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A Quantitative Study of Pinocytosis and Intracellular Proteolysis in Rat Peritoneal Macrophages

By MARGARET K. PRATTEN, KENNETH E. WILLIAMS and JOHN B. LLOYD
Biochemistry Research Unit, University of Keele, Keele, Staffordshire ST5 5BG, U.K.

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A method for the culture of rat peritoneal macrophages *in vitro* is described, in which pinocytic uptake of colloidal [^{198}Au]gold, ^{125}I-labelled poly(vinylpyrrolidone) and [^{14}C]sucrose proceeds at constant and fairly reproducible rates for several hours. The rate of uptake of colloidal [^{198}Au]gold, which exhibited some inter-batch variation, was approx. 100 times that of the other two substrates. Colloidal gold did not affect the rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone) and therefore its own high rate of uptake could not be attributed to a stimulation of the formation of pinocytic vesicles. It is concluded that uptake of colloidal gold is highly dependent on adsorption on binding sites on the plasma membrane. Uptake of formaldehyde-treated ^{125}I-labelled bovine serum albumin was followed by the release of [^{125}I]iodo-L-tyrosine into the culture medium and took place at a rate intermediate between those of colloidal [^{198}Au]gold and the other two non-digestible substrates, ^{125}I-labelled poly(vinylpyrrolidone) and [^{14}C]sucrose.

Interest in the mechanism and the pharmacology of pinocytosis has led to many attempts to quantify pinocytic uptake of solutes by mononuclear phagocytes (macrophages) in culture. Morphometric methods have been used (Cohn, 1966; Cohn & Parks, 1967*a*,*b*,*c*; Westwood & Longstaff, 1976), but these are of limited value, since the number of vacuoles visible in a cell with the optical microscope does not depend only on the rate at which vacuoles are formed by pinocytosis, but also on the longevity and size of the vacuoles. Also morphometric methods do not identify those substrates that are pinocytosed at a rapid rate because they enter by adsorbing on the cell membrane. Methods in which the uptake of radioactively labelled macromolecules by cells in culture is measured are potentially more powerful, but serious problems have been encountered in practice through the poor reproducibility of the rates of uptake. For example, Gosselin (1956, results republished Gosselin, 1967), in a detailed study of the uptake of colloidal gold by rabbit peritoneal macrophages, found the rate of uptake to decrease markedly during the course of an experiment and to vary between experiments. Moreover, much published work is marred by major methodological weaknesses that undermine the interpretation of the results. Some investigators calculate rates of uptake from measurements at only two time intervals and assume without evidence that uptake proceeds at a constant rate throughout the period. Another error occurs when a labelled protein is used as marker solute and no account is taken of its possible catabolism within the cells; yet post-ingestion catabolism of some proteins by macrophages is very rapid and ignoring it can give a completely false value for the true (higher) rate of uptake.

Nor is the interpretation of results without hazards. If two substrates are captured at demonstrably different rates, there are at least three possible explanations. Firstly, the more rapidly pinocytosed substrate may be captured by a cellular mechanism not available to the other substrate; for example, a solute may be able to enter by pinocytic invaginations too small to accommodate a colloid. Secondly, one solute may enter cells by adsorbing on binding sites on the plasma membrane being internalized, whereas another may enter only in the liquid phase. Thirdly, a substance may stimulate the rate of production of endocytic invaginations and thereby increase its own rate of uptake. It is not uncommon in published work for one explanation to be advanced without any evidence to exclude the others.

We describe here a method for culturing rat peritoneal macrophages in which the uptake of colloidal [^{198}Au]gold proceeds at a constant rate for several hours. The reproducibility of the rate of uptake was examined and comparisons made with the rates of uptake of ^{125}I-labelled poly(vinylpyrrolidone) and [^{14}C]sucrose. The rate of uptake of formaldehyde-denatured ^{125}I-labelled bovine serum albumin was measured by summing, at several time intervals, the radioactivity associated with the cells and that of

digestion products released into the medium. We believe the methods described will prove valuable for further studies on pinocytosis in macrophages.

Materials and Methods

Materials

Tissue-culture medium 199 (single strength, preparation TC20) was obtained from Wellcome Reagents, Beckenham, Kent, U.K. Swine serum (membrane-filter sterilized; catalogue no. 4-070D) was from Flow Laboratories, Irvine, Ayrshire, U.K. The serum was stored at −20°C for at least 3 days before use. Heparin (mucous) B.P. was from The Boots Company, Nottingham, U.K. Colloidal [^{198}Au]gold (particle size less than 20nm; preparation GCS.1P), ^{125}I-labelled poly(vinylpyrrolidone) (average mol.wt. 30000–40000; preparation IM.33P), [U-^{14}C]sucrose (preparation CFB.146) and [^{125}I]-iodide (preparation IMS.4) were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (type II) and poly(vinylpyrrolidone) (average mol.wt. 40000) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were of analytical grade. Where appropriate, glassware was silicone-treated with Repelcote (Hopkin and Williams, Chadwell Heath, Essex, U.K.).

Culture of peritoneal macrophages in vitro with radioactive substrates

Inbred male Wistar rats weighing 300–400g were killed with chloroform (a method found not to cause internal bleeding) and the abdominal fur was wetted with ethanol before reflexion of a skin flap. The peritoneal cavity was then injected with a mixture of medium 199 (8ml) and swine serum (2ml) containing 250i.u. of heparin. After vigorous massage of the abdomen the peritoneal contents were withdrawn with a sterile silicone-treated Pasteur pipette and placed in a sterile polycarbonate centrifuge tube. After centrifugation at 200*g* for 10min at room temperature, the supernatant was discarded and the pellet gently resuspended in a mixture of medium 199 (4ml) and swine serum (1ml). The suspension of cells was then recentrifuged, the supernatant discarded and the pellet resuspended with minimal agitation in a mixture of medium 199 (5ml) and swine serum (5ml). The number of cells present in the suspension was determined by using a haemacytometer slide with improved Neubauer ruling [Weber and Sons (Lancing), Sussex, U.K.]. Portions (1ml) of the suspension (which contained 1×10^6–4×10^6 cells/ml) were pipetted into individual sterile silicone-treated Leighton tubes each containing a sterile non-silicone-treated glass coverslip (9mm×35mm, no. 1½; Chance Propper, Smethwick, U.K.). The tubes were gassed with air/CO_2 (19:1) and placed in a water-jacketed incubator at 37°C for 30min. The culture medium was then removed and replaced with 1ml of medium 199/swine serum (1:1, v/v), thus removing non-adherent cells. (The number of such cells was measured, and regularly found to be approx. 10% of the total.) The tubes were regassed and the incubation was continued at 37°C for approx. 45h before incubation in the presence of radioactively labelled substrate.

In many experiments cells were collected from four to eight rats and pooled before determining the number of cells present and placing portions of the suspension in individual Leighton tubes. This permitted uptake of different substrates, or of the same substrate present at different concentrations, or the effect of different culture conditions, to be assessed with wholly comparable cell populations.

Culture in the presence of non-digestible gamma-emitting substrate

The medium in each Leighton tube was removed and replaced by 1ml of medium (comprising equal volumes of medium 199 and swine serum) in which either colloidal [^{198}Au]gold or ^{125}I-labelled poly-(vinylpyrrolidone) was dispersed. The tubes were then regassed and incubated at 37°C for various times up to 10h. At the end of the chosen incubation period duplicate 0.1ml samples of the medium were taken from each Leighton tube, diluted to 1.0ml with water in disposable polystyrene tubes (Luckham, Burgess Hill, Sussex, U.K.) and their radioactivity was measured under defined geometry with a Selektronic gamma spectrometer (Packard Instrument, Caversham, Berks., U.K.). Each coverslip with its adhering cell monolayer was gently washed six times with 2ml of Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954), pH7.4, placed in a Packard gamma-counter tube, and its radioactivity measured. Coverslip radioactivities were corrected for the difference in counting geometry. The short half-life of ^{198}Au required coverslips and media to be counted consecutively.

In another series of experiments release of radioactivity from cells subsequent to culture was measured after incubation by the normal method (see above) for 3h in the presence of colloidal [^{198}Au]gold (8μg/ml) or ^{125}I-labelled poly(vinylpyrrolidone) (100μg/ml) and the normal washing procedure. The coverslips were reincubated in substrate-free medium (1ml). At intervals over 3h (colloidal [^{198}Au]gold) or 7h [^{125}I-labelled poly(vinylpyrrolidone)] duplicate samples (0.1ml) of medium were withdrawn and replaced by 0.2ml of fresh culture medium. The samples of medium and the cell-bearing coverslip were assayed for radioactivity and the values obtained used to calculate the cumulative amount of radioactivity released (T_n), by the equation:

$$T_n = 10C_{t(t=n)} + 2\sum_{t=1}^{t=n-1} C_t$$

where n is the number of times samples were taken and C_i is the radioactivity of 0.1ml of the ith sample of medium. The total radioactivity present in the cells at the commencement of reincubation was determined by adding the total radioactivity released by the end of the reincubation to the residual radioactivity associated with the cells.

Culture in the presence of digestible gamma-emitting substrate

The macrophage monolayer was incubated as for a non-digestible gamma-emitting substrate except that the medium contained formaldehyde-treated ^{125}I-labelled bovine serum albumin, prepared by the method of Moore *et al.* (1977). The radioactivities of media and coverslips were determined as above. When the radioactivity of a diluted sample of medium (1.0ml) had been measured ('total radioactivity'), aq. 20% (w/v) trichloroacetic acid (0.5ml) was added and the precipitated protein sedimented by centrifuging at 1000*g* for 20min. The supernatant was then transferred to a clean disposable polystyrene tube and its radioactivity measured ('trichloroacetic acid-soluble radioactivity'); this value was corrected for the decrease in counting efficiency that accompanies an increase in sample volume. The 'trichloroacetic acid-insoluble radioactivity' was calculated by subtracting the acid-soluble radioactivity from the total radioactivity. Coverslips without cells were incubated with ^{125}I-labelled albumin to measure the amount of trichloroacetic acid-soluble radioactivity in the albumin preparation and to correct for any proteolytic activity associated with the culture medium.

Samples of media containing ^{125}I-labelled albumin, taken before and after culture for 24h with macrophages, were analysed to identify the radioactively labelled digestion products by column chromatography with Sephadex G-25 and its copper complex (Williams *et al.*, 1971, 1975*b*).

Culture in the presence of non-digestible beta-emitting substrate

The cells in this case were incubated in medium containing [^{14}C]sucrose. At the end of the incubation, the medium was assayed by using a slightly modified form of the method of Roberts *et al.* (1977). Duplicate samples (0.2ml) of medium were placed in scintillation vials; to each was added 0.8ml of water and 10.0ml of scintillation 'cocktail' [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 6g/l in toluene/Triton X-100 (2:1, v/v)]. Coverslips, with their adherent macrophages, were washed as above, and placed in scintillation vials with 1.0ml of water; scintillation 'cocktail' (10ml) was added to each vial, which was then briefly sonicated to disperse the coverslip-bound radioactivity. Radioactivity was measured for 10min in a Packard 2425 liquid-scintillation spectrometer three times over a period of several days. Counting efficiencies were determined by the internal-standardization method, a standard [^{14}C]sucrose solution being used to obtain relative counting efficiencies (see Roberts *et al.*, 1977). The presence of coverslips, with or without cells, had no demonstrable effect on the relative counting efficiencies.

Results

Uptake of non-digestible substrates

The amount of substrate accumulated by a given cell monolayer was expressed as the volume (μl) of culture medium whose contained substrate had been captured, per 10^6 cells. This quantity is given by the expression $C/(M \cdot N)$ where C is the total radioactivity of the cell monolayer (c.p.m., corrected for background and counting geometry), M is the radioactivity in 1μl of medium (c.p.m., corrected for background) and N is the number of cells (in millions) present in the monolayer. The uptake in a series of equivalent monolayers incubated for different times was then plotted against the incubation time. If uptake appeared to be linear with time, the rate of uptake was calculated by linear regression analysis and, following Williams *et al.* (1975*a*), a rate of uptake so expressed is termed an Endocytic Index. Although the units of Endocytic Index are μl/h per 10^6 cells, this does not imply that Endocytic Index is a measure of the rate of uptake of liquid (see below).

Expressing uptake in this way has a number of advantages (see Williams *et al.*, 1975*a*). It eliminates variability within an experiment arising from differences in the amount of radioactive substrate added to each culture vessel. It normalizes for the effects of variation in the specific radioactivity of substrates and in the number of cells present, and thus permits direct numerical comparison of rates of uptake, both in a series of experiments with the same radioactive substrate and in experiments with different substrates.

Uptake of colloidal [^{198}Au]gold

Fig. 1 shows the results of a typical experiment in which nine coverslips, each bearing cells from the same population, were incubated at 37°C with colloidal [^{198}Au]gold (8μg/ml) for periods of up to 6h. The accumulation of the substrate was linear with time, and the Endocytic Index was 2.31μl/h per 10^6 cells. This value indicates that only about 2.5% of the total radioactivity in the medium became associated with the cells within 6h; thus the radioactivity in the medium was effectively constant during the experiment, and no modification of the method of calculation was necessary to correct for the effects

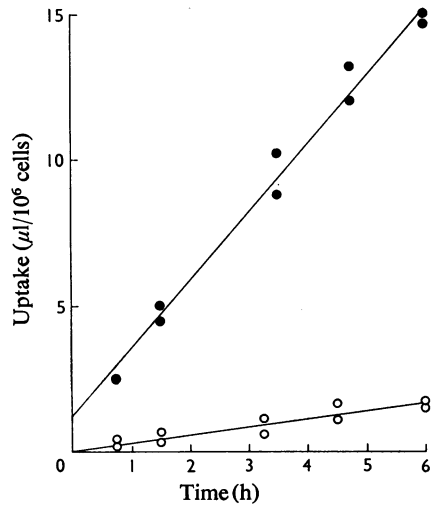

Fig. 1. *Accumulation of radioactivity by macrophages when incubated in the presence of colloidal [^{198}Au]gold*
Each point represents data derived from a single macrophage culture from the same population of cells, incubated for periods of up to 6h, at 37°C (●) or 4°C (○). The gradient of each plot gives a single value for the Endocytic Index.

Table 1. *Endocytic Indices of ten different batches of colloidal [^{198}Au]gold*
Each value for the Endocytic Index was derived from the plot of uptake against time by using data from an experiment in which nine or ten individual cultures from a single population of cells were incubated for intervals up to 6h in the presence of 8 μg of colloidal [^{198}Au]gold/ml. Results are expressed as means ±S.D. for the numbers of experiments given in parentheses.

Batch of colloidal [^{198}Au]gold	Endocytic Index (μl/h per 10^6 cells)
I (7)	5.58±1.51
II (7)	4.38±2.72
III (6)	1.21±0.46
IV (7)	8.33±1.98
V (14)	1.49±0.75
VI (9)	2.84±1.01
VII (13)	2.38±1.01
VIII (8)	2.11±0.93
IX (12)	2.93±1.95
X (6)	3.45±2.14

of substrate depletion. Other experiments showed that accumulation of colloidal gold continued at a constant rate for up to 17h, but normally incubations were of approx. 6h duration. Fig. 1 also shows that the rate of accumulation of label was much slower in incubations at 4°C. Table 1 shows the Endocytic Indices of several batches of colloidal gold.

Table 2. *Effect of increasing substrate concentration on rate of uptake of colloidal [^{198}Au]gold by macrophages*
Rates of uptake are expressed as Endocytic Indices. Each value for the Endocytic Index was derived from the plot of uptake against time by using results from a single experiment, as explained in the caption to Table 1. Results are expressed as means±S.D. for the numbers of experiments stated.

Concentration of colloidal gold (μg/ml)				
Radioactive	Non-radioactive	Total	No. of expts.	Endocytic Index (μl/h per 10^6 cells)
0.1	—	0.1	5	3.52±0.68
0.4	—	0.4	3	3.73±1.04
2	—	2	5	3.74±0.44
8	—	8	11	1.70±0.19
8	32	40	6	1.07±0.19
8	92	100	4	0.40±0.15
8	192	200	5	0.22±0.13

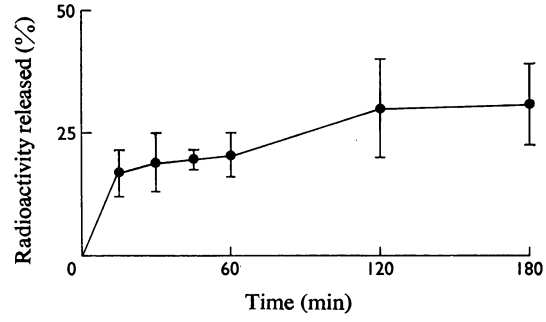

Fig. 2. *Release of radioactivity into the medium on culturing macrophages that had been preincubated in the presence of colloidal [^{198}Au]gold*
Released radioactivity is expressed as a percentage of the total radioactivity present in the cells at the commencement of reincubation. The graph shows the means±S.D. for four experiments.

Further experiments were performed with standard culture conditions but various concentrations (0.1–8 μg/ml) of colloidal [^{198}Au]gold. For higher concentrations (40–200 μg/ml), a colloidal [^{198}Au]gold preparation that had been stored for several months until no longer significantly radioactive was added in appropriate amount to media containing 8 μg of colloidal [^{198}Au]gold/ml. Two batches (V and VII) of colloidal [^{198}Au]gold with reasonably similar Endocytic Indices were used during the course of these experiments. Table 2 shows that the Endocytic Index decreased as the concentration increased. In a single experiment colloidal [^{198}Au]gold alone was used at 40, 100 and 200 μg/ml; results similar to those shown in Table 2 were obtained.

Neither poly(vinylpyrrolidone) at concentrations of 10–100 μg/ml nor sucrose at concentrations of up to 50 mg/ml had any effect on uptake of colloidal [^{198}Au]gold, although at sucrose concentrations above 25 mg/ml the macrophages tended to round up when examined by phase-contrast microscopy. At concentrations of sucrose above 100 mg/ml, uptake was much decreased and linearity of uptake with time was lost. Inhibition of pinocytosis by high concentrations of sucrose has been reported with Chang-strain human liver cells (Wagner *et al.*, 1971) and hamster fibroblasts (Warburton & Wynn, 1976).

Fig. 2 shows the release of radioactivity from macrophages cultured for 3 h in the presence of colloidal [^{198}Au]gold, washed and reincubated in substrate-free medium. An initial rapid loss of radioactivity was followed by a period of slower release.

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

Uptake of this substrate (10 μg/ml) was linear with time for 10 h, although the results showed more scatter than those with colloidal [^{198}Au]gold. There was no discernible difference between the Endocytic Indices of five different batches of ^{125}I-labelled poly(vinylpyrrolidone) and in 19 experiments the Endocytic Index was 0.034±0.006 (±S.D.) μl/h per 10^6 cells. The aged preparation of colloidal [^{198}Au]-gold with no residual radioactivity, at concentrations from 8 to 200 μg/ml, did not affect the rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone).

Fig. 3 shows the release of radioactivity from macrophages cultured for 3 h in the presence of ^{125}I-labelled poly(vinylpyrrolidone), washed and reincubated in substrate-free medium. Little radioactivity was released after the first 10 min, but the amount released in the initial period varied quite widely between experiments.

Uptake of [^{14}C]sucrose

One batch only of [^{14}C]sucrose was used, at a concentration of 1 μg/ml. Uptake was linear with time over 6 h and in four experiments the Endocytic Index was 0.020±0.004 (±S.D.) μl/h per 10^6 cells.

Uptake and digestion of ^{125}I-labelled bovine serum albumin

Fig. 4 shows the results of an experiment in which macrophages from the same population were incubated for various periods in the presence of formaldehyde-treated ^{125}I-labelled bovine serum albumin (10 μg/ml). The radioactivity associated with the cells was expressed as the volume of the associated culture medium in which was contained an equivalent amount of trichloroacetic acid-insoluble radioactivity, i.e. $C/(M \cdot N)$, where C is the radio-

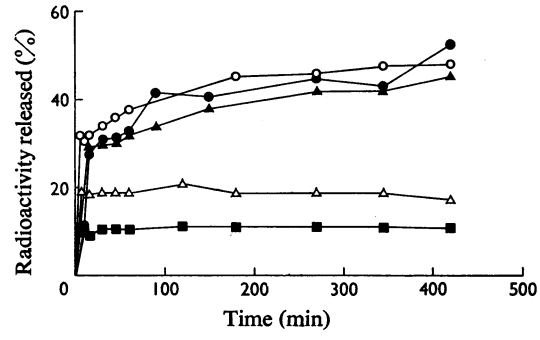

Fig. 3. *Release of ^{125}I-labelled poly(vinylpyrrolidone) by macrophages reincubated in fresh tracer-free medium*
Released radioactivity is expressed as a percentage of the total radioactivity present in the cells at the commencement of reincubation. The graph shows the individual results of five experiments.

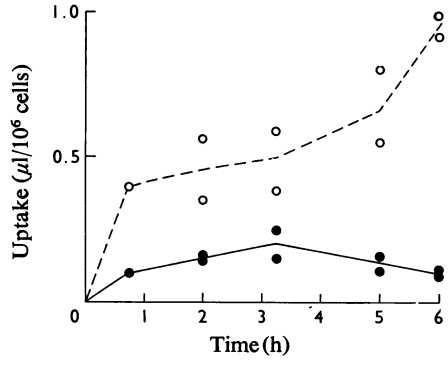

Fig. 4. *Accumulation of radioactivity by macrophages and appearance of trichloroacetic acid-soluble radioactivity in the culture medium on incubation of macrophages in the presence of formaldehyde-treated ^{125}I-labelled bovine serum albumin*
Each point represents results derived from a single culture, from the same population of cells, incubated separately for up to 6 h. The ordinate axis shows the volumes of culture medium whose content of trichloroacetic acid-insoluble radioactivity is found either associated with the tissue in trichloroacetic acid-insoluble or -soluble form (●), or in the culture medium as trichloroacetic acid-soluble radioactivity (○).

activity of the cell monolayer (c.p.m., corrected for background and geometry of counting), M is the trichloroacetic acid-insoluble radioactivity in 1 μl of medium (c.p.m., corrected for background) and N is the number of cells (in millions) present in the monolayer. The trichloroacetic acid-soluble radio-

activity of each sample of medium was expressed in the same way, $S/(M \cdot N)$, where S is the trichloroacetic acid-soluble activity in 1 ml of culture medium (c.p.m., corrected for background, hydrolysis by medium alone and geometry of counting).

The radioactivity associated with the cells became approximately constant after 2h, whereas the trichloroacetic acid-soluble radioactivity continued to rise during the entire incubation period. If it is assumed that the appearance of acid-soluble radioactivity in the medium results from pinocytic ingestion of labelled albumin, its subsequent intralysosomal digestion and release of digestion products into the medium, then the total uptake by each cell monolayer may be obtained by addition to the total cell-associated radioactivity of the acid-soluble radioactivity of the medium i.e. $(C+S)/(M \cdot N)$. Fig. 5 shows the data of Fig. 4 summed in this way. Uptake appeared to be linear with time, so that an Endocytic Index could be derived as before by linear-regression analysis. Table 3 shows the Endocytic Indices of five batches of labelled albumin. Considerable variation in the Endocytic Index was found between different batches of substrate, but intra-batch variation was small. It is noteworthy that the line in Fig. 5 has a positive intercept on the ordinate axis, which Fig. 4 shows is due to a high initial acid-soluble radioactivity. The size of this intercept varies markedly with small changes in the value used in the calculation for the percentage acid-soluble radioactivity of the albumin preparation, a value that is difficult to measure with great accuracy.

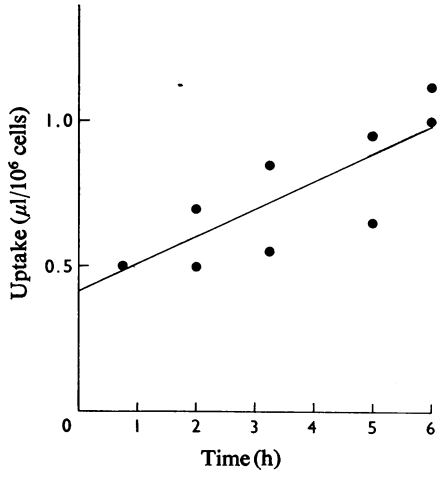

Fig. 5. *Uptake of ^{125}I-labelled bovine serum albumin by macrophages*

The ordinate axis shows the total volume of culture medium from which all the ^{125}I-labelled albumin has been ingested by the cells (calculated by summing the cell-associated radioactivity and the acid-soluble radioactivity released to the medium). Each point is derived from the results in Fig. 4 and relates to a single culture from the same population of cells, incubated for up to 6h.

Chromatography of pre- and post-culture media showed that the only major digestion product released was [^{125}I]iodo-L-tyrosine. This confirms the observation by Ehrenreich & Cohn (1967) with mouse peritoneal macrophages and ^{125}I-labelled human serum albumin as substrate.

Discussion

The system described here for the culture of rat peritoneal macrophages has been developed from methods of a number of previous investigators. One feature that deserves comment is that results are normalized to the number of cells rather than to their protein or DNA content. In measuring the rate of uptake of a non-digestible substrate, it would be adequate to determine radioactivity and protein (or DNA) contents on a representative sample of cells detached from the coverslip at the end of the incubation, quantitative detachment being experimentally impracticable. However, the method used here to derive the rate of uptake of ^{125}I-labelled albumin measures the digestion products released by the entire macrophage population and so requires a value for the entire radioactivity associated with all the cells on the coverslip. The number of cells must of course be determined before culture with substrate, but may be assumed to give a reliable estimate of adherent cells because only the coverslip is not silicone-treated and because a correction is made from a count of cells removed by the first wash at 30min.

Colloidal [^{198}Au]gold was accumulated by the macrophages at a constant rate throughout a culture period of 7h. To equate the rate of accumulation with the rate of endocytic uptake involves two assumptions, firstly that any adsorption on the macrophage surface reaches equilibrium rapidly (and so makes an equal contribution to cell-associated radioactivity at each time interval) and secondly that exocytosis of internalized substrate proceeds at a negligible rate compared with the rate of endocytic uptake. The first of these assumptions, although entirely reasonable, is difficult to confirm, and it is possible, albeit implausible, that a slow adsorptive process accounts for some of the accumulation of substrate seen. The much decreased, but still significant, accumulation of colloidal [^{198}Au]gold at 4°C could derive from either a slow adsorption or a residual endocytic activity. Loss of tightly bound radioactivity from macrophages previously exposed to colloidal [^{198}Au]-gold for 3h amounted to only 5% per h. Since this value must include release due to cell death

as well as to exocytosis, the rate of accumulation of colloidal [^{198}Au]gold may be taken as a close approximation to the rate of endocytic uptake. Davies *et al.* (1973*a*) have reported even slower release of colloidal [^{198}Au]gold from mouse macrophages.

Uptake of colloidal [^{198}Au]gold at a constant rate by (mouse) peritoneal macrophages cultured *in vitro* was reported by Davies *et al.* (1973*b*), who also obtained some estimate of the reproducibility of the rate between experiments by measuring the amount of radioactivity accumulated by the cells after a single defined incubation period. In the present experiments rates of uptake, expressed as Endocytic Indices to permit direct comparison between experiments (see above), were calculated by linear-regression analysis from values taken at five time intervals. Table 1 shows that the rate of uptake was reasonably reproducible for any one batch of colloidal [^{198}Au]-gold, indicating the method's applicability to investigations of the effects of modifiers of pinocytosis. Colloidal [^{198}Au]gold, as supplied by The Radiochemical Centre, has a spectrum of particle sizes (Radiochemical Centre, 1976), and the inter-batch differences in mean Endocytic Index may reflect differences in mean particle size. Similar inter-batch variation in the Endocytic Index of colloidal [^{198}Au]gold has also been observed in the rat yolk sac cultured *in vitro* (Roberts *et al.*, 1977).

The rate of uptake of ^{125}I-labelled poly(vinylpyrrolidone) was constant over a 10h incubation period and, as with colloidal [^{198}Au]gold, loss of tightly bound radioactivity from macrophages previously exposed to ^{125}I-labelled poly(vinylpyrrolidone) for 3h was very slow. The Endocytic Index of ^{125}I-labelled poly(vinylpyrrolidone) was about as reproducible between experiments as that of colloidal [^{198}Au]gold, but with no inter-batch variation. Roberts *et al.* (1977) found no inter-batch variation with ^{125}I-labelled poly(vinylpyrrolidone) in the yolk-sac system. The rate of uptake of [^{14}C]sucrose by macrophages was constant over a 6h incubation period and reproducible between experiments.

The Endocytic Indices of ^{125}I-labelled poly(vinylpyrrolidone) and [^{14}C]sucrose are two orders of magnitude lower than the Endocytic Index of colloidal [^{198}Au]gold. This result could be explained by colloidal gold acting as a powerful stimulator of pinosome formation, but this explanation is not compatible with the observed inability of colloidal gold to stimulate the uptake of ^{125}I-labelled poly-(vinylpyrrolidone). On the other hand, sucrose and poly(vinylpyrrolidone) could inhibit pinosome formation, but this is clearly not so since they had no effect at appropriate concentrations on uptake of colloidal [^{198}Au]gold. The remaining possibility is that colloidal [^{198}Au]gold is taken up more rapidly because it binds strongly to the plasma membrane, whereas sucrose and ^{125}I-labelled poly(vinylpyrrolidone) bind either weakly or not at all.

The Endocytic Index of colloidal [^{198}Au]gold decreased with increasing concentration of substrate (Table 2). This finding probably reflects a saturation of binding sites on the plasma membrane from which endocytic invaginations are forming. The alternative explanation, that high concentrations of colloidal gold either inhibit pinocytosis or increase mortality in the macrophage population, is excluded by the failure of colloidal gold to affect the Endocytic Index of ^{125}I-labelled poly(vinylpyrrolidone).

The results do not indicate whether uptake of colloidal [^{198}Au]gold is in the same vesicles that capture [^{14}C]sucrose and ^{125}I-labelled poly(vinylpyrrolidone) or in a separate class of endocytic invagination concerned with the capture of particulate matter and able to exclude liquid. It is, however, clear that the rate of uptake of liquid by the macrophages does not exceed the Endocytic Index ($0.02\,\mu$l/h per 10^6 cells) for [^{14}C]sucrose and that the Endocytic Index for colloidal [^{198}Au]gold indicates the the volume of culture medium whose substrate is captured (per h per 10^6 cells) and not the volume of liquid actually ingested.

Davies *et al.* (1973*b*) calculated the volume of the culture medium whose content of colloidal [^{198}Au]-gold was captured per h per 10^6 cells by mouse peritoneal macrophages cultured *in vitro*. Their values, which vary with the concentration of newborn-calf serum in the culture medium, are of the same order of magnitude as those seen with rat macrophages in the present work. However, they assumed that uptake of colloidal gold is entirely in the liquid phase and does not involve adsorption to membrane, and equated the volume of medium cleared of substrate with the volume of liquid captured. The present results contradict that interpretation, and it is noteworthy that Davies *et al.* (1973*b*) were aware that their calculated rate of uptake of liquid exceeded values obtained with other markers for endocytosis.

When macrophages were incubated with formaldehyde-denatured ^{125}I-labelled bovine serum albumin, trichloroacetic acid-soluble radioactivity appeared in the culture medium. The only radioactively labelled breakdown product detectable in the medium was [^{125}I]iodo-L-tyrosine, strong evidence that proteolysis is intracellular, for any breakdown of ^{125}I-labelled albumin on the external face of the plasma membrane would have led to the presence of radioactive peptides in the medium. Thus it is suggested that labelled albumin is ingested by pinocytosis and then digested within lysosomes. The permeability properties of the macrophage lysosomal membrane (Enrenreich & Cohn, 1969) would ensure that peptide fragments would be retained within lysosomes until proteolysis was complete. Whereas the amount of acid-soluble radioactivity in the medium continued

Table 3. *Endocytic Indices of five batches of formaldehyde-treated ^{125}I-labelled bovine serum albumin*

Each value for the Endocytic Index was derived from the plot of uptake against time by using results from an experiment in which nine or ten individual cultures from a single population of cells were incubated for intervals up to 6h in the presence of 10μg of formaldehyde-treated ^{125}I-labelled bovine serum albumin/ml. Results are expressed as means±S.D. for the numbers of experiments given in parentheses.

Batch of albumin	Endocytic Index (μl/h per 10^6 cells)
III (9)	0.441±0.285
V (6)	0.239±0.186
VI (3)	1.043±0.142
VII (3)	0.867±0.348
VIII (4)	0.627±0.185

to rise throughout the incubation, the radioactivity associated with the macrophages themselves did not (Fig. 4). This indicates that the proteolytic capacity of the macrophage lysosomes is adequate to degrade labelled albumin at the rate at which this substrate is being pinocytosed. Dingle *et al.* (1973) obtained a similar result by incubating rabbit alveolar macrophages with ^{3}H-labelled bovine haemoglobin, and further showed that incubation with an antiserum to rabbit cathepsin D decreased the rate of production of acid-soluble radioactivity and caused radioactivity to accumulate in the cells. It was concluded that the antiserum was acting as an intralysosomal inhibitor of proteolysis; the antiserum had no effect on the pinocytic uptake of [^{14}C]dextran.

The rate of uptake of ^{125}I-labelled albumin, calculated as described in the Results section and shown in Table 3, varied from one protein preparation to another. The values lay between those of [^{14}C]sucrose and ^{125}I-labelled poly(vinylpyrrolidone) and that of colloidal [^{198}Au]gold, indicating that adsorption plays a major role in pinocytic uptake of denatured albumin. This result parallels findings in the rat yolk sac (Williams *et al.*, 1975*b*), as does the inter-batch variability in Endocytic Index, which has been attributed to variations in the extent of denaturation (Williams *et al.*, 1975*b*; Moore *et al.*, 1977).

Several authors have demonstrated the ability of macrophages in culture to digest previously endocytosed proteins to amino acids (Ehrenreich & Cohn, 1967, 1968; Dingle *et al.*, 1973), and some have inferred the rate of endocytosis from the initial rate of accumulation of the protein by the cells (ignoring any loss by digestion). Ehrenreich & Cohn (1968), with mouse peritoneal macrophages and [^{3}H]haemoglobin as substrate, obtained results from which an Endocytic Index of 0.3μl/h per 10^6 cells may be deduced. This value is strikingly similar to the values reported in the present paper for ^{125}I-labelled bovine serum albumin. Steinman & Cohn (1972) obtained a value of 0.025μl/h per 10^6 cells with horseradish peroxidase as substrate. The similarity of this value to those obtained in the present investigation for ^{125}I-labelled poly(vinylpyrrolidone) and [^{14}C]sucrose supports the view of Steinman & Cohn (1972) that peroxidase enters only in the liquid phase. We have not, however, found previous reports in which the rate of uptake was calculated, as in the present paper, by summing the activity found in the cells and the digestion products released into the medium.

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