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Effect of molecular size of ^{125}I-labelled poly(vinylpyrrolidone) on its pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages

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Rates of pinocytosis of different molecular-weight distributions of ^{125}I-labelled poly(vinylpyrrolidone) by rat visceral yolk sacs and rat peritoneal macrophages were measured *in vitro*. Four preparations of mean molecular weights 50000, 84000, 700000 and 7000000, were used. Macrophages captured the highest-molecular-weight preparation more rapidly than the other preparations. In contrast, rate of capture by the yolk sac decreased with increasing molecular weight. Incubations with a very-high-molecular-weight fraction derived from the 7000000-average-mol.wt. preparation clearly demonstrated that very large polymer molecules are not accumulated by the yolk sac, but are preferentially captured by macrophages. Analysis of the ^{125}I-labelled poly(vinylpyrrolidone) internalized by the two cell types confirmed that low-molecular-weight material is preferred by the yolk sac, whereas the macrophage is less discriminating.

The rate at which macromolecular substrates are captured by pinocytosis depends on the nature of the substrate and the cell type in question (Pratten *et al.*, 1980). The properties of the substrate may be important in two ways: first, the size and configuration of a macromolecule must determine whether it can be trapped by the pinosomes forming at the plasma membrane; secondly, provided that the substrate is not so large as to be excluded from the nascent pinosomes, its affinity for the membrane greatly affects its rate of uptake. Using the rat yolk sac and rat peritoneal macrophages as model systems to study pinocytosis, we have previously shown that many different macromolecules are captured at rates that reflect their affinity for the plasma membrane (Williams *et al.*, 1975; Moore *et al.*, 1977; Roberts *et al.*, 1977; Pratten *et al.*, 1977), and we have also shown that it is possible to increase the affinity of colloidal [^{198}Au]gold for the plasma membranes of both cell types, by addition of polycations to the culture media (Pratten *et al.*, 1978; Duncan *et al.*, 1979).

The relationship between the size of a substrate and its rate of pinocytic uptake has been investigated by Bartholeyns & Baudhuin (1976) and Kooistra *et al.* (1977), who examined the pinocytosis of monomers and oligomers of ribonuclease A. Both groups reported a positive correlation between substrate size and rate of uptake. Although these data reveal an interesting relationship between size and membrane affinity, they do not help to elucidate the substrate-size limitations of a defined pinocytic system. The first study used two relatively small ribonuclease preparations (monomer and dimer) and the second, although employing a somewhat larger range of substrate sizes, was carried out *in vivo*, so that the substrate was exposed to cells with various endocytic capacities.

Here we have attempted to dissect the effect of molecular size from that of other substrate characteristics. In order to eliminate chemical differences between substrates, we have used the same polymer in several size ranges. The polymer chosen was poly(vinylpyrrolidone), which previous experiments (Roberts *et al.*, 1977; Pratten *et al.*, 1977) have shown does not adsorb to the plasma membrane of either yolk sac or macrophages, but enters these cells solely by fluid-phase pinocytosis. Four different size distributions of poly(vinylpyrrolidone) were labelled with [^{125}I]iodine, and their rates of capture by rat visceral yolk sacs and rat peritoneal macrophages were measured. After incubation of the two cell types with ^{125}I-labelled poly(vinylpyrrolidone), the material internalized was examined to assess

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whether cells could specifically select substrate from the size range to which they were exposed.

A preliminary report of some of this work has been published (Duncan *et al.*, 1980).

Materials and methods

^{125}I-labelled poly(vinylpyrrolidone) and Na^{125}I (preparations IM.33P and IMS.30) were from The Radiochemical Centre, Amersham, Bucks., U.K. Four different molecular-weight distributions of poly(vinylpyrrolidone), mean mol.wts. 50000, 84000, 700000 and 7000000, were from BASF, Ludwigshafen/Rhine, Germany. Pronase and papain were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Preparation of ^{125}I-labelled poly(vinylpyrrolidone)

The method of Regoeczi (1976) was used to label the four poly(vinylpyrrolidone) samples with [^{125}I]-iodide. The product was freed from excess [^{125}I]-iodide by adding solid KI (100mg) and dialysing for several days against 1% (w/v) NaCl.

Analysis of ^{125}I-labelled poly(vinylpyrrolidone) preparations

Electrophoresis on Whatman no. 1 paper, in 0.01M-barbitone buffer, pH 8.6, at 400V for 35min, was used to assess the free [^{125}I]iodide content of the preparations and thus the efficiency of the polymer-labelling procedure and the stability of the labelled polymers during storage at 4°C or incubation at 37°C.

The four radiolabelled poly(vinylpyrrolidone) preparations were subjected to column chromatography to assess their relative molecular-weight distributions. Samples were applied to Sepharose 6B columns and eluted with 0.02M-sodium acetate containing NaN_3 (0.05%, w/v).

Uptake of ^{125}I-labelled poly(vinylpyrrolidone) by rat visceral yolk sacs and rat peritoneal macrophages

Methods used to determine the uptake of ^{125}I-labelled poly(vinylpyrrolidone) by yolk sacs and macrophages have been previously described (Williams *et al.*, 1975; Pratten *et al.*, 1977). To cultures of rat yolk sacs was added ^{125}I-labelled poly-(vinylpyrrolidone) to a concentration of 20μg/ml, for material prepared in the laboratory, or 2μg/ml for that from The Radiochemical Centre. For experiments with macrophages it was necessary to increase these concentrations to 200 and 10μg/ml respectively. An exception to the regime was the 7000000-mean-mol.wt. material, which was used at one-fifth the above concentrations.

The rate of uptake of radiolabelled substrate was calculated as an Endocytic Index, defined as the volume of culture medium (μl) whose contained substrate is captured per mg of yolk-sac protein (or 10^6 macrophage cells) per h. Experiments were also carried out in which [^{125}I]iodide was added to both the yolk sac and macrophage culture medium and its accumulation by these cell types was estimated in the units defined above. The ^{125}I-labelled poly-(vinylpyrrolidone) of mean mol.wt. 7000000 was subfractionated by Sepharose 6B column chromatography to prepare a sample that contained only the very-highest-molecular-weight material. The rate of uptake of this fraction by both cell types was measured.

Analysis of ^{125}I-labelled poly(vinylpyrrolidone) captured by rat visceral yolk sacs and rat peritoneal macrophages

To determine whether these two cell types select material from the molecular-weight distributions presented to them, yolk sacs and macrophages were incubated in medium containing higher concentrations of ^{125}I-labelled poly(vinylpyrrolidone), either that supplied by The Radiochemical Centre (at 20μg/ml for yolk sacs and 100μg/ml for macrophages) or the highest-molecular-weight range (mean mol.wt. 7000000) produced in the laboratory (at a concentration of 200μg/ml and 500μg/ml respectively).

In these experiments three visceral yolk sacs were incubated together in a single flask (contrasting with the normal incubation conditions, where yolk sacs were incubated separately). Similarly macrophage cultures contained 2–3 times the usual number of cells per culture vesssel. Incubations of yolk sacs were for 6h and, after washing in ice-cold 1% NaCl, the tissues were dissolved by incubation for 3 days at 37°C in 5ml of an aqueous solution containing both Pronase and papain (each 1mg/ml). Macrophages were incubated overnight and washed in phosphate-buffered saline (Pratten *et al.*, 1977) before digestion in the solution of Pronase and papain (0.5ml was added to each Leighton tube).

Digests were analysed by applying to Sepharose 6B or Sephadex G-200 columns and eluting with 0.02M-sodium acetate containing NaN_3 (0.05%, w/v).

Results

Characteristics and stability of ^{125}I-labelled poly-(vinylpyrrolidone) preparations

Labelling efficiencies varied from 18 to 44%. After dialysis the preparations contained less than 3% free [^{125}I]iodide, except for the lowest-molecular-weight sample, which still retained over 12% after prolonged dialysis. During storage at 4°C there was a slow release of [^{125}I]iodide from all four size ranges, the rate of release being approx. 0.2%/day in each case. Although all the samples released

[^{125}I]iodide faster when incubated at 37°C in culture medium, the maximum increase in [^{125}I]iodide content was 0.2%/h and therefore not sufficient to necessitate any correction when estimating uptake of radiolabelled polymer by cells.

Fig. 1 shows the elution patterns of the various preparations of ^{125}I-labelled poly(vinylpyrrolidone) from Sepharose 6B. Each preparation was radiolabelled over a wide molecular-weight spectrum and, although there is some overlap of the sizes present within the different samples, the mean molecular weights are still distinctly different. The Sepharose-6B column was calibrated with proteins of known molecular weight, but the molecular weights of the ^{125}I-labelled poly(vinylpyrrolidone) preparations may not be confidently derived from these data, owing to uncertainty about the molecular configuration of the synthetic polymer.

Incubations with [^{125}I]iodide

When yolk sacs or macrophages were incubated in the presence of [^{125}I]iodide, there was no progressive accumulation of radioactivity (results not shown). Presumably [^{125}I]iodide crosses biological membranes with ease, and its intracellular concentration rapidly equilibrates with that in the extracellular environment. This result is important, since it justifies the use in subsequent experiments of samples of ^{125}I-labelled poly(vinylpyrrolidone) that contain some free [^{125}I]iodide.

Uptake of ^{125}I-labelled poly(vinylpyrrolidone) by rat yolk sacs

The uptake of all four sizes of ^{125}I-labelled poly(vinylpyrrolidone) by the rat yolk sac was linear with time. Table 1 shows the mean Endocytic Indices derived from several experiments with each size range. The Endocytic Index of the lowest-molecular-weight preparation was not significantly different from that of The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone). Samples containing the larger polymer molecules were internalized more slowly, the lowest relative Endocytic Index being observed with the sample of mean mol.wt. 700000.

A subfraction of the labelled preparation of mean mol.wt. 7000000 was separated so that it contained no low-molecular-weight material (see Fig. 1*e*). When yolk sacs were incubated with this very-high-molecular-weight material, little accumulation by the tissue was seen (Table 1).

None of the unlabelled poly(vinylpyrrolidone) samples, when present at 100μg/ml, changed the rate of uptake of The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone) (results not shown).

Uptake of ^{125}I-labelled poly(vinylpyrrolidone) by rat peritoneal macrophages

Uptake of all four preparations of ^{125}I-labelled poly(vinylpyrrolidone) was linear with time, and Table 1 shows the Endocytic Indices. A significantly increased rate of uptake was observed with the highest-molecular-weight sample. The high-molecular-weight fraction from the 7000000-mean-mol.wt. preparation was taken up even more avidly (Table 1).

As with yolk sacs, the presence of the samples of unlabelled poly(vinylpyrrolidone) (100μg/ml) did not greatly affect the rate of uptake of The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone) (results not shown).

Size distributions of the ^{125}I-labelled poly(vinylpyrrolidone) captured by rat yolk sacs and rat peritoneal macrophages

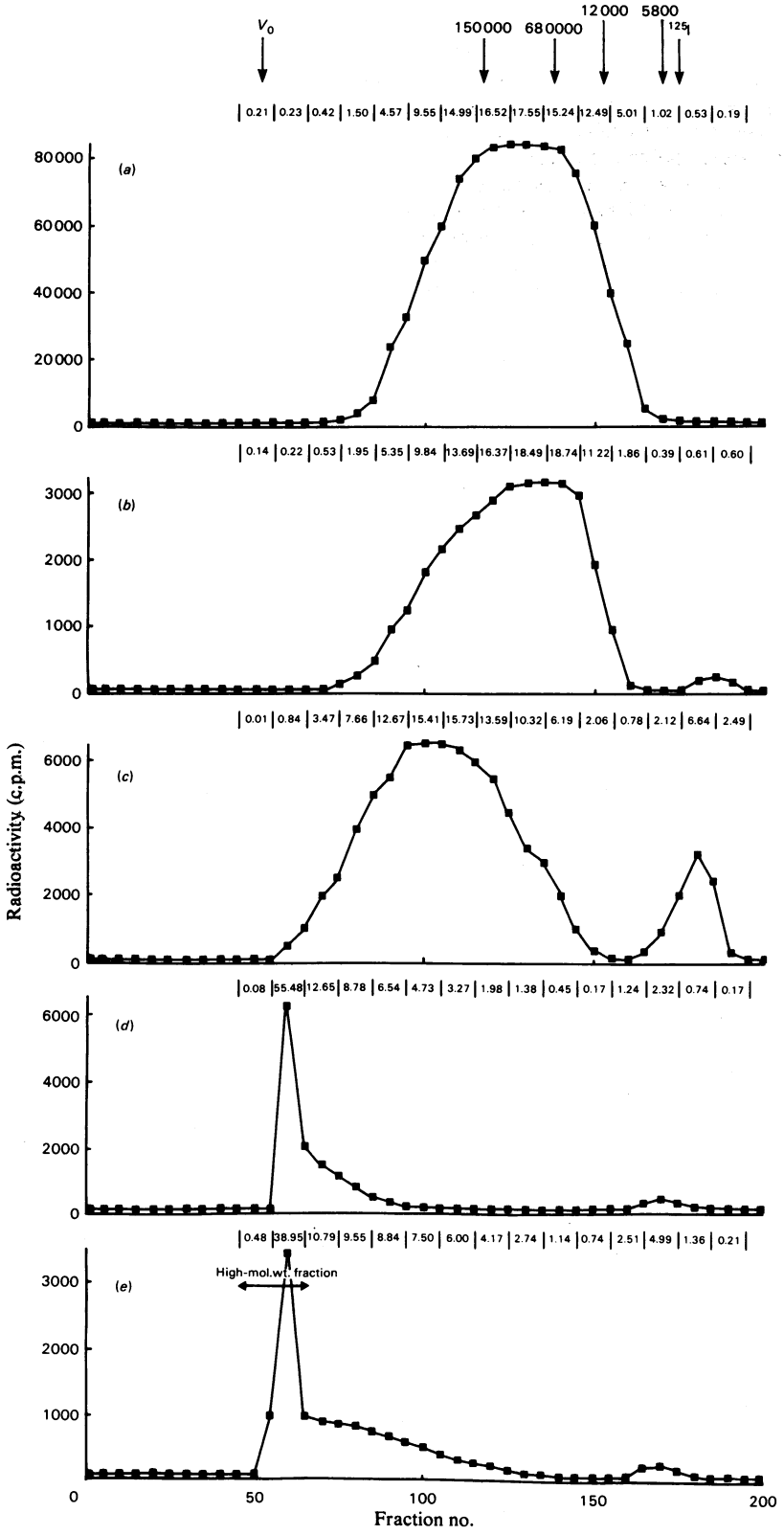
The elution profiles from Sephadex G-200 of

Table 1. *Uptake of four preparations of ^{125}I-labelled poly(vinylpyrrolidone) of different molecular-weight distribution by rat visceral yolk sacs and rat peritoneal macrophages*

Each experiment was conducted concurrently with a matched control performed with ^{125}I-labelled poly(vinylpyrrolidone) obtained from The Radiochemical Centre. Rates of uptake are expressed as Endocytic Indices (mean ± S.E.M. of at least three experiments). Each Endocytic Index was then expressed as a percentage of the Endocytic Index of the matched control, and the mean of these percentages is the value shown in parentheses.

Mean mol.wt. of ^{125}I-labelled poly(vinylpyrrolidone) preparation	Rate of uptake by yolk sacs (μl/mg of protein per h)	Rate of uptake by macrophages (μl/10^6 cells per h)
50000	2.14* ± 0.14 (104)	0.060* ± 0.016 (158)
84000	1.14 ± 0.18 (68)	0.061 ± 0.009 (184)
700000	1.13 ± 0.13 (53)	0.052 ± 0.015 (149)
7000000	1.73 ± 0.15 (68)	0.109 ± 0.036 (331)
High-mol.wt. fraction from 7000000-mean-mol.wt. sample	0.18 ± 0.23 (9.05)	0.130 ± 0.041 (686)

* Since this preparation contained a significant amount of [^{125}I]iodide, it was necessary when calculating Endocytic Indices to adjust the measured values of radioactivity in media, in order to determine the radioactivity attributable to ^{125}I-labelled poly(vinylpyrrolidone).


V_0
150000
68000
12000
5800
^{125}I
0.21 | 0.23 | 0.42 | 1.50 | 4.57 | 9.55 | 14.99 | 16.52 | 17.55 | 15.24 | 12.49 | 5.01 | 1.02 | 0.53 | 0.19
(a)
80000
60000
40000
20000
0
0.14 | 0.22 | 0.53 | 1.95 | 5.35 | 9.84 | 13.69 | 16.37 | 18.49 | 18.74 | 11.22 | 1.86 | 0.39 | 0.61 | 0.60
(b)
3000
2000
1000
0
0.01 | 0.84 | 3.47 | 7.66 | 12.67 | 15.41 | 15.73 | 13.59 | 10.32 | 6.19 | 2.06 | 0.78 | 2.12 | 6.64 | 2.49
(c)
6000
4000
2000
0
Radioactivity (c.p.m.)
0.08 | 55.48 | 12.65 | 8.78 | 6.54 | 4.73 | 3.27 | 1.98 | 1.38 | 0.45 | 0.17 | 1.24 | 2.32 | 0.74 | 0.17
(d)
6000
4000
2000
0
0.48 | 38.95 | 10.79 | 9.55 | 8.84 | 7.50 | 6.00 | 4.17 | 2.74 | 1.14 | 0.74 | 2.51 | 4.99 | 1.36 | 0.21
(e)
High-mol.wt. fraction
3000
2000
1000
0
50
100
150
200
Fraction no.

material captured by yolk sacs or macrophages during incubation with The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone) are shown in Fig. 2. The size distribution of the material presented to the cells is also shown. Fig. 3 shows similar data, obtained by Sepharose 6B chromatography, when the ^{125}I-labelled poly(vinylpyrrolidone) preparation of mean mol.wt. 7000000 was used as substrate.

Visual inspection of Fig. 2 suggests that, although macrophages capture a representative distribution of polymer molecules from the size-range available, yolk-sac cells have some preference for smaller molecules. However, when the elution patterns of the captured material were compared, by χ-squared test, with that of the substrate, no significant differences were found.

The apparent selectivity of the yolk sac was even more striking when the 7000000-mean-mol.wt. preparation was used as a substrate (Fig. 3). In this case the distribution of material captured was

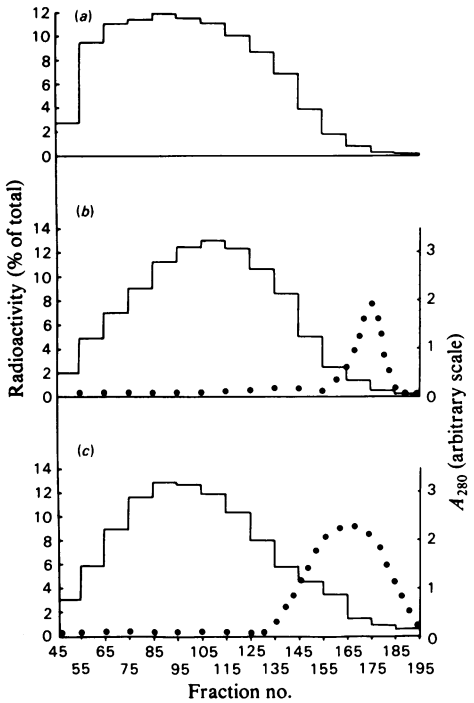


Fig. 2. *Elution from Sephadex G-200 of radioactivity from digests of yolk sacs and macrophages preincubated with ^{125}I-labelled poly(vinylpyrrolidone) from The Radiochemical Centre*

The histograms show the radioactivity measured in groups of ten consecutive fractions, expressed as a percentage of the total radioactivity recovered from the column. The elution profiles represent (*a*) The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone) incubated for 3 days with Pronase and papain, (*b*) a yolk-sac digest, (*c*) a macrophage digest. The curves shown by ● in (*b*) and (*c*) represent the A_{280}.

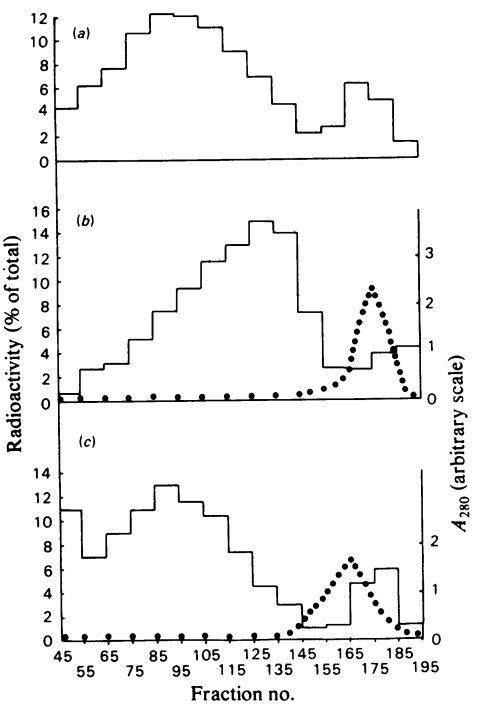


Fig. 3. *Elution from Sepharose 6B of radioactivity from digests of yolk sacs and macrophages preincubated with ^{125}I-labelled poly(vinylpyrrolidone) preparation of mean mol.wt. 7000000*

The histograms show the radioactivity measured in groups of ten consecutive fractions expressed as a percentage of the total radioactivity recovered from the column. The elution profiles represent (*a*) preparation of mean mol.wt. 7000000 incubated for 3 days with Pronase and papain, (*b*) a yolk-sac digest, (*c*) a macrophage digest. The curves shown by ● in (*b*) and (*c*) represent the A_{280}.

Fig. 1. *Elution from Sepharose 6B of ^{125}I-labelled poly(vinylpyrrolidone) preparations*

The elution profiles represent (*a*) sample from The Radiochemical Centre, (*b*) sample of mean mol.wt. 50000, (*c*) sample of mean mol.wt. 84000, (*d*) sample of mean mol.wt. 700000 and (*e*) sample of mean mol.wt. 7000000. Each point represents the radioactivity (c.p.m.) measured in a 2ml fraction. The summed radioactivities of groups of ten consecutive fractions, expressed as a percentage of the total radioactivity recovered, is also shown. The column was calibrated by determining the elution positions of bovine immunoglobulin G (mol.wt. 150000), rabbit haemoglobin (mol.wt. 68000), horse heart cytochrome *c* (mol.wt. 12000), bovine insulin (mol.wt. 5800) and [^{125}I]iodide. The very-high-molecular-weight fraction derived from the sample of 7000000 mean mol.wt. is also indicated (in Fig. 1*e*).

significantly different ($P < 0.001$) from the distribution of that presented to it, the yolk sac selecting against the higher-molecular-weight material. In contrast, macrophages concentrated material from the highest-molecular-weight fractions, although the distribution as a whole was not significantly different from that of the substrate.

Discussion

In general, the Endocytic Index for the yolk sac decreased with increasing mean molecular weight of ^{125}I-labelled poly(vinylpyrrolidone). However, the Endocytic Index for the substrate of mean mol.wt. 700000 was 53% of that for The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone), whereas the Endocytic Index for the preparation of mean mol.wt. 7000000 was 68% of the relevant control value. This apparent anomaly can be explained by the data shown in Fig. 1, which indicate a higher percentage of radiolabel associated with material of low molecular weight in the latter preparation.

Macrophages captured each of the three lower-molecular-weight preparations of ^{125}I-labelled poly(vinylpyrrolidone) at a somewhat higher rate than that for The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone). However, the 7000000-mean-mol.wt. preparation and its high-molecular-weight subfraction were captured much more efficiently.

Differences in rate of pinocytic uptake with increasing substrate size could be attributable to a substrate-induced change in the rate of vesicle formation at the plasma membrane. As the four unlabelled preparations of poly(vinylpyrrolidone) all failed to alter the rate of capture of The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone), this explanation is unlikely to be correct. The results more probably indicate that the two cell types studied can discriminate between molecules of the same substrate with different molecular weight.

The observation that very-high-molecular-weight material is excluded by the yolk sac is not surprising in view of the ultrastructure of this tissue. Plate 1 shows an electron micrograph of the apical region of a rat visceral yolk-sac epithelial cell treated with Ruthenium Red to delineate the extensive extracellular canalicular system which arises from the bases of the microvilli. It is from this intricate network of tubules that pinosomes originate, so that material internalized by pinocytosis must be sufficiently small to penetrate the narrow crypts. The poly(vinylpyrrolidone) molecule probably assumes a loosely coiled helical configuration in solution, and the largest molecules may well have difficulty in reaching the site of pinocytic invaginations.

The results indicate that macrophages can ingest the entire molecular-weight range of poly(vinylpyrrolidone) and specifically concentrate the very large molecules. The Radiochemical Centre ^{125}I-labelled poly(vinylpyrrolidone) is a fluid-phase marker in this system, but the data obtained here indicate some uptake of the highest-molecular-weight preparation by adsorptive pinocytosis. It is therefore necessary to postulate that very large ^{125}I-labelled poly(vinylpyrrolidone) molecules have some affinity for the macrophage cell surface that is not manifest in their smaller counterparts. It is possible that the affinity of the vinylpyrrolidone residue for the plasma membrane is so slight as to be ineffective until the molecule can provide a sufficient number of attachment sites. Ryser & Hancock (1965) showed that the binding, and consequently the rate of capture, of polycations by sarcoma-180 cells was proportional to molecular weight.

It is generally accepted that particles greater than 1 μm in diameter are only captured by phagocytosis (Allison & Davies, 1974). Here we have demonstrated that selection according to size also operates in pinocytosis. Synthetic macromolecules have, like liposomes, been proposed as potential carriers for the transport of pharmacologically active compounds to cells where therapeutic effect is desired (Ringsdorf, 1975; Kopeček, 1977). Size or molecular weight of a carrier affords a potential mechanism for preliminary targeting of the complex.

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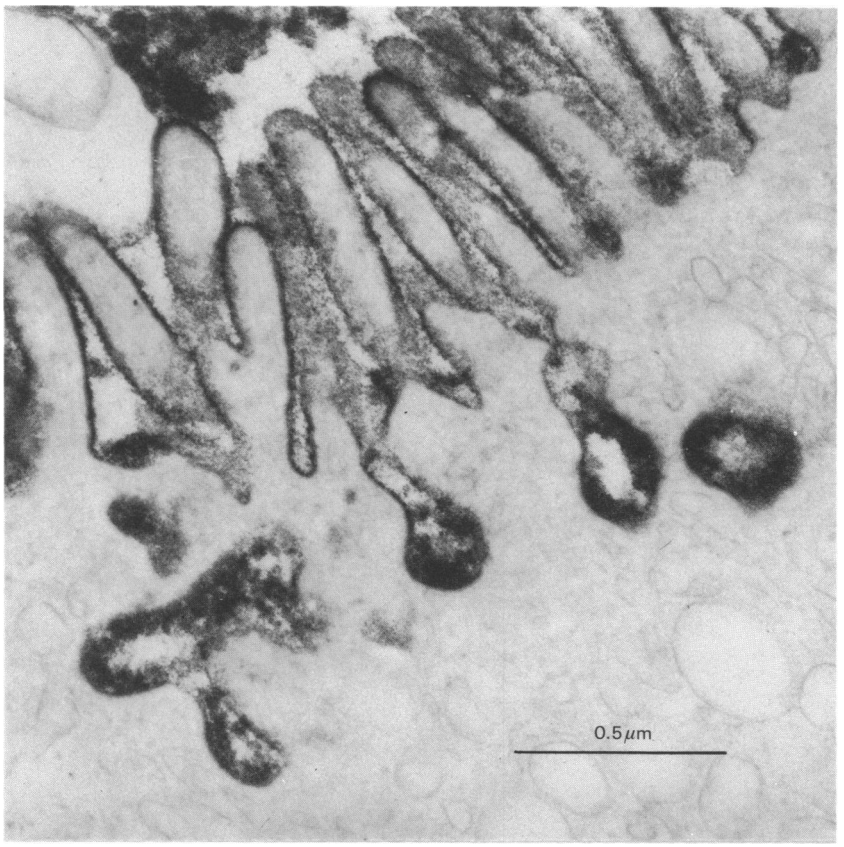

EXPLANATION OF PLATE 1

Electron micrograph showing the apical region of the rat visceral yolk sac

Immediately after removal from the rat, pieces of visceral yolk sac (at 17.5 days gestational age) were fixed with 1.5% glutaraldehyde in cacodylate buffer (pH 7.2) for 2h. After washing with buffer, they were post-fixed in OsO_4 (1% in cacodylate buffer) for 2h. All solutions contained 2mM-$MgCl_2$ and Ruthenium Red (0.5%). Electron-dense deposits of Ruthenium Red can be seen outlining the extracellular apical canalicular system.

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