rate of formation of pinocytotic vacuoles. Selectivity can therefore be achieved through substrates having different degrees of adsorption to the plasma membrane that is being internalized. Thus, the rate of entry of a substrate will depend on two factors, the rate of pinocytotic vesicle formation and the affinity of the substrate for the plasma membrane. It is important to remember that the rate of vesicle formation may itself be altered by the presence of the substrate. Although the substrates used in the present experiments did not change the rate of vesicle formation within the range of substrate concentration studied, Cohn and Parks (26, 27) have provided morphological evidence that many substances can stimulate or inhibit pinocytosis in macrophages. It is our opinion that the technique described in this and the preceding paper offers opportunities not hitherto available for studying quantitatively the uptake of a variety of substances by micropinocytosis, and the effects of modifiers which could alter the rate of vesicle formation, compete with the substrate for binding sites on the membrane or change the rate of intracellular catabolism. Some effects of trypan blue on pinocytosis in this system have been reported by us (21), and the effects of other modifiers are currently being investigated.

The aim of this work has been to investigate pinocytosis as a general phenomenon. The rat yolk sac was chosen for study because of the ease with which it may be dissected free from adjacent tissues and cultured *in vitro*. However, the results do have some bearing on the functional significance of pinocytosis in the rodent and lagomorph yolk sac, a subject that has recently been reviewed by Wild (28). In the rabbit and guinea pig, and to some extent in the rat and mouse, passive immunity is conferred on the fetus by passage of immunoglobulin from the maternal to the fetal circulation through the yolk sac in the last few days before parturition. There is strong evidence that antibodies pass through and not between the yolk sac cells, and do so by entering pinocytotic vacuoles which are transported across the cell, presumably releasing their contents at the mesenchymal border. It is not clear how antibodies can pass across cells within the vacuolar system without being digested by the lysosomal proteinases. Heterologous antibodies or even excess homologous antibodies are taken in by the cells but they are digested. Wild (28) following Brambell (29), considers the evidence to favor a scheme in which

homologous antibodies attach to specific receptors on the cell surface which bear them into the cell through pinocytosis and in some way protect them from protease attack. Other proteins and excess antibody enter in the liquid phase of the pinocytotic vesicle and, not being protected, are digested within heterolysosomes. The present results add to the plausibility of this receptor hypothesis, because they show that uptake by adsorption to the walls of pinocytotic vacuoles is quantitatively very important in the near-term rat yolk sac at least for some proteins, and also that the strength of adsorption can vary widely with minor changes in the structure of a protein molecule. However, at least with [ $^{125}$ I]BSA, entry by attachment to receptors affords no protection against proteolytic degradation. These experiments, and our earlier ones (18), also confirm the ability of the yolk sac to digest proteins ingested by endocytosis, and are compatible with the observations of Fridhandler and Zipper (30) on the *in vitro* ingestion of [ $^{14}$ C]rat hemoglobin by the same tissue.

Because of the yolk sac's intense endocytic activity towards a wide range of substances, it has long been supposed that the tissue plays an important role in embryonic nutrition, particularly in early stages of development before the development of a functional chorioallantoic placenta (31). This view has recently received support from other types of experiment (32, 33). The present experiments show that the yolk sac can select between macromolecules presented to it and digest at least proteins to their monomeric building blocks. Thus the yolk sac could effectively serve a nutritional purpose by capturing macromolecules, digesting them, and passing on the products to the embryo for its anabolic purposes by way of the vitelline vessels which lie in the mesenchyme subjacent to the endocytic epithelial cells.

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## REFERENCES

1. RYSER, H. J.-P. 1968. Uptake of protein by mammalian cells: an underdeveloped area. *Science (Wash. D. C.)* **159**:390-396.
2. JACQUES, P. J. 1968. Epuration Plasmatique des Protéines Etrangères, leur Capture, et leur Destinée dans l'Appareil Vacuolaire du Foie. Librairie Universitaire, Louvain.
3. GORDON, A. H., and P. J. JACQUES. 1966. Distribution of injected <sup>131</sup>I- or <sup>125</sup>I-labelled homologous plasma proteins among subcellular particles of rat liver. *In* Labelled Proteins in Tracer Studies. L. Donato, G. Milhaud, and J. Sirchis, editors. Euratom. 127-133.
4. WILD, A. E. 1973. Fluorescent protein tracing in the study of endocytosis. *In* Lysosomes in Biology and Pathology, Volume 3. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 511-526.
5. BECK, F., J. B. LLOYD, and C. A. SQUIER. 1972. Histochemistry. *In* Lysosomes- a Laboratory Handbook. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 200-239.
6. STRAUS, W. 1964. Cytological observations on the relationship between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase. *J. Cell Biol.* **20**:497-507.
7. SCHUMAKER, V. N. 1958. Uptake of protein from solution by *Amoeba Proteus*. *Exp. Cell Res.* **15**:314-331.
8. EHRENREICH, B., and Z. A. COHN. 1967. The uptake and digestion of iodinated human serum albumin by macrophages *in vitro*. *J. Exp. Med.* **126**:941-958.
9. GABATHULER, M.-P., and H. J.-P. RYSER. 1969. The digestive function of lysosomes as studied by the turnover of ingested foreign macromolecules. *Proc. R. Soc. Lond. B. Biol. Sci.* **173**:95-98.
10. MEGO, J. L., F. BERTINI, and J. D. MCQUEEN. 1967. The use of formaldehyde-treated <sup>131</sup>I-albumin in the study of digestive vacuoles and some properties of these particles from mouse liver. *J. Cell Biol.* **32**:699-707.
11. LEE, D. 1971. The relative permeability of lysosomes from *Tetrahymena pyriformis* to some amino acids and dipeptides. *Biochim. Biophys. Acta.* **225**:108-112.
12. LLOYD, J. B. 1971. A study of permeability of lysosomes to amino acids and small peptides. *Biochem. J.* **121**:245-248.
13. EHRENREICH, B. A., and Z. A. COHN. 1969. The fate of peptides pinocytosed by macrophages *in vitro*. *J. Exp. Med.* **129**:227-243.
14. JACQUES, P. J. 1974. The endocytic uptake of macromolecules. *In* Pathologic Aspects of Cellular Membranes. B. J. Trump and A. Arstilla, editors. Academic Press, Inc., New York. In press.
15. WILLIAMS, K. E., E. M. KIDSTON, F. BECK, and J. B. LLOYD. 1975. Quantitative studies of pinocytosis. I. Kinetics of uptake of [<sup>125</sup>I]polyvinylpyrrolidone by rat yolk sac cultured *in vitro*. *J. Cell Biol.* **64**:113-122.
16. HOLTER, H. 1962. Pinocytosis. *In* Enzymes and Drug Action (Ciba Symposium). J. L. Mongar and A. V. S. de Reuch, editors. J. & A. Churchill Ltd., London. 30-42.
17. JACQUES, P. J. 1973. Endocytosis. *In* Lysosomes in Biology and Pathology. Volume 2. J. T. Dingle and H. B. Fell, editors. North-Holland Publishing Co., Amsterdam. 395-420.
18. WILLIAMS, K. E., J. B. LLOYD, M. DAVIES, and F. BECK. 1971. Digestion of an exogenous protein by rat yolk-sac cultured *in vitro*. *Biochem. J.* **125**:303-308.
19. MOUGEY, E. H., and J. W. MASON. 1963. Separation of some iodoamino acids and iodide by gel filtration. *Anal. Biochem.* **5**:223-233.
20. FAZAKERLEY, S., and D. R. BEST. 1965. Separation of amino acids, as copper chelates, from amino acid, protein, and peptide mixtures. *Anal. Biochem.* **12**:290-295.
21. WILLIAMS, K. E., J. B. LLOYD, M. E. KIDSTON, and F. BECK. 1973. Biphasic effect of trypan blue on pinocytosis. *Biochem. Soc. Trans.* **1**:203-206.
22. GORDON, A. H. 1973. The role of lysosomes in protein catabolism. *In* Lysosomes in Biology and Pathology, Vol. 3. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 89-137.
23. KIRSCH, R. E., L. O' C. FRITH, and S. J. SAUNDERS. 1972. Albumin catabolism *in vitro* by cultured peritoneal and pulmonary mononuclear phagocytes. *Biochim. Biophys. Acta.* **279**:87-91.
24. MORELL, A. G., G. GREGORIADIS, M. SCHEINBERG, J. W. HICKMAN, and G. ASHWELL. 1971. The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Biol. Chem.* **246**:1461-1467.
25. LLOYD, J. B., K. E. WILLIAMS, F. BECK, and M. E. KIDSTON. 1972. A kinetic analysis of pinocytosis in rat yolk-sac. *Biochem. J.* **128**:144 P-145 P.
26. COHN, Z. A., and E. PARKS. 1967. The regulation of pinocytosis in mouse macrophages. II. Factors inducing vesicle formation. *J. Exp. Med.* **125**:213-230.
27. COHN, Z. A., and E. PARKS. 1967. The regulation of pinocytosis in mouse macrophages. III. The induction of vesicle formation by nucleosides and nucleotides. *J. Exp. Med.* **125**:457-466.
28. WILD, A. E. 1973. Transport of immunoglobulins and other proteins from mother to young. *In* Lysosomes in Biology and Pathology. Vol. 3. J. T. Dingle, editor. North-Holland Publishing Co., Amsterdam. 169-215.
29. BRAMBELL, F. W. R. 1970. The Transmission of Passive Immunity from Mother to Young. North-Holland Publishing Co., Amsterdam.

30. FRIDHANDLER, L., and J. ZIPPER. 1964. Studies *in vitro* of rat yolk sac: biosynthetic activities, respiration and permeability to hemoglobin. *Biochim. Biophys. Acta.* **93**:526-532.
31. LLOYD, J. B., and F. BECK. 1969. Teratogenesis. In *Lysosomes in Biology and Pathology*, Vol. 1. J. T. Dingle and H. B. Fell, editors. North-Holland Publishing Co., Amsterdam. 433-449.
32. NEW, D. A. T., and R. L. BRENT. 1972. Effect of yolk sac antibody on rat embryos grown in culture. *J. Embryol. Exp. Morphol.* **27**:543-553.
33. PAYNE, G. S., and E. M. DEUCHAR. 1972. An *in vitro* study of functions of embryonic membranes in the rat. *J. Embryol. Exp. Morphol.* **27**:533-542.