# QUANTITATIVE STUDIES OF PINOCYTOSIS

## I. Kinetics of Uptake of [$^{125}$I]Polyvinylpyrrolidone by Rat Yolk Sac Cultured In Vitro

KENNETH E. WILLIAMS, ELIZABETH M. KIDSTON, FELIX BECK,

and JOHN B. LLOYD

From the Biochemistry Research Unit, Keele University, Staffordshire ST5 5BG, and the Anatomy Department, University of Leicester, LE1 7RH, England

ABSTRACT

A method is described for the in vitro culture of 17.5-day rat visceral yolk sac. Tissue survival was good as judged by light and electron microscopy. The rate of pinocytic uptake of $^{125}$I-labeled polyvinylpyrrolidone by the tissue was constant both within and between experiments. Within the concentration range 0.15–24 $\mu$g/ml, the $^{125}$I-labeled polyvinylpyrrolidone neither stimulated nor inhibited pinocytosis. The system offers many advantages in the quantitative study of the physical basis of pinocytosis.

Many important questions concerning pinocytosis remain unanswered. For example, do pinocytically ingested solutes enter chiefly in free solution, or adsorbed to the plasma membrane? By what means and to what extent can pinocytosis be stimulated and inhibited? Such questions can be answered only by experiments in which pinocytosis of substances is studied quantitatively. Unfortunately, although theoretical analyses of the pinocytic process have been made (1, 2), the experimental data available are inadequate to test their validity. Some data, such as those gathered on the clearance of macromolecules from the bloodstream of intact animals or from organ perfusates, are difficult to interpret because of the many unmeasurable physiological variables inherent in such systems. Studies with isolated cells or unicellular organisms cultured in vitro, although attractive because potentially simpler to interpret, have in general proved disappointing since the rate of pinocytic uptake has frequently been observed to change markedly within the course of a single experiment and the reproducibility of experiments has been poor. This is true of Gosselin's (1) data on the uptake of colloidal gold by rabbit peritoneal macrophages, regarded by Jacques (2) as the most complete analysis available, and of the data of Chapman-Andresen (3, 4) on uptake of several solutes by *Amoeba* species. Some authors have approached the quantitation of pinocytosis by measuring morphological parameters such as the number of pinocytic vesicles visible in a cell. In this way, Cohn (5, 6) has produced evidence on the stimulation of pinocytosis in macrophages by a range of chemicals, and Chapman-Andresen (3, 4) has published similarly on *Amoeba*. But, as Ryser (7) and Jacques (2) point out, changes in the rate of pinocytosis of a solute do not necessarily parallel changes in morphological parameters.

In this and the accompanying paper we present an experimental method that appears to have considerable advantages over previously described methods for studying quantitative aspects of pinocytosis. The tissue used is the visceral yolk sac of

the 17.5-day pregnant rat, maintained in organ culture. Some of the work has been reported briefly elsewhere (8, 9, 10).

## MATERIALS AND METHODS

### Organ Culture of Yolk Sac

Wistar rats, aged 3-6 mo, from an inbred laboratory colony were brother-sister mated overnight. If sperm were detected in the vagina next morning, pregnancy was timed from midnight of the night of the mating. At 17.5 days, rats were killed by cervical dislocation, conceptuses removed under sterile conditions, and yolk sacs dissected free from fetus, amnion, and placenta under culture medium, which consisted of 9 vol of medium 199 mixed with 1 vol of inactivated calf serum (both obtained from Wellcome Reagents, Beckenham, Kent, U.K.; preparations TC 20 and CS 07, respectively). The yolk sacs were opened out by small incisions from the cut edge originally adjacent to the chorioallantoic placenta (see Fig. 1), rinsed in three changes of sterile culture medium, and incubated in sterile Erlenmeyer flasks (50 ml) containing 9.0 ml of culture medium. The air in each flask was displaced by a mixture of oxygen and carbon dioxide (95:5) and the vessel stoppered with a sterile silicone-rubber bung. Flasks were placed in a Unitemp water bath (Baird & Tatlock [London] Ltd., Romford, Essex, U.K.) maintained at 37.0° ± 0.2°C, and the shaker attachment was regulated to a stroke of 3.4 cm at a frequency of 100 ± 5 strokes per min. After 15-20 min, 30 $\mu$g of $^{125}$I-labeled polyvinylpyrrolidone ([$^{125}$I]PVP; mean mol wt 30,000-40,000; preparation IM 33P from Radiochem-



FIGURE 1   Dissection of a 17.5-day rat yolk sac for organ culture.

ical Centre, Amersham, Bucks., U.K.) was added to each flask as a solution in 1.0 ml of culture medium. The flasks were then regassed, stoppered, and replaced in the water bath.

In experiments to determine the uptake of [$^{125}$I]PVP, yolk sacs were removed after a period of incubation, agitated three times for 2 min in changes of ice-cold 0.9% NaCl (30 ml) to remove extracellular substrate, and stored at $-20°C$ until assay. Before assay, each yolk sac was homogenized in water (2 ml) with a Virtis 45 homogenizer (Techmation Ltd., Edgware, Middx., U.K.) at top speed, and the suspension was diluted to 5.0 ml with water. Portions of the yolk sac homogenates (1.0 ml) and of the corresponding culture media (1.0 ml), each in a 3-ml disposable tube, were assayed for radioactivity with a gamma spectrometer (Packard Instruments Ltd., Caversham, Berks., U.K.). The protein content of each yolk sac was determined by the method of Lowry et al. (11) with bovine serum albumin (Sigma [London] Chemical Company Limited, London SW5; type II) as reference protein.

In experiments to measure the release of [$^{125}$I]PVP from cultured yolk sac, three yolk sacs were incubated separately for 6 h in the presence of [$^{125}$I]PVP (40 $\mu$g/ml), and each was transferred into a 10-ml portion of sterile, substrate-free culture medium and incubated for 2 min to remove extracellular substrate. This process was repeated twice more before the yolk sacs were incubated separately for a further 6 h in 10.0 ml of substrate-free culture medium. In another such experiment, 1% wt/vol PVP of average mol wt 40,000 (PVP-40; Sigma [London] Chemical Co. Ltd.) was added to the substrate-free culture medium used in both the wash and the reincubation. In both series of experiments, samples of culture media (two, each of 1.0 ml) were withdrawn in sterile pipettes and replaced by an equal volume of the appropriate fresh culture medium which had been warmed to 37°C and gassed. The activity in the tissue itself at the end of the 12-h overall culture period was assayed as described above, and the total activity released into the culture medium over the second 6-h period was calculated by the following formula:

$$T_n = 10C_{i(i=n)} + 2 \Sigma_{i=0}^{i=(n-1)} C_i$$

where $T_n$ is the radioactivity (counts per minute) released up to the time of the $n^{th}$ sampling, and $C_i$ the counts per minute per milliliter of culture medium of the $i^{th}$ sample after correcting for background.

### Light Microscopy

Yolk sacs were fixed in Bouin's fluid or Zenker's fixative, dehydrated, and embedded in paraffin wax. Bouin-fixed sections were stained with hematoxylin and eosin, and Zenker-fixed material was stained by Mallory's trichrome method (12).
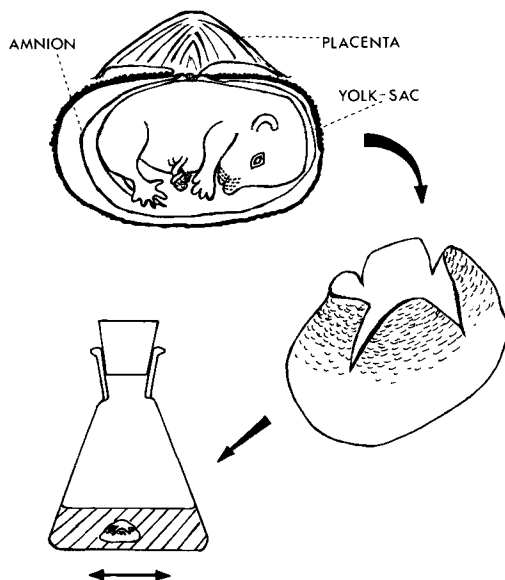
## Electron Microscopy

Small pieces of yolk sac were fixed at room temperature for 3 h in glutaraldehyde solution (4% vol/vol), washed at 4°C for 16 h in sucrose solution (7% wt/vol), then postfixed for 1.0 h in osmium tetroxide (1% wt/vol); all three solutions were made up in the same buffer (sodium cacodylate-HCl, pH 7.3). Araldite-embedded ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I.

## RESULTS

### Light Microscopy

Examination of sections from four uncultured yolk sacs showed the epithelium to be thrown into folds containing a sparse connective tissue core and resting upon a mesothelium with a well-developed basement membrane. The epithelial cells, which clearly formed the bulk of the tissue, showed basally located vesicular nuclei and extensive supranuclear vacuolation (Fig. 2). Extensive areas of five yolk sacs that had been incubated for 6 h in culture medium were also examined, along with a third group of sections from 16 yolk sacs incubated for the same time but in the presence of [$^{125}$I]PVP. Cultured tissue closely resembled uncultured tissue controls and the presence of [$^{125}$I]PVP had no detectable effect on the morphology.

Even after 24 h of culture (Fig. 3), when the occasional pycnotic nucleus could be observed, tissue survival was generally good and remained uninfluenced by the presence of [$^{125}$I]PVP.

### Electron Microscopy

Examination of several areas from two uncultured yolk sacs revealed well-developed microvilli at the apical border of the epithelial cells. The plasma membrane was invaginated into numerous caveolae lying between the microvilli and bearing a markedly thickened fuzzy coating on their external surface. The caveolae seemed to give rise to relatively electron-lucent vesicles lined with this same fuzzy coat. These vesicles lie in the same region as do smaller electron-dense microvesicles and canaliculi. The latter appear to fuse, giving rise to large electron-dense heterophagosomes (Fig. 4).

Sections from four separate yolk sacs, each incubated for 6 h in culture medium, showed detectable changes only in the vacuolar system of the epithelial cells, as compared with uncultured

tissue. The microvesicles and heterophagosomes were far less electron-dense, and the latter were larger, filled a greater portion of the apical cytoplasm, frequently contained myelin figures, and sometimes appeared to coalesce (Fig. 5). Surprisingly, the vacuolar system of tissue cultured for 24 h resembled more closely that of control tissue than that of tissue cultured for 6 h; the electron density of the heterophagosomes was restored, their size was reduced. and myelin figures were less numerous (Fig. 6).

The presence of [$^{125}$I]PVP in the culture medium was without influence on the ultrastructure at either 6 or 24 h.

### Method of Expressing Quantitative Data: Definition of Endocytic Index

It is desirable to express the amount of [$^{125}$I]PVP accumulated by a yolk sac in a form in which the reproducibility of the results between experiments can be assessed. The factors whose effects must be eliminated are the variable quantity of tissue in individual yolk sacs and the effect of radioisotope decay on the specific radioactivity of the substrate. For this reason uptake is expressed as the volume of culture medium whose contained substrate is captured by unit quantity of the yolk sac tissue, protein content being taken as a measure of the latter parameter. This volume (in microliters per milligram of tissue protein) is given by the expression $Y/MP$ where $Y$ is the total radioactivity (in counts per minute corrected for background) in the whole yolk sac, $M$ is the radioactivity (in cpm, corrected for background) per microliter of culture medium, and $P$ is the protein content (in milligrams) of the yolk sac. The rate of uptake of [$^{125}$I]PVP is derived from the plot of uptake (obtained as described above) against the durations of the respective incubations. The rate of uptake is here termed the endocytic index; its units are microliters per milligram of yolk sac protein per hour of incubation.

In all the experiments reported below, uptake of [$^{125}$I]PVP by the yolk sac resulted in a rate of fall of the [$^{125}$I]PVP concentration in the culture medium equivalent to approximately 0.1% per hour. Thus the concentration of [$^{125}$I]PVP in the medium is effectively constant for the duration of the incubations and the measured value of the radioactivity in the culture medium at the end of a culture period is used for $M$ in the above expression.

50μm

50μm

4μm

1μm
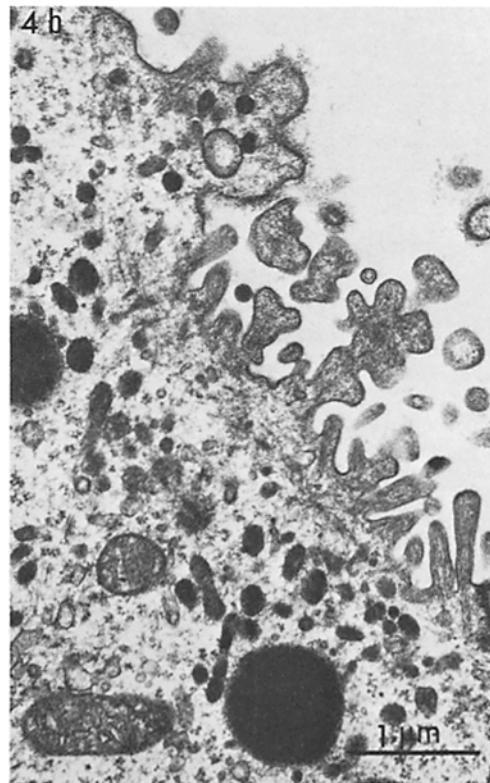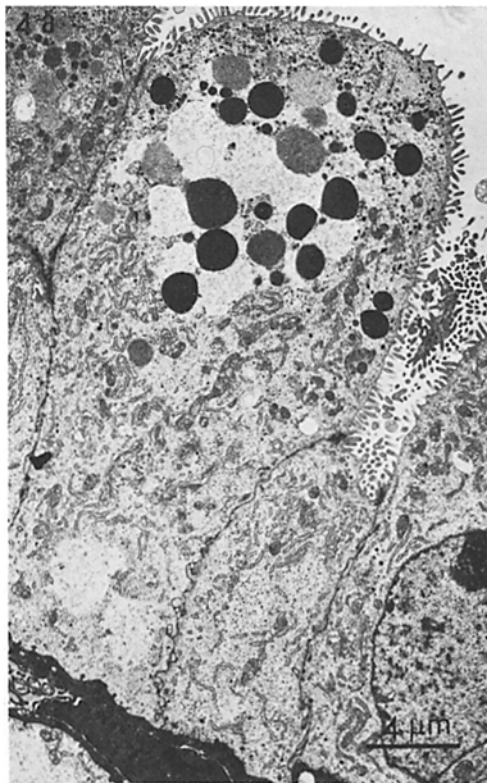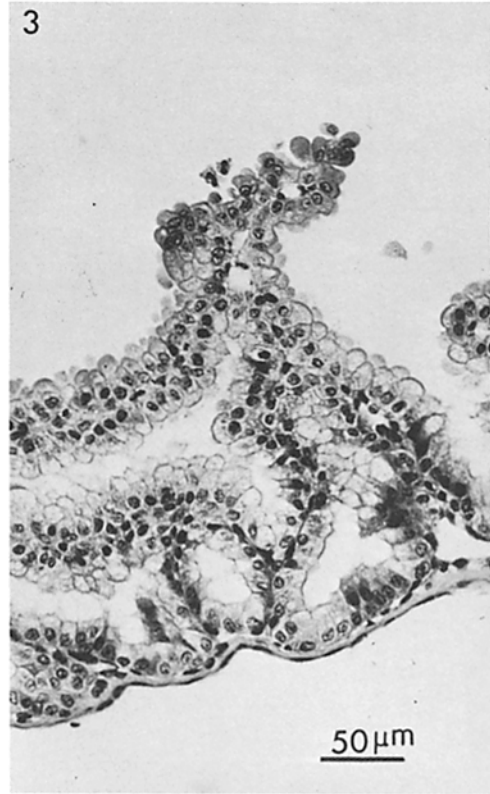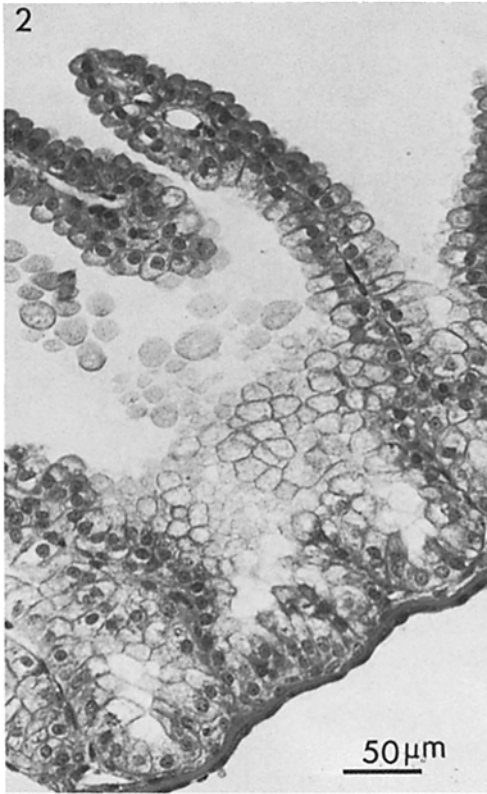
## Quantitative Data on the Uptake of [$^{125}I$]Labeled Polyvinylpyrrolidone

Fig. 7 shows the result of a typical experiment in which six yolk sacs from the same rat were incubated in culture medium containing 3 μg [$^{125}I$]PVP/ml, incubations being terminated at intervals up to 8 h. The rate of uptake is constant over this period. The results of 12 similar experiments were each subjected to a linear regression analysis and Table I indicates the correlation coefficient, the slope, and the intercept on the ordinate axis of the computer-fitted regression line through each set of experimental points. The slope of the regression line gives the endocytic index, i.e. the rate of uptake of [$^{125}I$]PVP from the culture medium by the tissue.

A series of statistical tests (13) was applied to the data of Table I to determine whether the variation between the 12 sets of data, each of which had been derived from observations on tissues from a single pregnant animal, was greater than the variation within each of the separate sets of data considered individually. The tests employed constituted an analysis of covariance in which variance ratios were calculated and used to determine whether either the slope or the intercept from each of the 12 separate plots of accumulation against time possessed individually distinct values for these two parameters, or whether interexperimental variation was no greater than intraexperimental variation. The variance ratios calculated indicated that, at the 10% significance level, there was no detectable difference in values of the slopes and intercepts. Hence it was legitimate to pool the uptake data from the 12 experiments to give a single overall plot, from which a single value of both the slope and intercept could be derived, along with associated confidence limits.

The regression line for the 64 experimental points (Fig. 8) of the 12 experiments had a slope of 1.71 μl/h/mg yolk sac protein (95% confidence limits 1.61, 1.83) and an intercept on the ordinate axis of 0.73 μl/mg yolk sac protein (95% confidence limits 0.26, 1.20).

## Relationship of Rate of Accumulation of [$^{125}I$]PVP to Rate of Its Endocytic Uptake

The rate of accumulation of [$^{125}I$]PVP as determined in the above experiments will be equal to the rate of endocytic uptake only if captured [$^{125}I$]PVP is not released from the tissue. Fig. 9 shows the release of radioactivity from yolk sacs that had accumulated [$^{125}I$]PVP during a 6-h period in culture, been washed three times for 2 min in substrate-free culture medium, then been reincubated for 6 h in substrate-free culture medium. From 2 to 3% of the total radioactivity remaining in the tissue after the washing procedure was released within 30 min and a further 2-3% was released progressively over the next 5.5 h. Even when a high concentration (1% wt/vol) of non-iodinated PVP was included in the reincubation culture medium (Fig. 10), the extent and pattern of release of radioactivity was essentially unchanged.

The 2-3% loss of radioactivity within 30 min of reincubation is most easily explained as the washing off of occluded or adsorbed extracellular radioactivity that remains after the standard washing procedure. This explanation is compatible with the observation that the regression line through the pooled data for uptake of [$^{125}I$]PVP (Fig. 8) has a small but positive intercept on the ordinate axis. The slow progressive release that followed must include the contributions from cell death or detachment, from exocytosis, and from further desorption, although it is unlikely that the latter is a significant component in view of the rate of release being unaffected by the presence of high concentrations of noniodinated PVP.

## Relationship of Endocytic Index to [$^{125}I$]PVP Concentration

Further experiments were performed in standard culture medium but with different concentrations of [$^{125}I$]PVP in the medium. At each substrate concentration, uptake proceeded at a constant rate up to 7 h, and Fig. 11 shows that the endocytic index is independent of substrate concentration within the range 0.15-24 μg/ml. The

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FIGURE 2   Unincubated visceral layer of yolk sac from 17.5-day pregnant rat. × 230.

FIGURE 3   Visceral layer of yolk sac from 17.5-day pregnant rat incubated for 24 h. Occasional pycnotic nuclei may be seen. × 230.

FIGURE 4   (a) Visceral yolk sac epithelial cell from unincubated tissue. × 3,050. (b) Apical portion of visceral yolk sac epithelial cell from unincubated tissue. × 17,000.
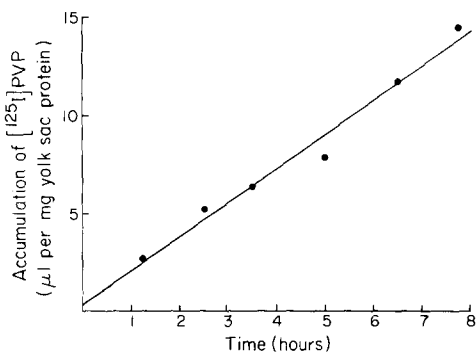
5a

3 μm

5b

2 μm

6a

3 μm

6b

1 μm
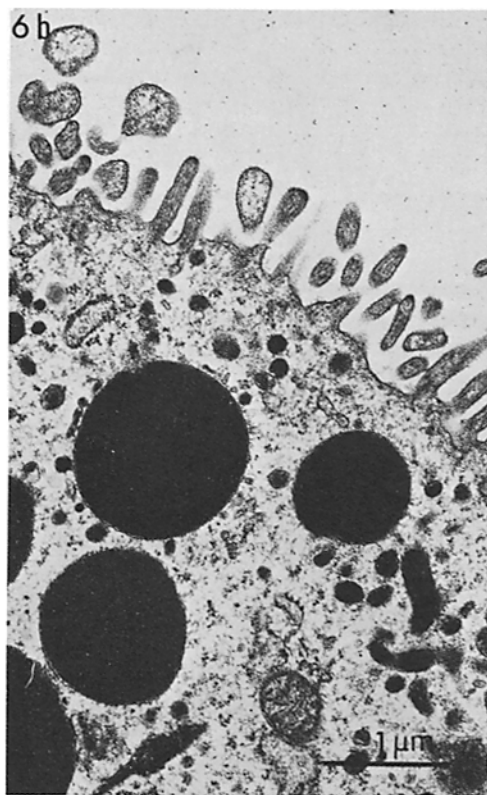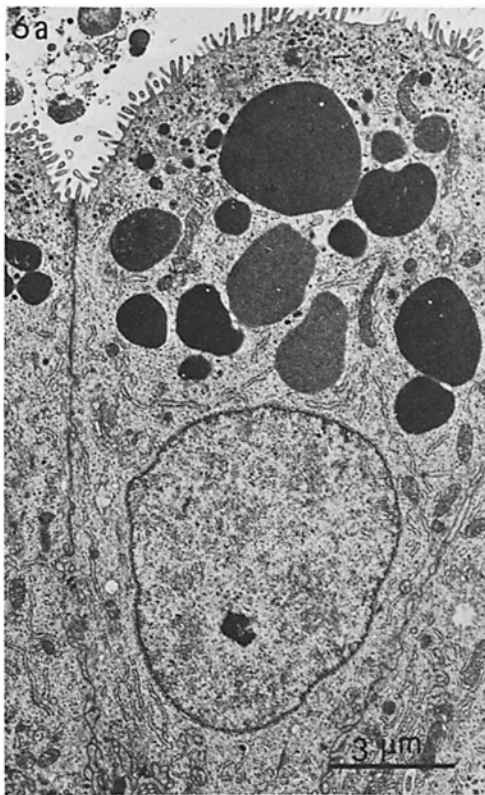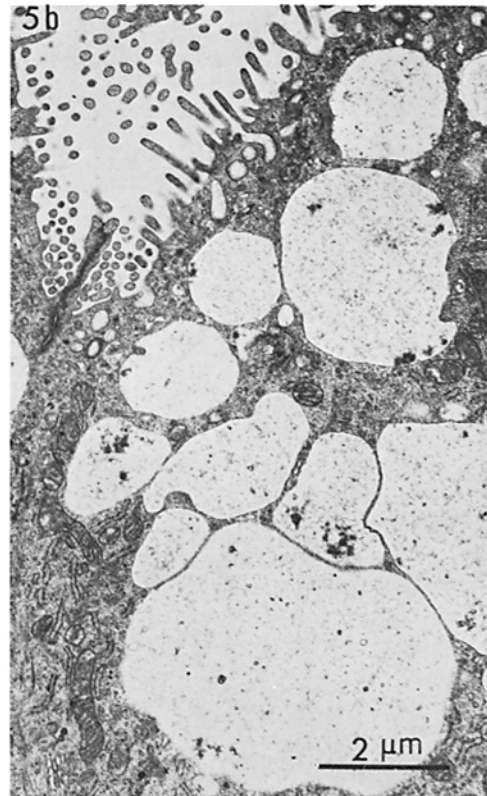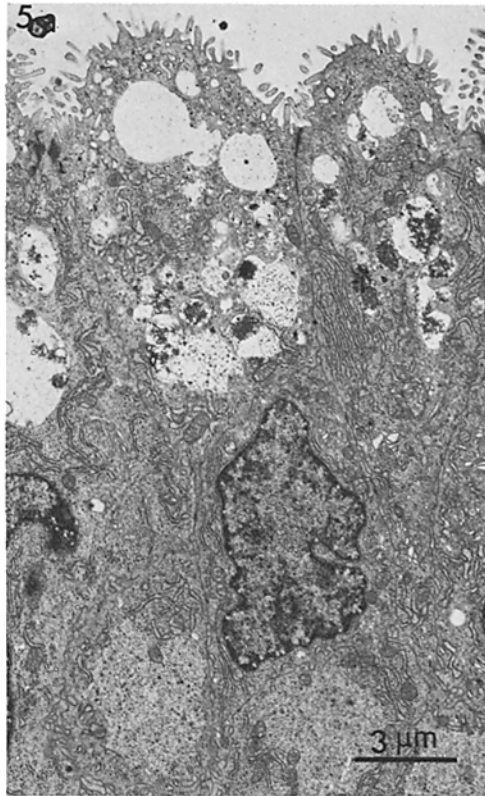
FIGURE 7 Accumulation of radioactivity by yolk sacs incubated in the presence of [125I]PVP (3.0 μg/ml). Each point is derived from data on a single yolk sac. Accumulation is expressed as the number of microliters of culture medium whose contained [125I]PVP has been ingested by unit quantity of tissue in a given time (see text).

TABLE I

*Correlation Coefficient, Slope, and Intercept on the Ordinate Axis for the Regression Lines Through Each Set of Data from 12 Separate Experiments on the Uptake of [125I]PVP by Rat Yolk Sac Cultured in Vitro*

| Experiment no. | Correlation coefficient | Slope | 95% Confidence limits of slope | Intercept on ordinate axis |
|---|---|---|---|---|
| | | μl/h/mg protein | μl/h/mg protein | μl/mg protein |
| 1 | 0.995 | 1.82 | 1.49–2.16 | 0.156 |
| 2 | 0.997 | 1.86 | 1.63–2.10 | 1.080 |
| 3 | 0.996 | 1.93 | 1.68–2.20 | -0.072 |
| 4 | 0.978 | 1.57 | 1.10–2.05 | 0.972 |
| 5 | 0.989 | 1.75 | 1.38–2.11 | 0.359 |
| 6 | 0.957 | 1.43 | 0.82–2.05 | 0.849 |
| 7 | 0.894 | 1.57 | 0.47–2.67 | 1.716 |
| 8 | 0.974 | 1.73 | 1.19–2.29 | 0.748 |
| 9 | 0.988 | 1.81 | 1.28–2.33 | -0.010 |
| 10 | 0.994 | 1.75 | 1.19–2.32 | 0.193 |
| 11 | 0.980 | 1.65 | 0.66–2.64 | 1.350 |
| 12 | 0.982 | 2.00 | 0.86–3.14 | 0.134 |

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 was determined at higher substrate concentrations (100 and 1,000 μg/ml), but from fewer experimental points because of the high cost of such experiments. The values obtained lay within the limits of those shown in Fig. 11.
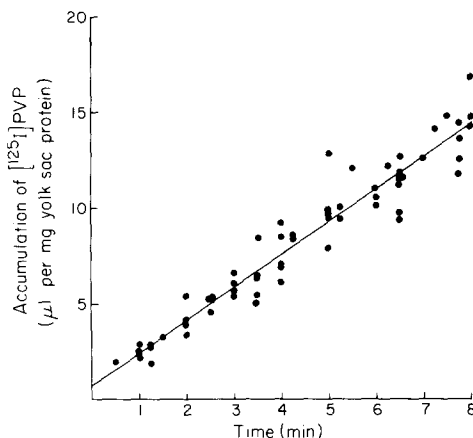


FIGURE 8 Accumulation of radioactivity by yolk sac incubated in the presence of [125I]PVP. The 64 points are derived from 12 separate experiments, each using yolk sacs taken from one animal only. Each of the points corresponds to data from a single yolk sac.
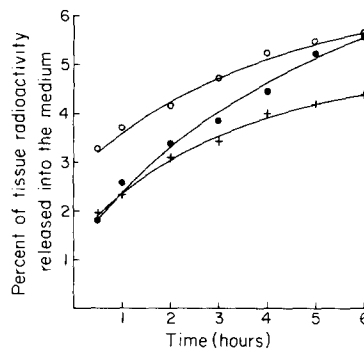


FIGURE 9 Release of [125I]PVP from yolk sacs on reincubation in culture medium containing no [125I]PVP. Each plot corresponds to a separate experiment in which release from a single yolk sac was monitored.

FIGURE 5 (a) Visceral yolk sac epithelial cells from tissue incubated for 7 h. × 4,500. (b) Apical portion of visceral yolk sac epithelial cell incubated for 7 h. × 8,620.

FIGURE 6 (a) Visceral yolk sac epithelial cells from tissue incubated for 24 h. × 4,600.

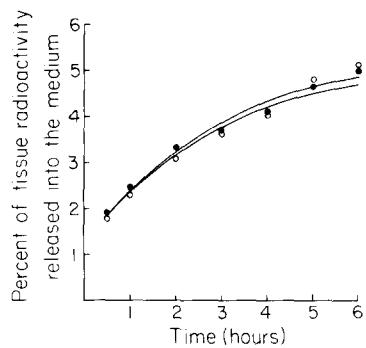FIGURE 6 (b) Apical portion of visceral yolk sac epithelial cell from tissue incubated for 24 h. × 17,350.

FIGURE 10   Release of [$^{125}$I]PVP from yolk sacs on reincubation in culture medium containing 1% (wt/vol) noniodinated PVP but no [$^{125}$I]PVP. Each plot corresponds to a separate experiment in which release from a single yolk sac was monitored.
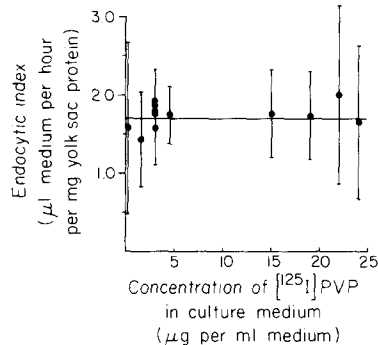


FIGURE 11   Endocytic index of [$^{125}$I]PVP at different concentrations of [$^{125}$I]PVP in the incubation medium. 95% confidence limits are shown.

## DISCUSSION

The histological and electron microscope appearances of the rat visceral yolk sac have been extensively documented (14, 15), and it is clear from these studies that micropinocytosis is a characteristic activity of all its epithelial cells. The fate of ingested compounds has been followed histochemically by both light and electron microscopy, and a variety of macromolecules, including horeseradish peroxidase (16, 17), Triton WR-1339 (18), Trypan Blue (19, 20, 21), and ferritin (21, 22), have been shown to congregate in secondary lysosomes of the epithelial cells. In an experiment in which $^{125}$I-labeled bovine serum albumin was adminsitered in vivo (23), it was shown that ingestion was followed by intracellular breakdown to the level of free amino acids and small peptides, providing further evidence of penetration into

lysosomes. Since [$^{125}$I]PVP is a macromolecule (mean mol wt 30,000–40,000), its uptake into yolk sac tissue is presumably accomplished by micropinocytosis, although direct evidence of this is lacking. Likewise, the site of intracellular accumulation may be supposed to be the vacuolar system, [$^{125}$I]PVP being resistant to digestion by lysosomal enzymes.

In this study, the electron microscope appearance of unincubated visceral yolk sac is consistent with the observations of Jollie and Triche (15) and adds nothing to their description and interpretation. Although the micropinocytic vesicles and the heterophagosomes situated more deeply in the cell appear larger and less electron dense after 6 h of incubation when compared with unincubated controls, there can be no doubt that active micropinocytosis is continuing throughout the culture period. It is legitimate, therefore, to use this in vitro system for quantitative studies of pinocytosis of the epithelial cells of visceral yolk sac although the measured values cannot be assumed to reflect those that obtain in vivo.

The quantitative results show that the yolk sac progressively accumulates [$^{125}$I]PVP when incubated in culture medium containing this substrate. The experiments in which the release of radioactivity from yolk sacs preincubated with [$^{125}$I]PVP was studied, while not excluding the possibility of limited exocytosis of ingested [$^{125}$I]PVP, certainly indicate that it is of no quantitative significance. Hence, the rate of accumulation of [$^{125}$I]PVP by yolk sacs is a true measure of the rate of endocytic capture.

In our experiments, in which each experimental point represents data from a single yolk sac taken from the same animal, the rate of uptake of [$^{125}$I]PVP was constant for an incubation period of 8 h. Additional experiments, not reported, showed that the rate of uptake did not measurably decrease in cultures maintained to 12 h. Furthermore, the rate of uptake was constant from one experiment to another. A comparable degree of linearity and reproducibility has not previously been reported in any organ or cell culture system. The system makes possible quantitative assessment of the effects of substances that stimulate or inhibit pinocytosis, and indeed the system has already been used in this way (24, 25).

The rate of uptake of [$^{125}$I]PVP has been expressed in this paper as the volume of external liquid whose substrate content has been internal-

ized by unit quantity of tissue in unit time, a function we have designated endocytic index. It must be stressed that the endocytic index does not necessarily correspond to the rate of ingestion of liquid by the cells. If any [$^{125}$I]PVP enters the tissue by virtue of adsorption on the plasma membrane, the measured endocytic index will be greater than the rate of ingestion of liquid. Thus, endocytic indices derived from experiments with different substrates would not necessarily be identical even if the rate of internalization of membrane and external liquid were the same for each substrate. This possibility is examined experimentally in the following paper (26).

If the endocytic index derived from experiments with a particular substrate is unaffected on varying the concentration of substrate, it means that the rate of uptake of substrate is directly proportional to the substrate concentration. In the experiments on [$^{125}$I]PVP reported here, the endocytic index was constant over the concentration range 0.15–24.0 $\mu$g/ml medium. It appears, therefore, that within this range [$^{125}$I]PVP neither stimulates nor inhibits pinocytosis.

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