

Fluid endocytosis by rat liver and spleen

Experiments with ^{125}I -labelled poly(vinylpyrrolidone) *in vivo*

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1. Rates of fluid endocytosis of rat liver, spleen, hepatocytes and sinusoidal liver cells have been determined, by using ^{125}I -labelled poly(vinylpyrrolidone) as marker. Poly(vinylpyrrolidone) was injected intravenously into rats, and plasma clearance and uptake by liver and spleen were estimated. From these data, rates of fluid endocytosis of 1.2 and 1.8 ml of plasma/g of protein per day were calculated for liver and spleen respectively. Essentially the same results were found in nephrectomized rats. 2. Hepatocytes and sinusoidal cells were separately isolated by the collagenase/Pronase method, and sinusoidal cells were further fractionated by centrifugal elutriation. Hepatocytes, sinusoidal cells, Kupffer cells and endothelial cells showed rates of fluid endocytosis of 0.96, 9.0, 19 and 13 ml of plasma/g of cell protein per day respectively. Total-body X-irradiation did not influence uptake of poly(vinylpyrrolidone) by spleen, indicating that spleen lymphocytes are not significantly involved in fluid endocytosis. 3. For liver a rate constant of exocytosis of 5% per day was found, whereas for spleen no significant loss of accumulated label could be demonstrated during a 21-day period. 4. Distribution of label over a great number of organs and tissues was measured 9 days after the injection. Liver, skin, bone and muscle together contained about 70% of the label present in the carcass; only spleen and lymph nodes contained more label per g fresh weight of tissue than liver.

Endocytosis is a widespread process of cell-surface invagination and subsequent internalization of plasma membrane as vacuoles, which is associated with transport of extracellular solutes into the cell. A substrate can enter a cell dissolved in extracellular fluid (fluid-phase endocytosis), or mainly adsorbed on the plasma membrane in a process called adsorptive endocytosis (Jacques, 1975; Silverstein *et al.*, 1977).

Determinations of fluid endocytosis are complicated by the fact that it is difficult to prove that the marker substance used is not to some degree adsorbed on the plasma membrane. One way to detect adsorptive endocytosis is by measuring the rate of uptake as a function of substrate concentration: adsorptive endocytosis shows, in contrast with fluid endocytosis, saturation kinetics. Studies of this kind are limited by the substrate concentrations that are practicable. Another approach is to compare the rate of uptake of several

chemically unrelated markers. Those substances that are internalized by fluid-phase endocytosis only will be taken up at the same rate, which will be lower than that found for any molecule that is adsorbed (Roberts *et al.*, 1977).

Most determinations of the rate of fluid endocytosis have been made on cells or tissues in culture, where substrate concentrations, and other conditions, can be fairly easily controlled (Steinman & Cohn, 1972; Edelson & Cohn, 1974; Williams *et al.*, 1975; Kaplan, 1976; Pratten *et al.*, 1977; Ose *et al.*, 1980). Studies on fluid endocytosis *in vivo* are, to our knowledge, limited to the work on liver by Wattiaux (1966), which was later extended by Boman & Berg (1975). In these studies, Triton WR-1339 was used as a marker. Since this substance, being a detergent, might bind to the plasma membrane of endocytosing cells, the values might have been too high. Also, the results might have been influenced by the fact that Triton

WR-1339 has affinity for a plasma protein (Henning *et al.*, 1971).

We have carried out similar studies, using a chemically entirely different compound, poly(vinylpyrrolidone) (labelled with ^{125}I), a synthetic, metabolically stable, hydrophilic polymer, which, at least in a number of cells *in vitro*, is taken up solely by fluid-phase endocytosis (Roberts *et al.*, 1977; Pratten *et al.*, 1977; Ose *et al.*, 1980). Our results support those of Wattiaux (1966) and Boman & Berg (1975). In addition, we have studied the effect of nephrectomy, and we have obtained data on Kupffer and endothelial cells from liver, on spleen and on other tissues.

Materials and methods

Materials

^{125}I -labelled poly(vinylpyrrolidone) (average mol.wt. 33000; range 8000–84000) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Collagenase, deoxyribonuclease and bovine serum albumin were from Sigma, St. Louis, MO, U.S.A. Pronase (B grade) was from Calbiochem, San Diego, CA, U.S.A. Fluothane [2-bromo-2-chloro-1,1,1-trifluoroethane with 0.01% (w/w) thymol added] was from I.C.I., Macclesfield, Cheshire, U.K. Sephadex G-25 and G-200 were from Pharmacia, Uppsala, Sweden. Ready-Solv VI was from Beckman, Fullerton, CA, U.S.A. Nylon gauze (110 mesh) was from Stokvis and Smits Textiel Maatschappij, Haarlem, The Netherlands. All other chemicals used were analytical grade and were purchased from E. Merck, Darmstadt, Germany.

Animals

Male rats of an inbred Wistar strain, average body weight about 200 g, were used for all experiments except the fractionation of sinusoidal cells. Anaesthesia was induced and maintained with fluothane in a mixture of NO and O_2 . Rats were injected via the vena femoralis or the vena penis, usually at a dose of 0.2 ml/100 g body wt. Unless stated otherwise the injected dose contained 0.6 mg of poly(vinylpyrrolidone)/ml of 0.15 M-NaCl. At the time of injection the labelled poly(vinylpyrrolidone) had a sp. radioactivity of approx. $40 \mu\text{Ci}/\text{mg}$. Blood samples were obtained from the orbital plexus, and were prepared for counting as described by Kooistra *et al.* (1979). Rats were nephrectomized by ligating the blood vessels of the kidneys or by removing the kidneys immediately before injection of poly(vinylpyrrolidone). Livers were perfused with iso-osmotic 0.15 M-NaCl at room temperature until they became yellowish. Spleens, kidneys and liver were homogenized in 5, 10 and 35 ml of an ice-cold 0.25 M-sucrose solution respectively. Intestines and

stomach, skin and whole carcass were dissolved in respectively 50, 100 and 300 ml of 60% (w/v) KOH at 90°C for 1 h as described by Gruber (1953). Urine and faeces were separately collected daily during most experiments. For the fractionation of sinusoidal cells female BN/BiRij rats of about 150 g were used.

Liver fractionations and determination of protein, DNA and enzymes

Liver fractionations were carried out as described by Bouma & Gruber (1966). Protein concentrations were determined by the Lowry method with bovine serum albumin as standard. DNA was determined as described by Bouma & Gruber (1964). Acid phosphatase was measured by the method of Gianetto & de Duve (1955) with β -glycerol phosphate as substrate. Acid deoxyribonuclease was determined as described by de Duve *et al.* (1955).

Assay of radioactivity

Radioactivity was measured as described by Kooistra *et al.* (1979).

In our calculations, we have used a volume of 3.13 ml of plasma/100 g body wt. (Altman, 1961). Uptake in spleen, kidney and perfused liver was corrected for the appropriate amount of radioactivity in residual plasma, present in the tissue at the time of sampling. Values for spleen ($47 \mu\text{l}$ of plasma/g fresh weight of tissue) and for kidney ($50 \mu\text{l}$ of plasma/g fresh weight of tissue) were taken from Altman (1961). A value for the amount of plasma left behind after perfusion of liver with saline ($35 \mu\text{l}$ of plasma/g fresh weight of tissue) was determined by measuring the radioactivity of liver that had been perfused with saline a short time after injection of labelled poly(vinylpyrrolidone).

Isolation of parenchymal and non-parenchymal cells

Isolation of parenchymal and non-parenchymal cells was done as described previously (Kooistra *et al.*, 1979). About 30% of both parenchymal and non-parenchymal cells was recovered. The non-parenchymal cell fraction was further purified with the Beckman JE-6 elutriating system as described by Knook & Sleyster (1976) (see also Sleyster *et al.*, 1977), with the following modifications. At a constant rotor speed of 2500 rev./min, the non-parenchymal cell suspension was separated into three fractions: lymphocytes, endothelial cells and Kupffer cells were successively elutriated at flow rates of 13.7, 21.7 and 40 ml/min respectively.

Kinetics

When a rat is injected with a radioactively labelled undegradable macromolecule, the amount of radioactivity present in tissues and organs is the

resultant of two opposing processes: endocytosis and exocytosis. If we assume that the macromolecule is exclusively taken up by fluid-phase endocytosis, the rate of its uptake will be proportional to its concentration in plasma. If, in addition, it is assumed that exocytosis is a first-order process, we can describe the change in organ radioactivity by:

$$\frac{dL}{dt} = (k' \cdot P) - (k'' \cdot L) \quad (1)$$

where L is the amount present in the organ (% of dose), t is time (days), k' is the rate of (fluid) endocytosis by the organ (ml of plasma/day), P is the plasma concentration (% of dose per ml of plasma), and k'' is the rate constant of exocytosis (day^{-1}).

If plasma clearance of poly(vinylpyrrolidone) were a first-order process, it could be described by:

$$P = P_0 \cdot e^{-kt} \quad (2)$$

where P_0 is the initial plasma concentration (% of dose/ml of plasma) and k is the rate constant of plasma clearance (day^{-1}). Combination of eqns. (1) and (2) gives:

$$\frac{dL}{dt} = (k' \cdot P_0 \cdot e^{-kt}) - (k'' \cdot L) \quad (3)$$

Integration between 0 and t gives, supposing $L = 0$ at $t = 0$:

$$L = \frac{k' \cdot P_0}{k - k''} (e^{-k''t} - e^{-kt}) \quad (4)$$

It turned out that the clearance of poly(vinylpyrrolidone) from plasma could not be described by a single exponential function, but, by using the residual method of Gibaldi & Perrier (1975), we could describe the plasma clearance of poly(vinylpyrrolidone) in untreated rats up to 21 days by the sum of four exponential functions. In nephrectomized rats two exponential functions were required to describe the clearance of poly(vinylpyrrolidone) up to 8 h. Eqn. (4) was modified accordingly.

Rates of fluid endocytosis (k') were calculated from the amounts of radioactivity found in the organs up to 2 days after injection. We assumed that in the initial phase of uptake (when the plasma concentration is relatively high, and the concentration of poly(vinylpyrrolidone) in the organ is still low), exocytosis is negligible compared with endocytosis.

It should be stressed that our calculations are independent of any interpretation of the clearance data. We assume that the molecules are taken up from plasma (or extravascular fluids that rapidly

equilibrate with plasma) only, and it is the concentration in plasma that is actually measured. The only purpose of the mathematical treatment of the clearance data is to obtain the integral of plasma concentration and time up to the moment that the target tissue is extirpated.

The rate constant of exocytosis in liver was determined as follows. When the amount of poly(vinylpyrrolidone) in liver reaches a maximum, the amount of poly(vinylpyrrolidone) entering the liver via endocytosis is equal to the amount of poly(vinylpyrrolidone) leaving the liver via exocytosis, i.e. $dL/dt = 0$. It follows from eqn. (1) that:

$$k'' = \frac{k' \cdot P(L = \text{max.})}{L_{\text{max.}}} \quad (5)$$

where $L_{\text{max.}}$ is the maximal value of L and $P(L = \text{max.})$ the corresponding value of P . Introducing the visually apparent value of $L_{\text{max.}}$ and the corresponding value of P in eqn. (5) gives an approximate value of k'' . This approximate value of k'' allows us to calculate the content of liver radioactivity as a function of time by introducing it into eqn. (4). To find the most probable value of k'' , successive approximations of k'' were tried. The value of k'' giving the curve best fitting the experimental results was assumed to be the actual rate constant of exocytosis.

Results

Plasma clearance of poly(vinylpyrrolidone)

The clearance of ^{125}I -labelled poly(vinylpyrrolidone) from plasma after intravenous injection is shown in Fig. 1. Almost 70% of the injected label is removed from the bloodstream within 1 h. This is partly due to renal excretion: about 55% of the injected dose was found in the urine after 1 day. This value increased only slightly in the following days: additional amounts of 3 and 7% had been excreted after 7 and 21 days respectively.

The excreted material probably consists of molecules with relatively low molecular weight. To avoid excretion of these molecules, we did the same experiments with nephrectomized rats (see Fig. 1). In this way it became possible to measure endocytosis of poly(vinylpyrrolidone) of relatively low molecular weight (see below). Furthermore, the results might indicate whether the rate of fluid endocytosis is affected by nephrectomy. This information might be relevant because studies on endocytosis of some low-molecular-weight proteins have been done in nephrectomized rats (Brown *et al.*, 1978; Kooistra *et al.*, 1977, 1979).

It seems likely that in nephrectomized rats plasma clearance is mainly caused by distribution of poly(vinylpyrrolidone) over extravascular spaces

and only to a small extent by endocytosis. Leakage to the extravascular compartment will, of course, also contribute to the clearance observed in animals that have not been nephrectomized.

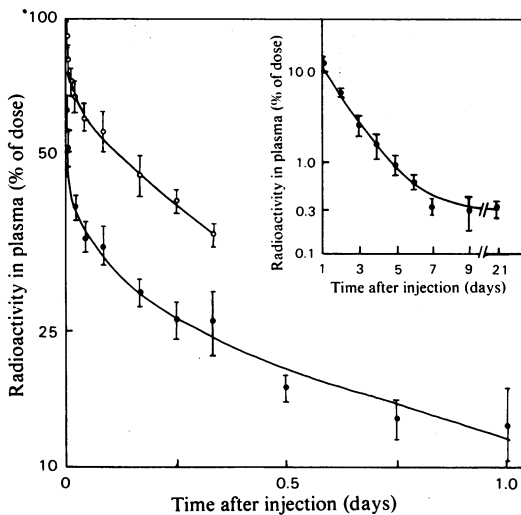


Fig. 1. Clearance of radioactivity from plasma after intravenous injection of ^{125}I -labelled poly(vinylpyrrolidone)

The amount of radioactivity in plasma of untreated (●) or nephrectomized (○) rats is expressed as a percentage of the injected dose assuming a plasma volume of 3.13 ml/100 g body wt. Values represent means \pm s.d. for at least three animals. The curves have been calculated after mathematical evaluation of the clearance data (see the Materials and methods section).

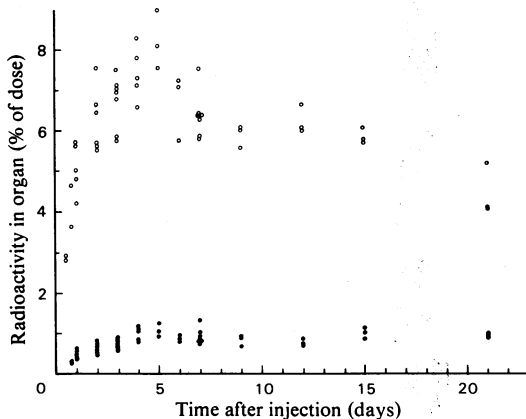


Fig. 2. Uptake of ^{125}I -labelled poly(vinylpyrrolidone) by liver and spleen of normal rats

The amount of radioactivity in liver (○) and spleen (●) is expressed as a percentage of the injected dose. Values were corrected for the amount of plasma assumed to be present in the tissues at the time of sampling. Each point represents one liver or spleen.

Uptake of poly(vinylpyrrolidone) by liver and spleen

The amount of radioactivity found in liver and spleen at different times after injection of radioiodinated poly(vinylpyrrolidone) is shown in Fig. 2. The maximal amount of radioactivity in liver (about 8% of the dose) as well as in spleen (about 1% of the dose) is reached after about 5 days. Thereafter, the amount of label present in liver slowly decreases, whereas radioactivity in spleen does not decrease significantly. Similar values for uptake of poly(vinylpyrrolidone) in liver have been found by Madnick *et al.* (1978). If results are expressed per g of tissue protein, spleen is found to be more active than liver: liver contained 6.2% of the dose/g of protein after 5 days, whereas for spleen this value was 14.3%. Radioactivity in liver and spleen was found to be systematically higher in nephrectomized rats, as might have been expected from the higher plasma values (results not shown).

Subcellular localization of poly(vinylpyrrolidone) in liver

The intracellular localization of the endocytosed poly(vinylpyrrolidone) was investigated by differential fractionation. Fig. 3 shows the distribution of radioactivity and the lysosomal marker enzymes acid phosphatase and acid deoxyribonuclease in the various subcellular liver fractions 24 h after injection of labelled poly(vinylpyrrolidone). The distribution of radioactivity resembles that of the lysosomal markers, and in particular that of acid deoxyribonuclease, an enzyme that is present at a relatively high concentration in lysosomes of sinusoidal cells (Munthe-Kaas *et al.*, 1976).

Distribution of poly(vinylpyrrolidone) between parenchymal and non-parenchymal liver cells

The cellular distribution of radioiodinated poly(vinylpyrrolidone) in the liver of untreated and nephrectomized rats was determined by separating parenchymal cells from non-parenchymal cells, and determining protein and radioactivity in either cell fraction. The ratio of the specific radioactivities in the non-parenchymal cell and the parenchymal cell fractions was calculated. The isolation of liver cells of nephrectomized rats was carried out 4 h, and the isolation of liver cells of untreated rats about 24 h, after injection of poly(vinylpyrrolidone). The ratios were 8.2 ± 2.4 (mean \pm s.d. for five separate experiments) for nephrectomized rats, and 10.3 ± 2.5 (mean \pm s.d. for seven separate experiments) for untreated rats. These results, presented in Table 1, show that non-parenchymal cells endocytose poly(vinylpyrrolidone) more efficiently than parenchymal cells, and that nephrectomy does not result in an alteration of the cellular distribution of poly(vinylpyrrolidone).

The non-parenchymal cell fraction consists of a

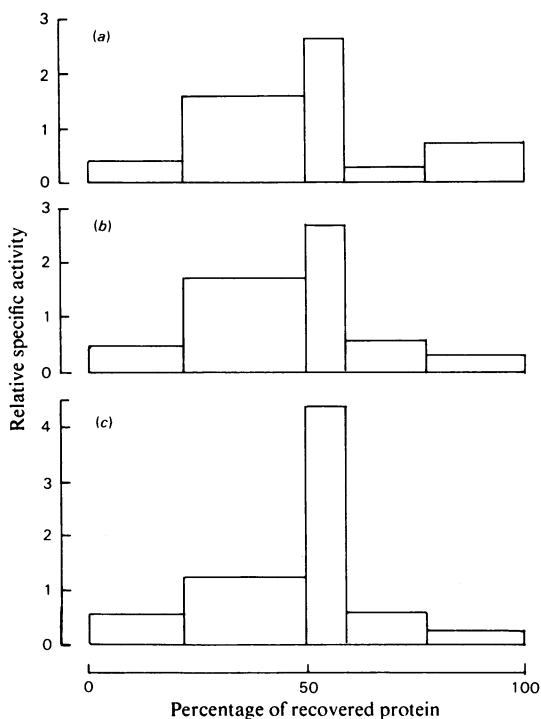


Fig. 3. Distribution pattern of radioactivity (a), acid deoxyribonuclease (b) and acid phosphatase (c) over sub-cellular fractions after differential fractionation of liver

Distribution of radioactivity (a), acid deoxyribonuclease (b) and acid phosphatase (c) over sub-cellular fractions after intravenous injection of ^{125}I -labelled poly(vinylpyrrolidone) into untreated rats is shown. Livers were homogenized and fractionated by differential centrifugation 24 h after injection of the radiiodinated polymer. Blocks from left to right represent fractions in the order in which they were isolated: nuclear, mitochondrial, lysosomal, microsomal and final supernatant fractions. The relative protein content is given on the abscissa. The ordinate represents the mean relative specific activity (percentage of total recovered activity divided by the percentage of total recovered protein). Thus, the area of each block is proportional to the percentage of activity per fraction. Each Figure represents the result of four fractionations. Mean recoveries \pm S.D. were: protein, $97 \pm 7\%$; radioactivity, $97 \pm 2\%$; acid deoxyribonuclease, $102 \pm 6\%$; acid phosphatase, $92 \pm 3\%$.

mixture of cells (mainly Kupffer cells, endothelial cells and lymphocytes) that can be fractionated by centrifugal elutriation (Knook & Sleyster, 1976; Sleyster *et al.*, 1977). Since this technique had been developed for BN/BiRij rats, we have used rats of this strain for estimating the endocytosis of poly(vinylpyrrolidone) by different types of non-parenchymal cells. The ratio of the radioactivity per g of cell protein found in fractions enriched in Kupffer

cells, endothelial cells and lymphocytes 24 h after injection was about 1:0.7:0.4. Since the Kupffer-cell fraction was contaminated with about 25% endothelial cells in these experiments the actual difference between Kupffer and endothelial cells is probably somewhat greater than this ratio suggests.

Rates of fluid endocytosis

Since poly(vinylpyrrolidone) is taken up by fluid-phase endocytosis (see the evidence discussed below), the rate of fluid endocytosis of liver, various types of liver cells and spleen could be calculated from the concentration of poly(vinylpyrrolidone) in plasma and the amount taken up by the organs and cells during a certain time interval (see the Materials and methods section). The results, which are presented in Table 1, show that nephrectomy does not affect the rates of fluid endocytosis by liver and spleen, and that non-parenchymal cells, and Kupffer cells in particular, take up poly(vinylpyrrolidone) much more efficiently than parenchymal cells.

Effect of total-body X-irradiation on uptake and retention of poly(vinylpyrrolidone) in spleen

Table 1 also shows that, if calculated per g of tissue protein, spleen endocytoses poly(vinylpyrrolidone) more rapidly than liver. Lymphocytes constitute a high proportion of the cells of the spleen. To estimate their contribution to the uptake of poly(vinylpyrrolidone), we determined the effect of total-body X-irradiation, which selectively removes lymphocytes from the spleen. One group of animals was irradiated 24 h before the injection of poly(vinylpyrrolidone), and the animals were killed 6 days after the injection. Another group of rats was irradiated 5 days after the injection, 1 day before excision of the spleen (Table 2). In both series of experiments, spleens of the irradiated rats had lost about 40% of the wet weight, and some 80% of their DNA, and showed much lower DNA/protein ratios, reflecting selective loss of lymphocytes (Bouma & Gruber, 1964). In spite of the loss of 80% of their DNA, spleens of previously irradiated rats did not contain significantly less poly(vinylpyrrolidone) than the controls. In rats that had been irradiated 5 days after injection of poly(vinylpyrrolidone), spleens contained even somewhat more poly(vinylpyrrolidone) than those of controls.

These results indicate that the contribution of lymphocytes to endocytosis of poly(vinylpyrrolidone) is negligible, and suggest that poly(vinylpyrrolidone) found in the 'lymphocyte fraction' of liver (Table 1) may actually be present in contaminating endothelial cells and/or Kupffer cells.

Rate constant of fluid exocytosis by liver

The most probable rate constant of exocytosis was estimated as described in the Materials and

Table 1. *Rates of fluid endocytosis*

Rates of fluid endocytosis (ml of plasma cleared of poly(vinylpyrrolidone)/organ per day) have been calculated as described in the Materials and methods section, assuming that during the first days after injection exocytosis is negligible. Time between injection of poly(vinylpyrrolidone) and removal of the organs varied for nephrectomized rats between 1 and 8 h, and for untreated rats between 8 h and 2 days. Cell isolations were started 4 h after injection of poly(vinylpyrrolidone) into nephrectomized rats, and about 24 h after injection of poly(vinylpyrrolidone) into untreated rats. All experiments were done with Wistar rats except for the isolation of fractions enriched in Kupffer cells, endothelial cells or lymphocytes, where BN/BiRij rats were used. Statistics refer to the mean \pm s.d., with the numbers of experiments in parentheses. Abbreviations used: PC, parenchymal cells; NPC, non-parenchymal-cell fraction; KC, Kupffer-cell fraction; EC, endothelial-cell fraction; LC, lymphocyte fraction.

| | Fluid endocytosis | | | |
|--------|----------------------|---------------------------|----------------------|---------------------------|
| | Untreated rats | | Nephrectomized rats | |
| | (ml/day) | (ml/day per g of protein) | (ml/day) | (ml/day per g of protein) |
| Spleen | 0.13 \pm 0.04 (19) | 1.86 \pm 0.47 (19) | 0.12 \pm 0.03 (18) | 1.82 \pm 0.48 (18) |
| Liver | 1.60 \pm 0.29 (17) | 1.18 \pm 0.18 (17) | 1.51 \pm 0.27 (17) | 1.16 \pm 0.37 (17) |
| PC | | 0.95 \pm 0.21 (7) | | 0.97 \pm 0.13 (5) |
| NPC | | 9.95 \pm 3.52 (7) | | 8.02 \pm 2.67 (5) |
| KC | | 18.56 \pm 4.31 (4) | | |
| EC | | 12.61 \pm 1.81 (4) | | |
| LC | | 8.25 \pm 2.00 (4) | | |

Table 2. *Effect of total-body X-irradiation on uptake and retention of poly(vinylpyrrolidone) in spleen*

All rats were injected with labelled poly(vinylpyrrolidone) and the radioactivity of the spleen was determined 6 days after the injection. Results in the second column refer to animals that had been irradiated with 500 R (Müller MG 300 X-ray apparatus, 200 kV, 12 mA, dose rate 27.5 R/min) 24 h before the injection. Data in the third column were obtained from rats that had been irradiated in the same way 5 days after the injection (and, consequently, 1 day before excision of the spleen). Values given are means \pm s.e.m.

| | Control (not irradiated) | First irradiated, then injected | First injected, then irradiated |
|--|-----------------------------|------------------------------------|------------------------------------|
| Number of animals | 5 | 5 | 5 |
| Fresh weight (mg/spleen) | 516 \pm 40 | 287 \pm 21 | 343 \pm 32 |
| DNA (mg/spleen) | 6.70 \pm 0.38 | 1.36 \pm 0.13 | 1.53 \pm 0.18 |
| DNA/protein ratio (mg of DNA/g of protein) | 98 \pm 5 | 36 \pm 4 | 34 \pm 2 |
| Poly(vinylpyrrolidone) (% of dose/spleen) | 0.66 \pm 0.05 | 0.61 \pm 0.05 | 0.88 \pm 0.05 |

Table 3. *Rate constant of exocytosis in liver*

The rate constant of exocytosis, k'' , was estimated as described in the Materials and methods section. For a number of values of k'' the amount of radioactivity per liver (L) was calculated as a function of time, and the differences between this calculated value (L_c) and each of the 52 measured values of L (L_m) were determined, squared and averaged. Results are means \pm s.e.m.

| k'' (day ⁻¹) | $\frac{1}{52} \cdot \sum_0^{52} (L_m - L_c)^2$ |
|-------------------------------|--|
| 0.000 | 4.50 \pm 0.91 |
| 0.020 | 2.76 \pm 0.87 |
| 0.030 | 1.49 \pm 0.47 |
| 0.040 | 0.98 \pm 0.31 |
| 0.047 | 0.89 \pm 0.28 |
| 0.055 | 0.91 \pm 0.28 |
| 0.064 | 1.13 \pm 0.33 |
| 0.075 | 1.39 \pm 0.41 |

methods section. First, the amount of radioactivity per liver was calculated as a function of time by introducing an approximate value of k'' in eqn. (4). Thereafter the differences between this calculated value and each of the 52 measured values of L were determined, squared and averaged. Results obtained for a number of k'' values are given in Table 3. The discrepancy between measured and calculated radioactivity in liver was found to be minimal at a rate constant of exocytosis of about 5% per day.

Endocytosis of high- and low-molecular-weight poly(vinylpyrrolidone)

Several reports have shown that an increase in the number of binding sites per molecule enhances uptake (Bartholeyns & Baudhuin, 1976; Kooistra *et al.*, 1977, 1979). If poly(vinylpyrrolidone) shows

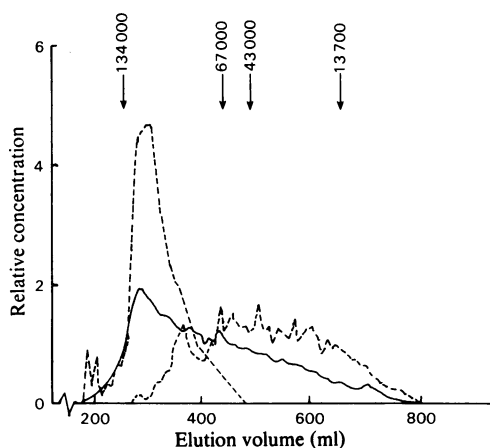


Fig. 4. Gel filtration of ^{125}I -labelled poly(vinylpyrrolidone)

A solution (2.5 ml) containing 6 mg of ^{125}I -labelled poly(vinylpyrrolidone) (sp. radioactivity about $40\mu\text{Ci}/\text{mg}$) was applied to a column (3.2 cm \times 93 cm) of Sephadex G-200 (fine). The column was eluted with 0.1M-ammonium acetate, pH 7, at a flow rate of 15 ml/h. Fractions (4.5 ml) were collected in silicone-treated glass tubes and monitored for radioactivity (—). High-molecular-weight (fractions 50–75) and low-molecular-weight (fractions 76–150) fractions were pooled, and, after freeze-drying, used for further experiments. Samples of both the high- and low-molecular-weight fractions were re-chromatographed under the same conditions as described above (----). Recoveries were at least 90%. The column was calibrated by determining elution volumes of a dimer and monomer of bovine serum albumin (mol.wts. 134 000 and 67 000 respectively), ovalbumin (mol.wt. 43 000) and ribonuclease (mol.wt. 13 700).

affinity to the plasma membrane the degree of adsorption, and consequently also the rate of uptake, will increase with increasing molecular weight. This was checked in the following way. A batch of ^{125}I -labelled poly(vinylpyrrolidone) was subjected to gel filtration on Sephadex G-200 as shown in Fig. 4. Rates of endocytosis by liver and spleen were determined in nephrectomized rats for both pooled high-molecular-weight fractions and pooled low-molecular-weight fractions that were resolved on Sephadex G-200. The rates of endocytosis (corresponding to 1.34 ± 0.35 and 1.94 ± 0.52 ml of plasma per day/g of protein for high- and low-molecular-weight poly(vinylpyrrolidone) respectively; values are means \pm s.d. for five separate experiments) were actually higher for low-molecular-weight poly(vinylpyrrolidone). For spleen essentially the same results were obtained: 1.39 ± 0.50 and 2.03 ± 0.86 ml of plasma/day per g of protein for the high- and low-molecular-weight fractions respectively. It is possible that a greater

Table 4. Tissue distribution of poly(vinylpyrrolidone)

The tissue distribution of label 9 days after intravenous injection of ^{125}I -labelled poly(vinylpyrrolidone) into rats was determined as described in the Materials and methods section. Samples from the femora, the epididymal fat-pads and from hind-leg muscles were counted for radioactivity. Bone was assumed to account for 6% of the total body weight, and fat for 10% (Lippman & Finch, 1972); for muscle a value of 45% was used (Keeler, 1970). At the moment of sampling about 25% of the injected dose was present in the carcass. Values are means \pm s.e.m. for three animals.

| Tissue or organ | Radioactivity in tissue or organ (% of total in carcass) | Radioactivity in tissue or organ (% of total in carcass/g of tissue or organ) |
|-----------------------------------|--|---|
| Adrenal | 0.10 ± 0.01 | 1.51 ± 0.26 |
| Bladder | 0.19 ± 0.03 | 1.15 ± 0.24 |
| Bone | 17.56 ± 2.93 | 1.36 ± 0.19 |
| Brain | 0.03 ± 0.01 | 0.02 ± 0.01 |
| Fat-tissue | 6.53 ± 1.33 | 0.31 ± 0.09 |
| Heart | 0.55 ± 0.05 | 0.65 ± 0.01 |
| Kidney | 1.64 ± 0.17 | 1.02 ± 0.04 |
| Liver | 23.94 ± 3.28 | 3.07 ± 0.26 |
| Lung | 1.24 ± 0.31 | 1.12 ± 0.15 |
| Lymph nodes | 1.58 ± 0.56 | 4.16 ± 0.43 |
| Muscle | 12.57 ± 2.24 | 0.13 ± 0.01 |
| Pancreas | 0.28 ± 0.08 | 0.76 ± 0.11 |
| Plasma | 1.06 ± 0.25 | 0.16 ± 0.04 |
| Salivary glands | 0.97 ± 0.38 | 1.47 ± 0.38 |
| Skin | 18.72 ± 1.78 | 0.46 ± 0.12 |
| Seminal vesicles + prostate gland | 0.43 ± 0.09 | 0.49 ± 0.08 |
| Spleen | 3.25 ± 0.30 | 7.47 ± 0.65 |
| Stomach + intestines | 9.02 ± 2.37 | 0.70 ± 0.27 |
| Testis + epididymis | 1.81 ± 0.08 | 0.47 ± 0.03 |
| Thymus | 0.33 ± 0.10 | 0.81 ± 0.23 |

part of the surface area of the endocytosing cells is accessible to the lower-molecular-weight material or that this material penetrates further into the cell coat that might be present in some endocytotic vesicles.

Tissue distribution

Table 4 shows the distribution of radioactivity in rat tissues 9 days after injection. The data suggest that only spleen and lymph nodes endocytose poly(vinylpyrrolidone) somewhat more rapidly than liver. A high percentage of total radioactivity was found in liver, skin, bone (including bone marrow) and muscle. Most (or all) of the activity found in skin and muscle may actually be present in extracellular fluid and/or tissue macrophages. In calculating radioactivity in bone, we have extrapolated from measurements made on the femora.

It should be kept in mind that these data are not corrected for exocytosis, which might have occurred at different rates in the various tissues. Also, some

cells might take up poly(vinylpyrrolidone) to a certain extent by adsorptive endocytosis.

Discussion

An essential requirement for any fluid endocytosis marker is that it is not adsorbed on the cell surface. Poly(vinylpyrrolidone) seems to fulfil this requirement in rat yolk sac (Williams *et al.*, 1975; Roberts *et al.*, 1977), in macrophages (Pratten *et al.*, 1977) and in parenchymal and non-parenchymal liver cells *in vitro* (Ose *et al.*, 1980). The following observations suggest that poly(vinylpyrrolidone) *in vivo* is also taken up exclusively by fluid-phase endocytosis, at least in liver and spleen. (1) If poly(vinylpyrrolidone) was taken up by adsorptive endocytosis, saturation kinetics would be observed. The uptake of ^{125}I -labelled poly(vinylpyrrolidone) by liver and spleen was not decreased in the presence of a 100-fold excess of cold poly(vinylpyrrolidone) (results not shown). Thus, there is no evidence for saturation kinetics in the range tested. (2) High-molecular-weight poly(vinylpyrrolidone) was endocytosed at a lower rate than low-molecular-weight poly(vinylpyrrolidone), indicating a lack of affinity to the plasma membrane, as an increase in molecular weight could lead to an increase in the number of binding sites per molecule and thus to an increase in uptake. (3) The rate of fluid endocytosis by liver calculated from our experiments with poly(vinylpyrrolidone) (1.6 ml of plasma/day) is very similar to that found by Wattiaux (1966) for the chemically unrelated marker Triton WR-1339 (1.15 ml of plasma/day). Boman & Berg (1975) found that, per g of cell protein, sinusoidal liver cells endocytose about ten times more Triton WR-1339 than hepatocytes, i.e. about the same ratio as we have found for poly(vinylpyrrolidone). This also points to uptake by fluid-phase endocytosis, for it is highly unlikely that these chemically very different components would show the same relative adsorption on the plasma membranes of both types of cells.

In our calculations, we have assumed that the molecules are taken up from plasma or from extracellular fluids that rapidly equilibrate with plasma. This seems a reasonable assumption in the case of a slowly endocytosed solute like poly(vinylpyrrolidone) and organs like liver that have an abundant blood supply.

Our data on the rate of fluid endocytosis by parenchymal and non-parenchymal liver cells *in vivo* (0.95 and 9.95 ml/day per g of protein respectively) are remarkably similar to recently published data on fluid endocytosis by these cells *in vitro*, namely 0.9 and 16.8 ml/day per g of cell protein (Ose *et al.*, 1980).

We have found that the rate of fluid endocytosis of Kupffer cells *in vivo* is about 19 ml/day per g of

cell protein. This value may be compared with data obtained with Kupffer cells and peritoneal macrophages *in vitro*. From results reported by Munthe-Kaas (1977) it can be calculated that isolated Kupffer cells endocytose the amount of $[^3\text{H}]$ sucrose present in 3 ml of medium/day per g of cell protein. Pratten *et al.* (1977), with ^{125}I -labelled poly(vinylpyrrolidone) and ^{14}C sucrose, found a rate of fluid endocytosis of 10 and 6 ml/day per g of cell protein respectively in experiments with rat peritoneal macrophages. A value of about 13 ml/day per g of cell protein can be calculated from results obtained by Steinman & Cohn (1972) on uptake of horseradish peroxidase by mouse peritoneal macrophages. These values are all of the same order of magnitude. The differences found might be due to differences between the cell types and/or the experimental systems used. The values given above may be compared with data on some proteins that are taken up by adsorptive endocytosis. Sinusoidal liver cells *in vivo* endocytose lactate dehydrogenase isoenzyme 5 (Sinke *et al.*, 1979) and lysozyme (Kooistra *et al.*, 1980) at rates corresponding to the amount of enzyme present in 2700 and 1400 ml/day per g of cell protein respectively; labelled asialofetuin is taken up at a rate corresponding to 1300 ml/day per g of cell protein by parenchymal cells *in vitro* (Tolleshaug *et al.*, 1977).

If calculated per mass of protein, endocytosis by sinusoidal liver cells is about ten times as fast as that by hepatocytes, but per cell, endocytosis by a hepatocyte does not differ from the endocytosis by an average sinusoidal cell. If endocytosis is expressed per unit surface of plasma membrane [as can be done if stereological data by Blouin *et al.* (1977) are used], sinusoidal cells endocytose poly(vinylpyrrolidone) about twice as fast as hepatocytes.

In liver, the rate of exocytosis is low. As Fig. 2 shows, the amount of label present in liver decreases from about 8% of the dose on the fifth day after injection to about 4.5% after 21 days. It seems likely that a considerable proportion of this decrease is due to exocytosis in bile. Results obtained by LaRusso & Fowler (1979) suggest that roughly 3% of the content of hepatic lysosomes is discharged into the bile per day. Poly(vinylpyrrolidone) in bile will in time be eliminated from the body by way of the faeces. Ravin *et al.* (1952) have found that in rats where the bile flow was interrupted, no poly(vinylpyrrolidone) was found in the faeces at all. We found that about 3% of the injected dose was present in the faeces produced by the rats between 5 and 21 days after injection. Since only hepatocytes are situated along the bile canaliculi, it seems probable that exocytosis of poly(vinylpyrrolidone) in faeces is limited to these cells. In this context it may be relevant that we found no significant

exocytosis in spleen. Prolonged storage of poly(vinylpyrrolidone) in tissues of patients treated with poly(vinylpyrrolidone) (Tohill, 1965; Reske-Nielsen *et al.*, 1976), and the very existence of lysosomal storage diseases in general, also suggest that *in vivo* true exocytosis is at most a slow process.

Schneider *et al.* (1979) have shown that during endocytosis pieces of membrane are shuttled between plasma membrane and lysosomes: vesicles arise from the plasma membrane, fuse with lysosomes and return to the plasma membrane. Some adsorbed molecules can also be shuttled between the extracellular and intralysosomal compartments; our results suggest that movement of non-adsorbed macromolecules is strictly one-way.

Fluid-phase endocytosis by liver may contribute significantly to the turnover of some plasma proteins. As shown in Table 1, the liver of a rat of about 200 g body weight interiorizes about 1.6 ml of plasma/day, i.e. about 25% of the intravascular plasma volume. According to Schultze & Heremans (1966), serum albumin has a half-life of about 2.6 days in the rat. If this protein is distributed over a volume twice that of the intravascular compartment (Katz *et al.*, 1970), this will imply that fluid endocytosis by the liver contributes about one-half to the breakdown of this plasma protein.

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References

- Altman, P. L. (1961) *Blood and Other Body Fluids* (Dittmer, D. S., ed.), Federation of American Societies of Experimental Biology, Washington, DC
- Bartholeyns, J. & Baudhuin, P. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 573–576
- Blouin, A., Bolender, R. P. & Weibel, E. R. (1977) *J. Cell Biol.* **72**, 441–452
- Boman, D. & Berg, T. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 301–308
- Bouma, J. M. W. & Gruber, M. (1964) *Biochim. Biophys. Acta* **89**, 545–547
- Bouma, J. M. W. & Gruber, M. (1966) *Biochim. Biophys. Acta* **113**, 350–358
- Brown, T. L., Henderson, L. A., Thorpe, S. R. & Baynes, J. W. (1978) *Arch. Biochem. Biophys.* **188**, 418–428
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Edelson, P. J. & Cohn, Z. A. (1974) *J. Exp. Med.* **140**, 1364–1386
- Gianetto, R. & de Duve, C. (1955) *Biochem. J.* **59**, 433–438
- Gibaldi, M. & Perrier, D. (1975) *Pharmacokinetics*, vol. 1, pp. 281–292, M. Dekker Inc., New York
- Gruber, M. (1953) *Biochim. Biophys. Acta* **10**, 136–142
- Henning, R., Kaulen, M. D. & Stoffel, W. (1971) *Hoppe Seyler's Z. Physiol. Chem.* **352**, 1347–1358
- Jacques, P. J. (1975) in *Uptake of Informative Macromolecules by Living Cells* (Ledoux, L., ed.), pp. 277–294, North-Holland Publishing Co., Amsterdam
- Kaplan, J. (1976) *Nature (London)* **263**, 596–597
- Katz, J., Sellers, A. L., Bonorris, G. & Golden, S. (1970) in *Plasma Protein Metabolism* (Rothschild, M. A. & Waldmann, T., eds.), pp. 129–154, Academic Press, New York
- Keeler, R. (1970) *Can. J. Physiol. Pharmacol.* **48**, 131–138
- Knook, D. L. & Sleyster, E. Ch. (1976) *Exp. Cell Res.* **99**, 444–449
- Kooistra, T., Duursma, A. M., Bouma, J. M. W. & Gruber, M. (1977) *Acta Biol. Med. Ger.* **36**, 1763–1776
- Kooistra, T., Duursma, A. M., Bouma, J. M. W. & Gruber, M. (1979) *Biochim. Biophys. Acta* **587**, 282–298
- Kooistra, T., Duursma, A. M., Bouma, J. M. W. & Gruber, M. (1980) *Biochim. Biophys. Acta* **631**, 439–450
- LaRusso, N. F. & Fowler, S. (1979) *J. Clin. Invest.* **64**, 948–954
- Lippman, M. E. & Finch, S. C. (1972) *Yale J. Biol. Med.* **45**, 463–470
- Madnick, H. M., Winkler, J. R. & Segal, H. L. (1978) *Arch. Biochem. Biophys.* **191**, 385–392
- Munthe-Kaas, A. C. (1977) *Exp. Cell Res.* **107**, 55–62
- Munthe-Kaas, A. C., Berg, T. & Seljelid, R. (1976) *Exp. Cell Res.* **99**, 146–154
- Ose, L., Ose, T., Reinertsen, R. & Berg, T. (1980) *Exp. Cell Res.* **126**, 109–119
- Pratten, M. K., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **168**, 365–372
- Ravin, H. A., Seligman, A. M. & Fine, J. (1952) *N. Engl. J. Med.* **247**, 921–929
- Reske-Nielsen, E., Bojsen-Møller, M., Vetner, M. & Hansen, J. C. (1976) *Acta Pathol. Microbiol. Scand. Sect. A* **84**, 397–405
- Roberts, A. V. S., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **168**, 239–244
- Schneider, Y.-J., Tulkens, P., de Duve, C. & Trouet, A. (1979) *J. Cell Biol.* **82**, 466–474
- Schultze, M. E. & Heremans, J. F. (1966) *Molecular Biology of Human Proteins with Special Reference to Plasma Proteins*, vol. 1, p. 479, Elsevier, Amsterdam
- Silverstein, S. C., Steinman, R. M. & Cohn, Z. A. (1977) *Annu. Rev. Biochem.* **46**, 669–722
- Sinke, J., Bouma, J. M. W., Kooistra, T. & Gruber, M. (1979) *Biochem. J.* **180**, 1–9
- Sleyster, E. Ch., Westerhuis, F. G. & Knook, D. L. (1977) in *Kupffer Cells and Other Liver Sinusoidal Cells* (Wisse, E. & Knook, D. L., eds.), pp. 289–298, Elsevier/North-Holland Biomedical Press, Amsterdam
- Steinman, R. M. & Cohn, Z. A. (1972) *J. Cell Biol.* **55**, 186–204
- Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **499**, 73–84
- Tohill, P. (1965) *J. Nucl. Med.* **6**, 582–587
- Wattiaux, R. (1966) *Étude Expérimentale de la Surcharge des Lysosomes*, (Thèse), pp. 51–53, J. Duculot, Gembloux
- Williams, K. E., Kidston, E. M., Beck, F. & Lloyd, J. B. (1975) *J. Cell Biol.* **64**, 113–122