Rate of Pinocytic Capture of Macromolecular Substrates by Rat Yolk Sac Incubated in Serum-Free Culture Medium

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Pinocytic activity was quantified for rat yolk sacs incubated in a medium that was either serum-free or contained 10% (v/v) of calf serum. Absence of serum from the medium caused a small increase in the rate of pinosome formation, as determined by the rates of capture of both ^{125}I -labelled poly(vinylpyrrolidone) and $[^{14}\text{C}]$ sucrose. In contrast, the rates of uptake of substrates ingested by adsorptive pinocytosis were greatly enhanced when serum proteins, which compete for the same binding sites on the plasma membrane as used by adsorbing substrates, were absent. Elimination of such competition greatly simplifies the quantitative analysis of the binding process, and permitted a detailed study of the binding to the plasma membrane of formaldehyde-denatured bovine serum albumin, a protein that is rapidly digested within the lysosomal system after its pinocytic capture. Binding obeyed Michaelis–Menten kinetics and showed a dissociation constant of approx. $1\ \mu\text{M}$, indicating the high affinity of this protein for binding sites on the surface of actively pinocytosing yolk-sac cells.

The addition of between 10 and 50% (v/v) of mammalian serum to a chemically defined culture medium usually favours the survival and multiplication of mammalian cells *in vitro*, but the identity of the active components of the serum and their precise effects on cells remain unclear (Temin *et al.*, 1972). However, a number of published studies indicate that the endocytic activity of mouse peritoneal macrophages, as determined either by morphometric methods (Cohn & Parks, 1967) or by the rate of accumulation of colloidal $[^{198}\text{Au}]$ gold (Davies *et al.*, 1973), is markedly enhanced on increasing the amount of newborn-calf serum in the culture medium. It is not known whether such modulation of endocytic activity by components of serum is peculiar to macrophages from this source, or is a more general property of endocytic mammalian cells, since few systems *in vitro* are as reproducible and as well documented as the mouse peritoneal macrophage system (Williams, 1979).

Williams *et al.* (1975a), however, described a method of culturing rat visceral yolk sac, in medium 199 containing 10% (v/v) of heat-inactivated calf serum. By using this method, the rates of capture of radiolabelled substrates which are not hydrolysed by lysosomal enzymes, and hence accumulate within the lysosomal system after pinocytic uptake, have been measured (Williams *et al.*, 1975a; Roberts *et al.*, 1977). A method of analysis was also described (Williams *et al.*, 1975b) that permits the determination

of the rate of capture of ^{125}I -labelled bovine serum albumin even though this protein is hydrolysed rapidly within yolk-sac lysosomes after pinocytic capture (Williams *et al.*, 1971, 1975b; Moore *et al.*, 1977). The high rate of ingestion of this protein preparation, compared with that of ^{125}I -labelled poly(vinylpyrrolidone), was shown to arise from its adsorbing extensively to regions of the plasma membrane that invaginate to form the limiting membrane of pinocytic vesicles (Moore *et al.*, 1977).

Since, in all the above studies, the culture medium routinely contained 10% (v/v) of calf serum, it was not known whether substantial changes in the serum content of the medium were capable of modifying the rate of pinosome formation, in a manner similar to that observed with peritoneal macrophages. Moreover, in the initial studies with ^{125}I -labelled bovine serum albumin as substrate (Williams *et al.*, 1975b) only a limited quantitative analysis of the observed plasma membrane adsorption phenomenon was possible, since the serum proteins, present in high concentrations compared with the tracer protein, were all at least potentially capable of competing for binding sites on the surface of the yolk-sac cells. This confused the interpretation of the dependence of the rate of capture of this protein on its concentration in the medium and indicated that it was desirable to simplify the system by removing the serum. The rates of capture of a number of substrates, including formaldehyde-denatured ^{125}I -labelled albumin, were

therefore determined, by using rat yolk sacs incubated in medium 199 alone, and the associated kinetics analysed.

Materials and Methods

Non-radiolabelled poly(vinylpyrrolidone) (PVP-40; mean mol.wt. 40000) was purchased from Sigma (London) Chemical Co. (London S.W.6, U.K.). The details of other materials are given in Williams *et al.* (1975a), Moore *et al.* (1977) or Roberts *et al.* (1977).

Preparation of formaldehyde-denatured iodinated bovine serum albumin

The method of Moore *et al.* (1977) was used to prepare batches of both ^{125}I -labelled bovine serum albumin and (non-radioactive) [^{127}I]iodinated bovine serum albumin iodinated to the same extent as the radiolabelled analogue. The maximum working concentration of the latter was 2mg/ml, since higher concentrations resulted in precipitation during the dialysis procedure. Solutions of these preparations were stored at -20°C .

Incubation of rat yolk sac with radiolabelled substrates

In experiments using medium containing 10% (v/v) serum, yolk sacs were incubated for a maximum period of 6.5h. However, preliminary experiments in serum-free medium 199 showed that uptake was poorly reproducible beyond 5h; hence all such incubations were routinely terminated by this time.

The rates of uptake of ^{125}I -labelled poly(vinylpyrrolidone) and colloidal [^{198}Au]gold were determined by the method described by Williams *et al.* (1975a) and that of [^{14}C]sucrose by the method of Roberts *et al.* (1977). The rate of uptake of formaldehyde-denatured ^{125}I -labelled bovine serum albumin was determined by the method of Williams *et al.* (1975b), modified as described by Moore *et al.* (1977) and as described below.

Measurement of the amount of ^{125}I -labelled poly(vinylpyrrolidone) or colloidal [^{198}Au]gold present in the culture medium

The amount of radioactive substrate present in the culture medium at the end of an incubation was determined on a Selekttron gamma spectrometer (Packard Instruments Ltd., Caversham, Berks., U.K.) by counting radioactivity of duplicate samples (1.0ml) from each flask in 3ml disposable tubes positioned in a standard geometrical relation to the crystal detector.

Measurement of the hydrolysis products of ^{125}I -labelled albumin in the culture medium

A modification of the original method of Williams *et al.* (1975b) was used. Duplicate samples (1.0ml) of each medium were first counted for radioactivity

as described above. Those samples containing 10% (v/v) calf serum were then mixed directly with trichloroacetic acid (0.5 ml of 20%, w/v), to precipitate intact and incompletely hydrolysed ^{125}I -labelled albumin, whereas each sample containing no calf serum was first mixed with calf serum (0.1 ml), to provide carrier protein, before trichloroacetic acid precipitation in the above manner. All tubes were then centrifuged (40000g-min) and the clear supernatant solutions carefully decanted into disposable tubes and counted for radioactivity. After correction of the observed radioactivity for background, the trichloroacetic acid-soluble radioactivity was multiplied by the appropriate empirical correction factor to convert the observed value into that which would have obtained if the entire acid-soluble radioactivity, including that occluded within the precipitated protein, had been measured in 1.0ml of solution. This factor was determined by counting radioactivity of 1.0ml portions of a solution of [^{125}I]iodo-L-tyrosine (the known major hydrolysis product of ^{125}I -labelled albumin in the yolk-sac system; see Williams *et al.*, 1975b) in either medium 199 or medium 199 containing 10% calf serum, then precipitating with trichloroacetic acid by the appropriate method and counting the supernatants for radioactivity after centrifugation (40000g-min). The ratio of initial to supernatant radioactivity gave the corresponding correction factor.

On assaying the samples of medium, the differences between the initial and the corrected acid-soluble radioactivity gave the amount of trichloroacetic acid-insoluble radioactivity remaining in the culture medium at the end of an individual incubation period.

Measurement of [^{14}C]sucrose in the culture medium

The method described in Roberts *et al.* (1977) was used.

Measurement of the tissue-associated substrate radioactivity

For methods see Roberts *et al.* (1977).

Determination of protein

The protein content of each yolk sac was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as reference protein.

Expression of the accumulation of non-digestible substrates by yolk sacs

The amounts of ^{125}I -labelled poly(vinylpyrrolidone), [^{14}C]sucrose and colloidal [^{198}Au]gold captured by the yolk sacs in a given time were expressed as the volume of culture medium (μl) whose contained substrate had been captured by unit quantity of tissue. The merits of this form of

Fig. 1. Accumulation of radioactivity by rat yolk sacs incubated in serum-free medium 199 containing tracer quantities of radiolabelled substrates

The results of three representative experiments, each with a different substrate, are shown. In individual plots, points at each time-interval correspond to data derived from a single yolk sac incubated separately for up to 5.0 h. Substrates were: ●, ^{125}I -labelled polyvinylpyrrolidone (2–5 μg/ml); ○, $[U-^{14}\text{C}]$ -sucrose (0.1 μg/ml); □, colloidal $[^{198}\text{Au}]$ gold (1 μg/ml).

expression are detailed in Roberts *et al.* (1977). This quantity was calculated from the expression:

$$A = \frac{Y}{M \times P} \times 10^3 \text{ (}\mu\text{l/mg of yolk-sac protein)}$$

where Y is the radioactivity contained in the yolk sac (c.p.m.), P is the amount of protein (mg) in an individual yolk sac and M the mean amount of substrate radioactivity in 1.0 ml of medium during the incubation period (c.p.m.). M is equal to $T + \frac{1}{2}(Y/10)$, where T is the substrate radioactivity (c.p.m./ml of medium) as measured at the end of the incubation period and the term $\frac{1}{2}(Y/10)$ represents one-half of the substrate radioactivity (c.p.m./ml of medium) captured by the yolk sac from each ml of medium (total volume 10.0 ml) during the incubation period of the individual yolk sac.

All radioactivity counts are corrected for background, counting geometry and counting efficiency where appropriate.

Expression of the rate of ingestion of ^{125}I -labelled proteins by yolk sacs

The following expression was used to calculate the amount of ^{125}I -labelled protein captured over the period of time for which an individual yolk sac was incubated:

$$A = \frac{Y + 10\Delta S}{M' \times P} \times 10^3 \text{ (}\mu\text{l/mg of yolk-sac protein)}$$

where the numerator is equal to the sum of Y , the substrate radioactivity associated with the yolk sac (c.p.m.), and the total quantity of substrate radioactivity that has been rendered trichloroacetic acid-soluble by the tissue during the incubation period (c.p.m.). The latter is equal to $10\Delta S$, where ΔS is the difference (c.p.m./ml of medium) between the amount of trichloroacetic acid-soluble radioactivity in the medium (total volume 10.0 ml) at the end of the incubation period and that in 1.0 ml of the control medium that has been incubated in the absence of tissue for the same period of time.

M' is the mean amount of trichloroacetic acid-insoluble radioactivity (c.p.m./ml of medium) present during the individual incubation period of the tissue, and, assuming a constant rate of substrate depletion, is given by:

$$M' = T' + \frac{1}{2} \left(\frac{Y}{10} + \Delta S \right)$$

The ordinate axis shows the volume of culture medium whose contained radiolabelled substrate has been accumulated within a given time-interval; values are normalized on the basis of the protein content of each yolk sac.

Table 1. *Effects of removing calf serum from the incubation medium on the rates of pinocytosis of non-digestible radiolabelled substrates*

Each value of the Endocytic Index was obtained from a separate experiment in which yolk sacs (6–11) from a 17.5-day-pregnant rat were incubated in the presence of one of the radioactively labelled substrates at the tracer concentration indicated. For incubations in the presence of calf serum a maximum incubation period of 6.5 h was used; in the absence of calf serum the maximum incubation period was decreased to 5.0 h, since uptake was noticeably less reproducible beyond this time. Uptake (calculated as described in the text) was plotted against time; the Endocytic Index is equal to the gradient of the regression line through these 6–11 points. Values are expressed as means $\pm$ S.D. for the numbers of experiments given in parentheses.

Substrate	Substrate concn. in medium ($\mu\text{g/ml}$)	Endocytic Index ($\mu\text{l/h}$ per mg of yolk-sac protein)	
		Medium 199 + 10% calf serum	Medium 199 alone
^{125}I -labelled poly(vinylpyrrolidone)	2–5	1.70 ± 0.30 (55)	2.47 ± 1.00 (11)
[U- ^{14}C]Sucrose	0.1	3.69 ± 0.36 (3)	4.13 ± 0.88 (3)
Colloidal [^{198}Au]gold	1.0	4.56 ± 0.62 (3)	55.62 ± 3.40 (3)

Fig. 2. *Accumulation of radioactivity by yolk-sac tissue and appearance of trichloroacetic acid-soluble radioactivity in the serum-free medium on incubating yolk sacs in the presence of tracer quantities of formaldehyde-treated ^{125}I -labelled bovine serum albumin*

Points at each time-interval correspond to data derived from single yolk sacs incubated separately for up to 5.0 h. The ordinate shows the volume of culture medium whose contained trichloroacetic acid-insoluble radioactivity is found associated with the tissue in either trichloroacetic acid-soluble or -insoluble form ($\bullet$), or is found in the incubation medium as trichloroacetic acid-soluble radioactivity ($\circ$). These two quantities are summed for each yolk sac to give the total volume of culture medium whose contained ^{125}I -labelled albumin has been ingested by the tissue ($\blacksquare$).

where T' is the acid-insoluble radioactivity (c.p.m./ml of medium) measured at the end of the yolk-sac incubation period, and the second term represents one-half of the amount of acid-insoluble radio-

activity ingested from each ml of medium, in the same time.

The associated calculations were performed with the aid of an ICL 4130 computer.

Measurement of the rate of exocytosis of ^{125}I -labelled poly(vinylpyrrolidone) from yolk sacs

The method described by Williams *et al.* (1975a) was used.

Results

In agreement with the findings of Roberts *et al.* (1977), ^{125}I -labelled poly(vinylpyrrolidone), [U- ^{14}C]-sucrose and colloidal [^{198}Au]gold were all accumulated linearly with time when yolk sacs were incubated for up to 6.5 h with these substrates in medium 199 containing 10% (v/v) of calf serum. This permitted the rates of uptake to be expressed in the form of Endocytic Indices as defined by Williams *et al.* (1975a). Similar experiments, using these same substrates and serum-free medium 199, resulted in linear uptake up to 5.0 h (Figs. 1a and 1b), but the Endocytic Indices were significantly less reproducible even for data gathered within this shorter incubation period (see Table 1). The Endocytic Index of both ^{125}I -labelled poly(vinylpyrrolidone) and [U- ^{14}C]-sucrose rose slightly, but that of colloidal [^{198}Au]gold showed a 12-fold increase on removal of serum from the medium (Table 1). The rate of release of ^{125}I -labelled poly(vinylpyrrolidone) from yolk sacs re-incubated in medium 199 alone was approx. 1%/h, almost identical with the value obtained by Williams *et al.* (1975a) from tissues re-incubated in 10% calf serum.

In experiments in which yolk sacs were incubated in serum-free medium 199, containing both ^{125}I -labelled poly(vinylpyrrolidone) (2 $\mu\text{g/ml}$) and differing concentrations of unlabelled poly(vinylpyrrolidone) of a similar molecular weight to the radiolabelled

Table 2. *Effects of removing calf serum from the incubation medium on the rates of pinocytosis of different batches of formaldehyde-denatured ^{125}I -labelled bovine serum albumin*

Each value of the Endocytic Index was obtained from a separate experiment, as described in Table 1, in which the concentration of formaldehyde-denatured ^{125}I -labelled bovine serum albumin in the medium was 0.6–1.5 $\mu\text{g}/\text{ml}$. The amount of radioactivity associated with the tissue, expressed in the same units as uptake, is also shown. Results are expressed as means $\pm$ s.d. for the number of experiments given in parentheses.

Batch of ^{125}I -labelled bovine serum albumin	Medium 199+10% calf serum		Medium 199 alone	
	Endocytic Index ($\mu\text{l}/\text{h}$ per mg of yolk-sac protein)	Tissue radioactivity ($\mu\text{l}/\text{mg}$ of yolk-sac protein)	Endocytic Index ($\mu\text{l}/\text{h}$ per mg of yolk-sac protein)	Tissue radioactivity ($\mu\text{l}/\text{mg}$ of yolk-sac protein)
I	56.3 $\pm$ 10.3 (5)	70.2 $\pm$ 8.7 (5)	158.6 $\pm$ 44.6 (3)	70.1 $\pm$ 9.2 (3)
II	64.8 $\pm$ 5.9 (6)	93.3 $\pm$ 16.4 (6)	242.2 $\pm$ 31.9 (3)	124.6 $\pm$ 25.4 (3)
III	—	—	229.8 $\pm$ 5.4 (4)	80.0 $\pm$ 8.0 (4)
IV	69.5 $\pm$ 6.4 (7)	71.1 $\pm$ 14.8 (7)	381.5 $\pm$ 24.8 (3)	102.6 $\pm$ 5.6 (3)
V	81.8 $\pm$ 13.6 (4)	85.0 $\pm$ 9.9 (4)	—	—

Table 3. *Effects on the pinocytosis of formaldehyde-denatured ^{125}I -labelled bovine serum albumin of adding increasing concentrations of formaldehyde-denatured [^{127}I]iodinated bovine serum albumin to the serum-free medium 199*

Experiments were performed in medium 199 alone in essentially the same manner as described in Table 2 except that, in addition to formaldehyde-denatured ^{125}I -labelled bovine serum albumin (batch IV, 0.6 $\mu\text{g}/\text{ml}$ of medium), various quantities of formaldehyde-denatured [^{127}I]iodinated bovine serum albumin (0–170 $\mu\text{g}/\text{ml}$ of medium) were also present. Results are expressed as means $\pm$ s.d. for the numbers of experiments indicated.

Concn. of [^{127}I]iodinated bovine serum albumin ($\mu\text{g}/\text{ml}$ of medium)	No. of experiments	Endocytic Index ($\mu\text{l}/\text{h}$ per mg of yolk-sac protein)	Tissue radioactivity ($\mu\text{l}/\text{mg}$ of yolk-sac protein)
0	3	381.5 $\pm$ 24.8	102.5 $\pm$ 5.5
4.25	3	278.4 $\pm$ 64.8	77.9 $\pm$ 10.8
8.5	4	214.7 $\pm$ 30.7	61.6 $\pm$ 10.8
17.0	4	197.7 $\pm$ 24.2	55.7 $\pm$ 13.7
42.5	3	171.3 $\pm$ 51.6	53.0 $\pm$ 12.2
85.0	4	138.3 $\pm$ 27.6	30.5 $\pm$ 8.2
127.5	4	78.6 $\pm$ 26.9	20.7 $\pm$ 5.4
170.0	3	96.8 $\pm$ 24.9	23.7 $\pm$ 2.0

substrate, progressively raising the concentration of unlabelled poly(vinylpyrrolidone) from 0 to 1000 $\mu\text{g}/\text{ml}$ of medium did not cause the corresponding value of the Endocytic Index to change significantly [2.47 ± 1.00 (s.d.) and 2.34 ± 0.56 (s.d.) $\mu\text{l}/\text{h}$ per mg of yolk-sac protein respectively]. There appeared, however, to be a marginal increase in the degree of reproducibility of results of experiments in which high concentrations of the unlabelled polymer were used. The presence of formaldehyde-treated [^{127}I]iodinated bovine serum albumin, at concentrations of up to 170 $\mu\text{g}/\text{ml}$ of medium, had effects on the Endocytic Index of ^{125}I -labelled poly(vinylpyrrolidone) that were almost identical with those caused by the higher concentrations of unlabelled poly(vinylpyrrolidone).

When formaldehyde-denatured ^{125}I -labelled bovine serum albumin was used as substrate in medium containing 10% (v/v) of calf serum, uptake (calculated as described in the Materials and Methods section) was linear with time over a 6.5 h period and

the values of the Endocytic Index obtained were virtually identical with the value [65.0 ± 11.0 (s.d.) $\mu\text{l}/\text{h}$ per mg of yolk-sac protein] obtained by Moore *et al.* (1977) in similar experiments. On removal of calf serum from the incubation medium, uptake was again observed to be linear with time (Fig. 2) over a 5.0 h period, but frequently became noticeably more scattered beyond this time limit. The results of a number of experiments in the presence and absence of calf serum are summarized in Table 2 and show that a 3–5-fold rise in the Endocytic Index of radiolabelled albumin occurs on removing calf serum from the culture medium, but that this is accompanied by only a marginal rise (up to 44%) in the quantity of radioactivity associated with the tissue in the steady-state period, i.e. beyond 2.0 h of incubation.

Although increasing concentrations of formaldehyde-denatured [^{127}I]iodinated bovine serum albumin were without effect on the Endocytic Index of ^{125}I -labelled poly(vinylpyrrolidone), such increases

greatly decreased the Endocytic Index of formaldehyde-denatured ^{125}I -labelled bovine serum albumin (Table 3).

Discussion

The highly reproducible pinocytic activity of yolk sacs incubated in medium 199 containing 10% (v/v) of heat-inactivated calf serum is now well established (Williams *et al.*, 1975a,b; Roberts *et al.*, 1976, 1977; Moore *et al.*, 1977). The results of the current study show pinocytic activity to be maintained in the absence of serum; the amount of ^{125}I -labelled poly(vinylpyrrolidone) accumulated within the tissue rose linearly with time, but the rate of accumulation was less reproducible, even when the maximum incubation period was limited to 5.0 h, than that from equivalent experiments in medium containing calf serum (10%, v/v). The observation that addition of unlabelled poly(vinylpyrrolidone) to serum-free medium restored the degree of reproducibility to a value similar to that for a serum-containing medium is remarkably similar to the finding of Becker & Ashwood-Smith (1973) when equivalent changes were made in the culture conditions of Chinese-hamster fibroblasts pinocytosing ^3H sucrose. Such findings suggest that one effect of calf serum in culture media is somehow to stabilize the pinocytic behaviour of cells, but that an inert macromolecule such as poly(vinylpyrrolidone) can serve this purpose equally well.

The rate of loss of ^{125}I -labelled poly(vinylpyrrolidone) on re-incubating, in fresh medium, yolk sacs that have previously ingested this substrate in the absence of calf serum, remained very low [approx. 1%/h, the same value as reported from similar re-incubations in medium containing 10% (v/v) calf serum; Williams *et al.*, 1975a]. Hence accumulation is a measure of uptake and, relative to the rates of uptake in the presence of calf serum (10%, v/v), those in the absence of serum rose by 12% for $[\text{U-}^{14}\text{C}]$ sucrose and by 45% for ^{125}I -labelled poly(vinylpyrrolidone) respectively. These results imply either that the rate of pinosome formation is increased by up to 45% when serum is removed from the medium, or that pinosome formation continues at the same rate but some component of the calf serum binds strongly to the plasma membrane and displaces these low-affinity substrates from binding sites that can be occupied when serum is absent. This second explanation was rendered highly unlikely by the observed failure of increasing concentrations of non-radioactive poly(vinylpyrrolidone) to decrease the Endocytic Index of the radiolabelled form of the substrate in substrate-free medium. Thus, in agreement with the proposals of Roberts *et al.* (1977), we conclude that ^{125}I -labelled poly(vinylpyrrolidone) is internalized entirely in the fluid phase. Furthermore, we conclude

that the rate of pinosome formation is increased by up to 45% on removing calf serum from the medium. {The smaller increase in the Endocytic Index of $[\text{U-}^{14}\text{C}]$ sucrose on removing serum from the medium may be a consequence of an increase in the rate of leakage of sucrose from the tissue after pinocytic capture in serum-free medium 199, since Roberts *et al.* (1977) observed that, even in the presence of serum, $[\text{U-}^{14}\text{C}]$ sucrose is lost more rapidly, on re-incubating the washed tissue in fresh medium, than is either ^{125}I -labelled poly(vinylpyrrolidone) or colloidal ^{198}Au -gold.} The 45% rise in the rate of capture of ^{125}I -labelled poly(vinylpyrrolidone) by yolk sacs on removing calf serum (10%, v/v) from the medium 199 is in complete contrast with the results when similar changes in the culture conditions of mouse peritoneal macrophages are made (Davies *et al.*, 1973). These authors observed a 60% fall in the rate of capture of colloidal ^{198}Au gold on decreasing the calf serum concentration in the medium 199 from 10 to 1% (v/v) and a stimulation of uptake (up to 30%) on progressively raising the serum concentration from 10% to 50% (v/v). Becker & Ashwood-Smith (1973), however, observed less than a 10% fall in the rate of capture of ^3H sucrose by hamster fibroblasts when foetal-calf serum (10%, v/v) was removed from the medium. Thus removal of serum from the medium may not always have the same effect on pinocytosis as reported by Davies *et al.* (1973). The same conclusion was reached by Brunk *et al.* (1977), who observed that removal of calf serum (10%, v/v) from the culture medium greatly decreased the membrane-ruffling activity in human glia cells, but had a much smaller effect on this activity in malignant glioma cells.

In contrast with ^{125}I -labelled poly(vinylpyrrolidone) and $[\text{U-}^{14}\text{C}]$ sucrose, both colloidal ^{198}Au -gold and formaldehyde-denatured ^{125}I -labelled bovine serum albumin showed greatly increased rates of uptake by yolk sacs on removal of serum from the medium (Tables 1 and 2). Moreover, in contrast with the results of the equivalent experiments with ^{125}I -labelled poly(vinylpyrrolidone), the Endocytic Index of formaldehyde-denatured ^{125}I -labelled bovine serum albumin was decreased progressively (by up to 75%) on increasing the concentration of its non-radioactive analogue in the serum-free medium (Table 3). The possibility that this decrease resulted from a pharmacological effect of these concentrations of the iodinated protein on the rate of pinosome formation in serum-free medium can be dismissed, since the rate of capture of ^{125}I -labelled poly(vinylpyrrolidone) was not altered on adding increasing concentrations of formaldehyde-denatured ^{127}I -iodinated bovine serum albumin to the serum-free medium. The elevated rates of uptake of labelled gold and albumin, and the observed decrease in the rate of uptake of labelled albumin in the presence of its

unlabelled analogue, appear to result from binding of these compounds to the plasma membrane.

Jacques (1969) formulated an expression for Q , the rate of pinocytic capture of a solute, as the sum of two terms; one representing uptake by fluid capture, the other uptake by prior adsorption of substrate to the plasma membrane:

$$Q = Fc + \frac{SRc}{K+c}$$

where c is the concentration of solute in the extracellular fluid, K the dissociation constant of the substrate-surface complex and R is the number of substrate-binding sites found per unit area of plasma membrane. F and S are the rates of capture of extracellular fluid and plasma membrane respectively. From this relation Williams *et al.* (1975b) derived the corresponding expression for the Endocytic Index as:

$$\text{Endocytic Index} = \frac{Q}{c} = F + \frac{SR}{K+c}$$

from which it follows that, if there is no substrate adsorption, the second term can be ignored and, provided that the substrate itself does not alter the rate of pinosome formation, the Endocytic Index of a non-adsorbed substrate will be constant and equal to F . Knowing ^{125}I -labelled poly(vinylpyrrolidone) to be a non-adsorbing substrate, then, in serum-free incubations, F can be assigned a value of 2.47 ± 1.00 (s.d.) $\mu\text{l/h}$ per mg of yolk-sac protein. In contrast, the Endocytic Index of ^{125}I -labelled bovine serum albumin was far higher, but varied significantly between preparations (range 159–382 $\mu\text{l/h}$ per mg of yolk-sac protein). Moreover, the Endocytic Index of this substrate was greatly decreased on introducing small quantities of the [^{127}I]iodinated form of this protein into the serum-free medium (Table 3), but similar additions of the [^{127}I]iodinated protein had no effect on the rate of pinosome formation as determined by the rate of capture of ^{125}I -labelled poly(vinylpyrrolidone). It thus appears that the dominant (98.5–99.3%) route of uptake of this protein substrate in serum-free medium is by adsorption on the plasma membrane.

Assuming that the ^{125}I -labelled and [^{127}I]iodinated proteins are treated identically and that none of the components of medium 199 adsorbs to the binding sites on the plasma membrane that are occupied by this substrate, the membrane binding of this protein can be analysed more fully. Adsorptive uptake can be formulated as:

where B represents unoccupied membrane binding sites, X extracellular substrate, B·X occupied binding

sites and B·P adsorbed substrate that has been pinocytosed. Since internalization was found to be effectively irreversible, k_{-2} can be taken as zero, and, since under defined culture conditions the rate of pinocytosis of ^{125}I -labelled poly(vinylpyrrolidone) remained unaltered, k_{+2} appears to be constant and rate-limiting. Thus, replacing F , the rate of capture of fluid, in the expression $E = F + [SR/(K+c)]$ by 2.47 ± 1.00 (s.d.), the amount of substrate internalized adsorptively can be determined and the components of the adsorption term examined. Assuming a rapid and complete equilibration of the substrate with the binding sites on the plasma membrane, simple Michaelis-Menten kinetics should be observed so that v , the rate at which substrate is ingested via the adsorptive route (mol/h per mg of tissue protein), is given by the following expression:

$$v = (E - F)c = \frac{SRc}{K+c}$$

so that the expression analogous to the Lineweaver-Burk equation is:

$$\frac{1}{v} = \frac{K}{SR} \cdot \frac{1}{c} + \frac{1}{SR}$$

Fig. 3. Lineweaver-Burk plot for adsorptive-phase pinocytosis of formaldehyde-treated ^{125}I -labelled bovine serum albumin

The ordinate is the reciprocal of v , the rate at which radiolabelled albumin is ingested via the adsorptive route by yolk sacs incubated at 37°C in serum-free medium 199 (see the text for methods of calculating both the total rate of uptake of this digestible protein and the rate of uptake by adsorptive pinocytosis). The abscissa is the reciprocal of the total concentration, c , of iodinated formaldehyde-treated bovine serum albumin, both ^{125}I -labelled and [^{127}I]iodinated (non-radioactive), present in the culture medium.

where SR is equal to the maximum rate of uptake when all available binding sites are occupied. Thus, taking the molecular weight of bovine serum albumin to be 65000 and summing the concentrations of ^{125}I -labelled and [^{127}I]iodinated formaldehyde-denatured bovine serum albumin to give the substrate concentration, a double-reciprocal plot (Fig. 3) can be made from the data presented in Table 3. From this plot a value of K of $1.27\ \mu\text{M}$ was obtained, and also a value for SR of $0.31\ \text{nmol/h}$ per mg of yolk-sac protein. [In a parallel study in which different concentrations (1.5 , 7.7 , 38.4 and $76.8\ \mu\text{g/ml}$ of medium) of the formaldehyde-denatured ^{125}I -labelled bovine serum albumin (batch IV) alone were used, rather than a mixture of a constant amount of ^{125}I -labelled protein and a varying quantity of its [^{127}I]iodinated analogue, the results were essentially the same, being $1.26\ \mu\text{M}$ and $0.25\ \text{nmol/h}$ per mg of yolk-sac protein.]

The results show the dissociation constant of the substrate-plasma-membrane complex to be approx. $1\ \mu\text{M}$, a value which indicates that the affinity of formaldehyde-denatured albumin for binding sites on the yolk-sac plasma membranes is higher than the affinities of the majority of mitochondrial enzymes for their substrates (Altman & Katz, 1976). The affinity is, however, much lower than the affinities of lysosomal enzymes for adsorptive pinocytosis binding sites on human fibroblasts from patients with a congenital deficiency for a given lysosomal enzyme. Thus α -L-iduronidase (Sando & Neufeld, 1977), N -acetyl- α -D-glucosaminidase (von Figura & Kresse, 1974) and β -glucuronidase (Kaplan *et al.*, 1977) showed dissociation constants of $3\ \text{nM}$, $0.9\ \text{nM}$ and $6\ \text{nM}$ respectively, values that are similar to the concentration ($0.58\ \text{nM}$) of ^{125}I -labelled asialo-orosomucoid that irreversibly saturates 50% of the binding sites on plasma membranes isolated from rat liver (Van Lenten & Ashwell, 1972). Thus the affinity of a simple protein, formaldehyde-denatured ^{125}I -labelled bovine serum albumin, for adsorptive pinocytosis sites on the plasma membrane of yolk-sac tissue is high, but not as great as the affinities of lysosomal enzymes (that carry a phosphohexose-based recognition region) for fibroblasts, or asialoglycoproteins (carrying a galactose-based recognition region) for liver parenchymal cells. These glycoproteins have affinities that are similar in magnitude to those of glucagon ($4\ \text{nM}$) and insulin ($0.1\ \text{nM}$) for their receptor sites on target cells (Cuatrecasas, 1974).

Important features of the method of investigation reported above are that correction for the rate of fluid-phase ingestion is made and hydrolysis of the protein, after its pinocytic capture, is treated quantitatively. In the studies of the clearance of lysosomal enzymes by human fibroblasts quoted above these effects are ignored, but probably do not introduce significant errors into the interpretation of the data,

since the affinities of the lysosomal enzymes are so high that they almost certainly are ingested almost exclusively by way of adsorptive endocytosis, so that ignoring fluid-phase ingestion will introduce little error. Moreover, these lysosomal enzymes, unlike the majority of proteins, appear to be highly resistant to degradation, on passing into the lysosomal system after uptake, as judged by their half-lives within the tissue. However, the methods reported in the present paper should enable investigations of pinocytic rates to be extended with confidence to glycoproteins and proteins that are either less avidly ingested (hence fluid-phase pinocytosis cannot be ignored) or are rapidly digested on passing into the lysosomal system (so that major errors would arise if intracellular digestion was not treated quantitatively in a manner similar to that stated in the Materials and Methods section).

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