

## TEMPERATURE DEPENDENCE OF CERTAIN INTEGRATED MEMBRANE FUNCTIONS IN MACROPHAGES

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### SUMMARY

We have studied the effect of temperature on uptake and degradation of molecules entering mouse peritoneal macrophages by fluid-phase, adsorptive and receptor-mediated pinocytosis, and on degradation of their intracellular proteins. Uptake of [<sup>3</sup>H]sucrose and uptake and degradation of formaldehyde-treated <sup>125</sup>I-labelled human serum albumin and <sup>125</sup>I-labelled mannose-bovine serum albumin continued, but were progressively slowed as the temperature decreased from 37 °C to 20 °C. The uptake and degradation were completely abolished at approximately 15 °C. Arrhenius plots for adsorptive and fluid uptake were unilinear, whereas that for receptor-mediated endocytosis showed an inflection point at approximately 20 °C. The results did not indicate any distinction between adsorptive and fluid pinocytosis. An 'intracellular turnover time' calculated for mannose-bovine serum albumin taken up by the specific route is 19-24 min and this time calculated for human serum albumin is, in contrast, 99 min. Studies of the kinetics of degradation of both endocytosed and endogenous proteins showed similarity in the temperature cut-off of degradation of endocytosed and endogenous long-half-life proteins ( $\approx$  15 °C) and continuance of endogenous short-half-life degradation at much lower temperatures.

### INTRODUCTION

It has been proposed (Allison & Davies, 1974) that macrophages can internalize solutes by an energy-independent form of pinocytosis involving small vesicles, and a distinct, energy-dependent macro-pinocytosis, which requires microfilament-microtubule function and involves larger vesicles. All such endocytic processes involve membrane fusion. Both extracellular and intracellular proteins can be degraded within a lysosome, only after they have entered this hydrolytic compartment, by a second process requiring membrane fusion (de Duve & Wattiaux, 1966). Such physiological uptake of substrates into lysosomes is known to occur by two mechanisms, heterophagy and autophagy; these terms concern molecules originating from outside and inside the cell, respectively (Dean & Barrett, 1976). The second membrane fusion is for heterophagy and sometimes for autophagy, a vesicle-vesicle fusion, while for other forms of autophagy it is an intra-vesicle fusion.

The intracellular proteins can be experimentally subdivided crudely into two groups,

the short-lived class (those proteins labelled in approximately 1 h) and long-lived proteins (these proteins labelled in approximately 24 h). The breakdown of long-lived proteins has been reported to be diminished by specific inhibitors of lysosomal proteinases, while these substances apparently do not affect degradation of short-lived proteins (Hopgood, Clark & Ballard, 1977). Thus degradation of short-lived proteins seems to be mainly non-lysosomal: much of it may be processing of protein precursors (Mortimore & Schworer, 1980). Degradation of endocytosed proteins, in contrast, seems to be almost entirely a lysosomal function.

The aims of this investigation were twofold: firstly, to determine if macro- and micro-pinocytosis are distinct, by studying the effect of temperature on uptake of substrates entering by three different mechanisms (fluid-phase, adsorptive and receptor-mediated endocytosis). Secondly, to investigate the fate of various tracers inside the cell. It has been shown (Edelson & Cohn, 1974; Dunn, Hubbard & Aronson 1980) that, in the heterophagic function of lysosomes, fusion of pinocytic vacuoles with lysosomes is very sensitive to low temperature. If all intracellular protein molecules are broken down within the lysosome, having entered by processes that also depend on membrane fusion, their rate of digestion should also show a sensitivity to temperature. Accordingly, we studied and compared the degradation of endocytosed proteins, short- and long-lived protein in cultured macrophages as a function of temperature.

#### MATERIALS AND METHODS

Plastic multi-well dishes (35 mm diameter) were obtained from Costar, Cambridge, MA, U.S.A. Tissue-culture media, Hepes buffer, sera and antibiotics were from Flow Laboratories, Irvine, Ayrshire, U.K. The sera were inactivated by heating at 56 °C for 30 min. Cytochalasin B, bovine serum albumin (fraction V) and yeast mannan were from Sigma (London) Chemical Co., Poole, Dorset, U.K. [<sup>3</sup>H]sucrose, <sup>125</sup>I-labelled human serum albumin ([<sup>125</sup>I]dHSA) were all obtained from the Radiochemical Centre, Amersham, Bucks, U.K.; mannose-bovine serum albumin (Man-BSA) was kindly provided by Dr Philip Stahl, Department of Physiology and Biophysics, Washington University, School of Medicines, St Louis, Missouri (prepared according to the method of Lee, Stowell & Krantz, 1976). Other reagents were of the best available commercial grade.

Iodination of mannose-bovine serum albumin was accomplished using the iodogen method (Salacinski *et al.* 1981) to a specific activity of  $1.3 \times 10^8$  to  $2 \times 10^8$  c.p.m./ $\mu$ g. The reaction products were then dialysed for 48 h against five 4-litre changes of phosphate-buffered saline (PBS), after which less than 1% of the radioactivity was soluble in 10% trichloroacetic acid. <sup>125</sup>I-labelled human serum albumin treated with formaldehyde (prepared according to the method of Moore, Williams & Lloyd, 1977) was then dialysed for 48 h against five 4-litre changes of 0.9% NaCl, after which only 0.2% free iodine was detectable.

Mouse peritoneal macrophages were routinely cultured in Medium 199 (M199), 10% heat-inactivated swine serum and antibiotics, as previously described (Dean, Hylton & Allison, 1979). During incubation at various temperatures, M199 was buffered with 20 mM-Hepes, so that gassing with CO<sub>2</sub> could be omitted. NaHCO<sub>3</sub> was included at a lower concentration of 5 mM. Endocytosis was measured by adding radioactively labelled molecules directly to the medium and measuring their association with the cells. The standard amounts of tracers were used at the following concentrations: [<sup>3</sup>H]sucrose, 4  $\mu$ g/ml; [<sup>125</sup>I]dHSA, 5  $\mu$ g/ml; and Man-BSA, 5  $\mu$ g/ml. These concentrations were chosen to be in the linear part of the Endocytic Index profile (see below). All experiments have been done with standard amounts of tracers unless otherwise stated. At various times after addition of substrate the media were harvested and the cells were washed 4 times with PBS. Preliminary experiments showed that after 4 washes there

was no trace of marker in the 5th wash. The cells were either lysed by addition of 0.1% Triton X-100 in PBS for measurement of radioactivity, or the medium was changed and the cells incubated at the temperature chosen for investigating degradation of intracellular protein.

For measurement of degradation after the further incubation, the culture medium was removed, the cells were washed 4 times with PBS and lysed. The washing procedure was found to deplete the cells of radioactive low molecular weight substances; less than 2% of the radioactivity associated was found to be soluble in 10% (w/v) trichloroacetic acid. To measure degradation at each time chosen, 0.5 ml samples of the medium and cell suspension were then precipitated with 0.5 ml of 10% trichloroacetic acid; 50  $\mu$ l of 10% bovine serum albumin was added before the acid to act as carrier. The precipitates were then sedimented at 1300 g for 15 min, and a portion of the supernatant was collected for counting. Pellets from precipitation with acid were resuspended and washed twice with 5% trichloroacetic acid, and the final pellet redissolved in 1 ml of formic acid (98–100%) for counting radioactivity. Degradation is expressed as acid-soluble radioactivity, as a percentage of the total radioactivity in the system, and the pinocytotic uptake of a degradable substance is the sum of the tissue level of the substance and the digestion products. Zero-time values were always measured for both endocytosis and degradation.

Samples were counted either in Triton X-100/toluene (3:7, v/v) scintillant ( $\beta$  isotopes) or directly by a  $\gamma$  counter. Quench correction was by the internal-samples-channels ratio method. When uptake proceeds at a constant rate throughout the incubation, this rate is expressed as an Endocytic Index (EI), defined as either the volume of culture medium ( $\mu$ l) whose contained substrate has been captured, or the amount of substrate (ng) captured, per mg cell protein per hour.

To compare rates of degradation, we calculate an 'intracellular turnover time', which is the time taken for the tissue to digest, and return to the incubation medium as hydrolysis products, the amount of substrate present in the cells in the steady-state condition. To calculate this the quantity of the substrate label associated with the tissue during the steady-state period was divided by the rate of uptake of the substrate by the cells; and the result has the unit of time. In all mannose-bovine serum albumin experiments, non-specific uptake was estimated by adding an excess of yeast mannan (2.5 mg/ml) to the incubation media. Non-specific uptake was less than 30% of the total uptake.

Protein was measured by the Lowry, Roseborough, Farr & Randall (1951) procedure, with bovine serum albumin as standard. For experiments on degradation of endogenous proteins, macrophages ( $0.5 \times 10^6$  to  $1.0 \times 10^6$ ) were cultivated in 1 ml of the normal medium, in tissue-culture tubes (Sterilin). Cells were labelled with [ $^3$ H]leucine in leucine-free Minimal Essential medium (MEM) containing 10% heat-inactivated swine serum and antibiotics; to label short-half-life proteins 1  $\mu$ Ci/ml [ $^3$ H]leucine was supplied for 1 h, and to label long-half-life proteins, 0.5  $\mu$ Ci/ml [ $^3$ H]leucine for 24 h. Cells were then washed 4 times with PBS containing 10 mM-leucine (non-radioactive), placed in fresh M199 buffered with Hepes containing 10 mM-leucine and NaHCO<sub>3</sub> (as above), and incubated at the specified temperatures in water-baths. At the end of the incubation, degradation was measured as described above for endocytosed substrates. Preliminary experiments showed that degradation of pre-labelled proteins at 37 °C was indistinguishable, whether cells were in M199 with NaHCO<sub>3</sub> or Hepes, and whether or not the Hepes-containing cultures were gassed with 5% CO<sub>2</sub> in air. 10 mM-leucine suppresses reutilization maximally (Dean, 1979b). % Degradation was expressed as:

$$\frac{\text{Total trichloroacetic acid-soluble radioactivity}}{\text{Total radioactivity}} \times 100.$$

This value for cultures harvested immediately after labelling and washing was less than 5% for short-half-life proteins, and less than 2 for long. An upper estimate of % cell lysis obtained as  $100 \times$  (acid-precipitable radioactivity in the medium/total radioactivity). This was always less than 10% for experiments up to 24 h of degradation.

All the data quoted in Results are from experiments conducted with triplicate observations for each condition. They are usually quoted as mean  $\pm$  s.d., both in the text and on the figures and table; thus error bars on graphs also represent s.d.

## RESULTS

*Basic characterization of endocytosis*

In the initial experiments the essential characteristics of uptake and degradation of substrates were assessed in our experimental system. Uptake of [<sup>3</sup>H]sucrose was linear with time and concentration of tracer over 6 h and the EI for each experiment was between  $0.105 \pm 0.03$  and  $0.23 \pm 0.05$   $\mu\text{l}/\text{mg}$  cell protein per h (means  $\pm$  s.d. for the two experiments giving the extreme values). Uptake did not show saturation at high concentrations of sucrose, which was thus a satisfactory marker for fluid-phase endocytosis.

Experiments with formaldehyde-treated <sup>125</sup>I-labelled human serum albumin ([<sup>125</sup>I]dHSA) showed that the radioactivity associated with the cells became constant after 3 h, but acid-soluble radioactivity (digested products) continued to rise throughout the incubation period.

Uptake of [<sup>125</sup>I]dHSA was linear with time over 24 h and the EI was 6  $\mu\text{l}/\text{mg}$  cell protein per h (corresponding to 3 ng/mg cell protein per h). This is compatible with results obtained by Pratten, Williams & Lloyd (1977) using rat peritoneal macrophages.

The massive uptake of albumin (compared with that of sucrose) was most probably a function of the degree of membrane adsorption, but would also result if the substrate were stimulating pinosome formation at the plasma membrane. These alternatives were distinguished between by assessing the effect of [<sup>125</sup>I]dHSA on the uptake of the accepted fluid-phase marker [<sup>3</sup>H]sucrose and *vice versa*. Sucrose at concentrations of up to 20 mg/ml had no effect on the uptake of [<sup>125</sup>I]dHSA and uptake of sucrose in the presence of 2 mg/ml HSA was unchanged. Thus it was established that HSA did not stimulate pinosome formation and thus entered the cells associated with membrane, and was a useful tracer of adsorptive endocytosis.

It has been shown (Stahl *et al.* 1980) that mannose-bovine serum albumin is endocytosed by a receptor-mediated system in rat alveolar macrophages. We found that uptake of [<sup>125</sup>I]Man-BSA at a concentration of 0.5  $\mu\text{g}/\text{ml}$  is linear with time over 24 h. The radioactivity associated with cells became constant after 1 h. Allowing for non-specific binding and uptake, the EI for specific uptake was found to be 8.5 ng/mg cell protein per h (corresponding to a nominal fluid intake of 17  $\mu\text{l}/\text{mg}$  cell protein per h), which is approximately three times greater than the rate of uptake for [<sup>125</sup>I]dHSA and consistent with intake by rapid receptor-mediated endocytosis. This rate is lower than that reported by Stahl *et al.* (1980), presumably because of the lower number of available receptors on mouse peritoneal, compared with rat alveolar, macrophages. The EI for non-specific uptake of Man-BSA (5.9  $\mu\text{l}/\text{mg}$  cell protein per h) was equal to that of [<sup>125</sup>I]dHSA (6  $\mu\text{l}/\text{mg}$  cell protein per h).

*Temperature dependence of uptake*

To investigate the membrane activities involved in the endocytosis of the three substrates, we studied their rate of uptake at various temperatures between 4 and 37 °C (Figs. 1, 2). The effects of temperature on uptake of [<sup>3</sup>H]sucrose (fluid-phase marker),

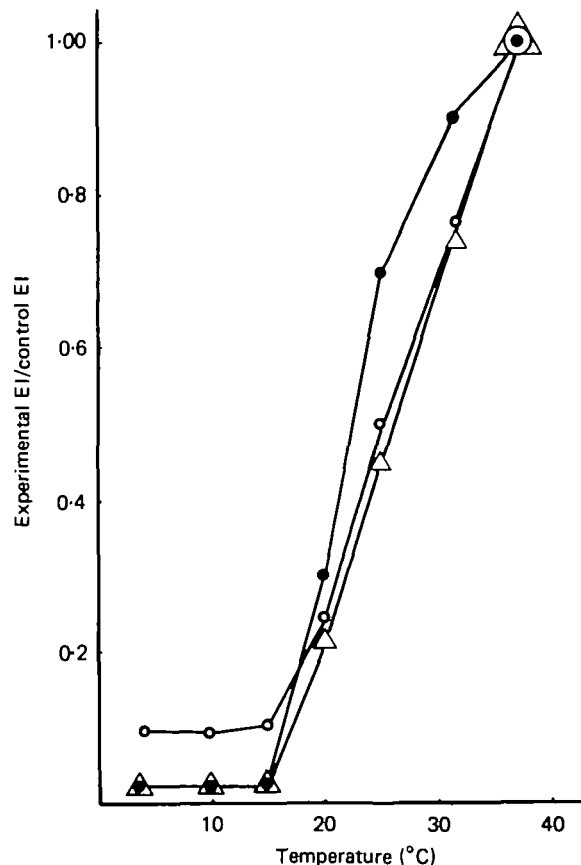


Fig. 1. Effect of temperature on rate of uptake of [<sup>3</sup>H]sucrose, [<sup>125</sup>I]dHSA and [<sup>125</sup>I]-Man-BSA. Each value is the average from three individual cultures from a single population of cells compared to cultures from the same population incubated at 37 °C. Tracers were used at standard concentrations. Standard deviation of the averages for temperatures above 20 °C were always less than 10%. None of the values obtained at temperatures of 15 °C and below were significantly different from their corresponding zero-time controls; and thus did not reflect true internalization. (Δ) [<sup>125</sup>I]Man-BSA; (●) [<sup>125</sup>I]HSA; (○) [<sup>3</sup>H]sucrose.

[<sup>125</sup>I]dHSA (adsorptive substrate) and the receptor-mediated substrate [<sup>125</sup>I]Man-BSA are very similar, in that for all three substrate capture was almost completely abolished below 20 °C.

#### *Studies with inhibitors*

It has been suggested frequently that microtubules and microfilaments play an important part in the movement of organelles in endocytic cells (Allison, Davies & de Petris, 1971). Cytochalasin B, which is believed to affect polymerization of microfilaments when used at a concentration of 10 μg/ml, decreased uptake of [<sup>3</sup>H]sucrose by 41% and of [<sup>125</sup>I]dHSA by 30% (Table 1), which is consistent with the report of Pratten & Lloyd (1979) that this inhibitor could not decrease uptake of labelled colloidal

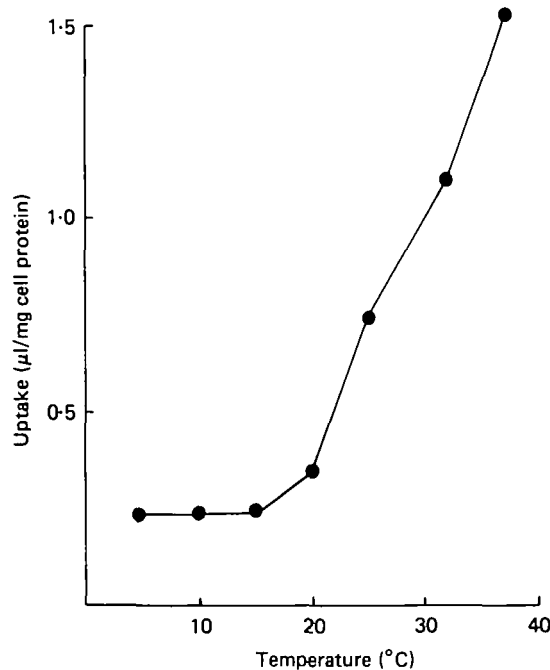


Fig. 2. Effect of temperature on the rate of uptake of [ $^3\text{H}$ ]sucrose. Cells were incubated with standard concentrations of sucrose at various temperatures (4 to 37 °C) for 6 h. None of the rates obtained at temperatures of 15 °C and below were significantly different from their corresponding zero-time control, and thus they did not reflect true internalization. Qualitatively, the differences between amounts of uptake at the different temperatures were also very similar after 24 h, but this period exceeded the linear uptake phase (6 h).

Table 1. Effect of cytochalasin B on uptake of [ $^3\text{H}$ ]sucrose and [ $^{125}\text{I}$ ]dHSA at various temperatures

Temperature (°C)	[ $^3\text{H}$ ]sucrose (μl uptake/mg cell protein per h)		[ $^{125}\text{I}$ ]dHSA (μl uptake/mg cell protein per h)	
	DMSO	Cytochalasin B	DMSO	Cytochalasin B
20	0.078 ± 0.01	0.045 ± 0.01	3.7 ± 0.5	2.1 ± 0.2
32	0.189 ± 0.02	0.111 ± 0.02	4.17 ± 0.14	3.0 ± 0.24
37	0.237 ± 0.02	0.142 ± 0.01	5.21 ± 0.18	3.6 ± 0.23

Cells were incubated with [ $^3\text{H}$ ]sucrose (4 μg/ml) or [ $^{125}\text{I}$ ]dHSA (0.5 μg/ml). Cytochalasin B (10 μg/ml) was dissolved in 0.1% dimethyl sulphoxide (DMSO). Controls contained the same amount of DMSO.

gold or  $^{125}\text{I}$ -labelled poly(vinylpyrrolidone) in rat peritoneal macrophages by more than 40%, and with previous work by one of us (Dean, 1979a). This effect could not be due to non-specific toxicity of cytochalasin B or dimethyl sulphoxide (0.1%, v/v), which was present in cultures containing cytochalasin B, because, although the cells looked slightly rounded in the presence of cytochalasin B, lactate dehydrogenase and protein assay showed they were viable, and dimethyl sulphoxide itself had no effect on

pinocytosis and/or degradation of either substrate. Our experiments showed that the effect of cytochalasin B on the uptake of sucrose and HSA was qualitatively similar (Table 1) at various temperatures.

#### Temperature dependence of degradation

In this series of experiments the temperature dependence of intracellular degradation of endogenous proteins and of endocytosed tracers was investigated.

*Degradation of [<sup>125</sup>I]Man-BSA.* Binding of [<sup>125</sup>I]Man-BSA requires the presence of Ca<sup>2+</sup>. In the absence of Ca<sup>2+</sup> and with EGTA added, binding is completely inhibited and surface-bound molecules can be removed with trypsin (Stahl *et al.* 1980).

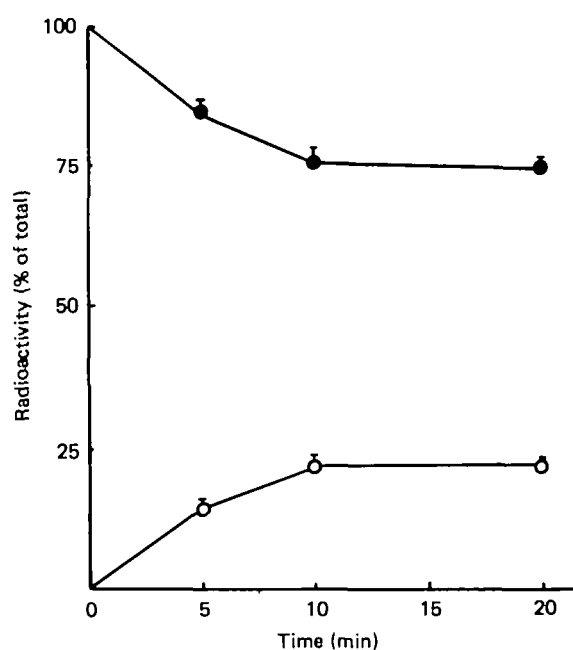


Fig. 3. Removal of prebound [<sup>125</sup>I]Man-BSA (4 °C) after a short period of internalization. Cells were incubated with 2.3 μg [<sup>125</sup>I]Man-BSA/ml for 60 min at 4 °C and then washed 4 times, incubated in ligand-free medium for 10 min at 37 °C, and then incubated with trypsin-EGTA at 4 °C. At the stated intervals after commencement of trypsin-EGTA treatment, cells were washed and harvested. (●) % Cell-associated radioactivity; (○) % radioactivity in the medium; both as % of the total radioactivity present at commencement of trypsin-EGTA treatment.

Use was made of this observation to remove surface label after binding at 4 °C and a short period of internalization in our system, so that the temperature dependence of degradation of internalized protein could be measured with minimal complication from continuing internalization of molecules bound to the plasma membrane.

Thus cells were incubated with [<sup>125</sup>I]Man-BSA at 4 °C for 60 min, after which they were washed free of excess ligand at 4 °C with cold PBS. The cells were then incubated at 37 °C for 15 min, allowing cells to internalize pre-bound ligand (that is, ligand

bound at 4 °C). Media were then removed, cells were incubated for 10 min in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS containing 10 mg/ml BSA, 0.1% trypsin and 10 mM-EGTA at 4 °C. Fig. 3 shows that the pre-bound ligands that had not been internalized (i.e. are still on the surface of cells after 10 min at 37 °C) were released almost quantitatively within 10 min incubation at 4 °C with trypsin/EGTA.

After such a 'stripping' the cells were washed 4 times with cold PBS and incubated at various temperatures between 4 °C and 37 °C for measurement of degradation.

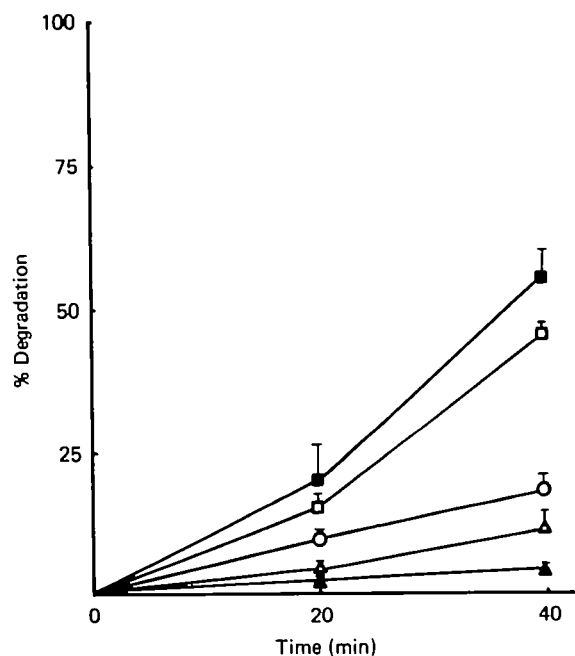


Fig. 4. Effect of temperature on rate of degradation of internalized [<sup>125</sup>I]Man-BSA. Cells were incubated with 2.3 µg [<sup>125</sup>I]Man-BSA/ml at 4 °C followed by 15 min at 37 °C and then trypsin-EGTA treatment, and finally incubated at: (▲) 15 °C, (△) 20 °C, (○) 25 °C, (□) 32 °C and (■) 37 °C for different periods of time.

It is clearly shown in Fig. 4 that after 40 min at 37 °C 60% of intracellular Man-BSA was degraded, while there was 12% degradation at 20 °C and insignificant degradation at 15 °C.

**Degradation of [<sup>125</sup>I] HSA.** Our experiments showed that a larger pool of internalized macromolecular [<sup>125</sup>I]HSA is maintained at 20 °C than at other temperatures. Thus cells were incubated at 20 °C for 24 h, media were then removed and cells were washed 4 times with PBS to remove the intracellular ligand and then incubated with ligand-free media at various temperatures (4 °C to 37 °C). Fig. 5 shows the result of this experiment: 80% of HSA was degraded in 4 h and degradation was completely abolished below 20 °C. The degradation of low-temperature cultures reverted to normal when the cultures were replaced at 37 °C, confirming the non-toxicity of the low-temperature regime.



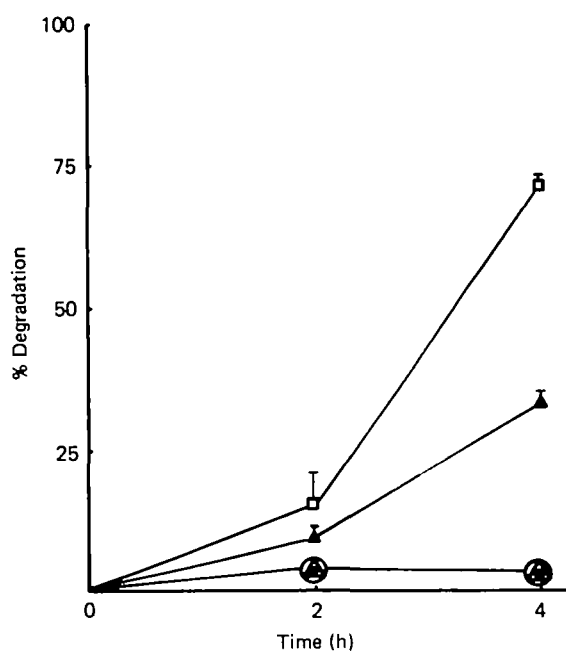


Fig. 5. Effect of temperature on rate of degradation of internalized [ $^{125}\text{I}$ ]HSA. Cells were incubated with standard concentration of [ $^{125}\text{I}$ ]HSA at 20 °C for 24 h and then washed 4 times, incubated in ligand-free medium at: (●) 4 °C, (▲) 10 °C, (○) 15 °C, (▲) 20 °C and (□) 37 °C, for different periods of time.

*Degradation of endogenous proteins.* The kinetics of degradation of both short and long-half-life proteins were studied up to 24 h of degradation. While that of short-half-life proteins was still significant (statistically greater than the relevant zero-time value) at 10 °C, that of long-half-life proteins was abolished between 15 °C and 10 °C. To illustrate this, Fig. 6 shows the degradation of short-half-life proteins achieved in 18 h, and that of long-half-life proteins achieved in 24 h. The reduction of degradation of both categories of protein at low temperatures could be reversed immediately when the cultures were replaced at 37 °C, thus confirming the observed viability of the cells during the temperature shifts.

#### DISCUSSION

Our experiments showed that all steps involved in both uptake and degradation of substrates by the mouse peritoneal macrophages were slower at temperatures lower than the physiological temperature; membrane internalization stopped completely at temperatures below 15 °C, which is consistent with the findings of Pratten & Lloyd (1979).

The activation energy ( $E_a$ ) calculated for fluid and adsorptive endocytosis was 18 kcal/mol, with temperature coefficient ( $Q_{10}$ ) around 2.8, consistent with other reported values (Pratten & Lloyd, 1979; Silverstein, Steinman & Cohn, 1979; Mahoney, Hamill, Scott & Cohn, 1977; Dunn *et al.* 1980).

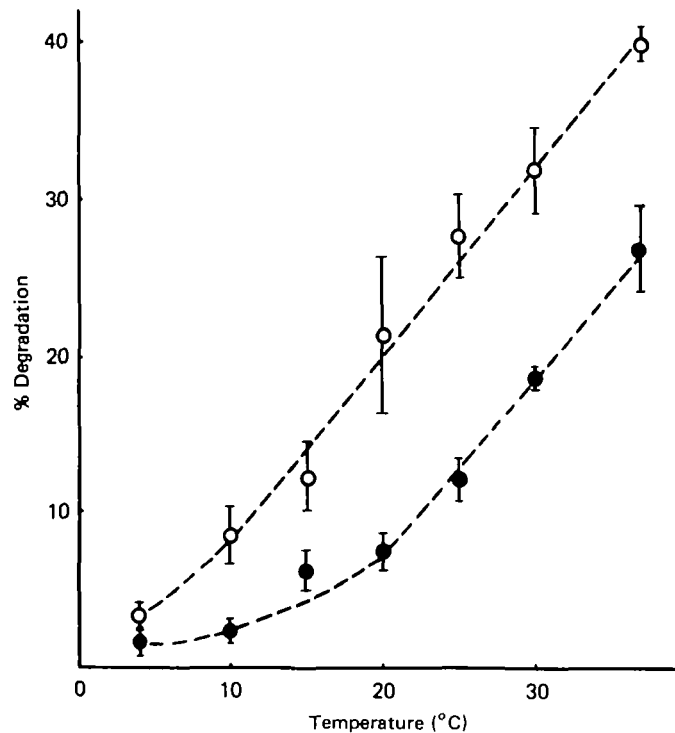


Fig. 6. Degradation (%) of short-half-life proteins in 18 h (○) and long-half-life proteins in 24 h (●) at various temperatures. Values are means  $\pm$  s.d. of triplicates. Zero-time degradation values were indistinguishable from the 4 °C values (and also from the 10 °C value for long-half-life proteins). Complete kinetic data were obtained in these experiments, and the data shown were simply chosen to represent comparable degrees of degradation for the two classes of substrates.

However, whereas the Arrhenius plots for adsorptive and fluid uptake were unilinear, that for receptor-mediated endocytosis of Man-BSA at temperatures between 4 °C and 37 °C showed an inflection point at approximately 20 °C with an  $E_a$  of 16.2 and  $Q_{10}$  of 2.3 for above 20 °C and an  $E_a$  of 27.5 and  $Q_{10}$  of 4.5 below 20 °C. Similarly, Weigel & Oka (1980) reported the same inflection point at 20 °C for the endocytosis of receptor-mediated  $^3\text{H}$ -labelled asialooros mucoid by isolated rat hepatocytes, reporting values of  $E_a = 17$  kcal/mol and  $Q_{10} = 2.6$  above 20 °C and  $E_a = 45.9$  kcal/mol and  $Q_{10} = 15.6$  for below 20 °C. The Arrhenius plot presented by Kaplan & Nielsen (1979) for the internalization of  $\alpha$ -macroglobin-trypsin complexes by rabbit alveolar macrophages also suggests an inflection point at about 20 °C.

This evidence of an inflection in the Arrhenius plot for Man-BSA uptake only suggests that receptor-mediated endocytosis involves two discrete stages; and that there may be a distinction between the membrane events it involves and those in adsorptive and fluid-phase pinocytosis. There are no such indications of distinction within or between adsorptive and fluid pinocytosis in our data. In spite of the association of HSA with the membrane, and the contrasting independent of sucrose from the membrane, the internalization of these two markers is qualitatively similar. These

results lend no support to the concept (Allison & Davies, 1974) that macrophage pinocytosis has two mechanistically distinct components, macro- and micro-pinocytosis; uptake of fluid and adsorbed tracers seems to be the result of a single process.

Comparing the results of experiments done with Man-BSA and HSA, it is clear that uptake of receptor-mediated Man-BSA is much greater than uptake of HSA, and in respect of degradation after internalization it is shown that 60% of internalized Man-BSA could be degraded in 40 min, while 60% of HSA is degraded in approximately 3.5 h. The intracellular turnover time calculated for Man-BSA taken up by the specific route is 19–24 min and this time for HSA is, in contrast, 99 min, which is approximately the same as that for Man-BSA taken up non-specifically. This may indicate that the intracellular route for catabolism of molecules taken up by receptor-mediated endocytosis is different from that for molecules taken in by fluid-phase or adsorptive pinocytosis.

We calculated the intracellular turnover times for specific uptake of Man-BSA by rat alveolar macrophages from the report of Stahl *et al.* (1980) and of BSA by rat peritoneal macrophages from the report of Pratten *et al.* (1977), which are 12 and 120 min, respectively.

Next, we investigated the temperature dependence of intracellular degradation of various tracers to find out if their intracellular fates vary, even though their mechanism of internalization is similar. In the present study we have eliminated effects due to the continuous uptake of substrate by removing the non-bound ligand before beginning an experiment, and we looked at degradation of receptor-mediated substrate and also adsorptively endocytosed proteins.

The striking inhibitory effect of low temperature was observed to be quite similar for both categories of substrate. Our results also indicate that degradation of long-lived protein, which is believed to occur in lysosomes, ceases at around 15 °C, while 1-h labelled proteins (short-lived protein) are still degraded even at 10 °C. The similarity in the temperature at which cut-off for degradation of both endocytosed and endogenous long-half-life proteins occurs to that for endocytosis itself, suggests that in all three cases it is a reflection of the same phenomenon, the temperature dependence of membrane fusion. The continuance of endogenous, short-half-life protein degradation at much lower temperatures indicates that proteolytic activity itself can proceed below the cut-off at 15 °C, and supports the view that lysosomes (and by extension, membrane fusion) are hardly ever involved in the turnover of such proteins (related evidence consistent with this view has also been presented by Neff, DeMartivo & Goldberg, 1979). Intracellular fusion has been studied directly in recent work, and the results are consistent with our interpretation. Thus, Kielian & Cohn (1980) reported that in mouse peritoneal macrophages fusion between phagosome and lysosome continued with decreasing rates, for temperatures from 37 °C to 20 °C. At 15 °C no further fusion was observed and the inhibition appeared as great as that seen at 2 °C. They reported an  $E_a$  of 16.4 kcal/mol and a  $Q_{10}$  of 2.5, which is comparable to the  $E_a$  for pinocytosis, 18–25 kcal/mol. Similarly, Dunn *et al.* (1980) observed that fusion between internalized pinocytic vesicles and lysosomes failed to

occur below 20 °C, despite the demonstrable activity of lysosomal enzymes *in vitro* at these temperatures.

Thus, it seems logical to suggest that the temperature-sensitive fusion between pinocytic vesicles or autophagosomes and lysosomes is the reason for discontinuation of degradation below 15 °C, and to expect that degradation processes of all proteins that are catabolized within lysosomes would stop at or around 15 °C.

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