Sugar solutions were circulated in the mouth for a 5 min test period in a glycylglycine buffer (pH 6.0).

The uptake of D-glucose (5 mM), D-xylose (5 mM), L-xylose (10 mM) and 3-O-methyl-D-glucose (10 mM) through the buccal mucosa was significantly increased by the addition of CaCl₂ (2.6 mM) to the incubation medium, whereas the uptake of 5 mM D-fructose, 10 mM D-arabinose and 10 mM D-ribose remained unaffected (Table 1). MgCl₂ (2.0 mM) produced no significant rise in uptake of any of the sugars tested. The uptake of 5 mM D-xylose rose steeply with increasing Ca²⁺ concentration to reach a maximum at 2.6 mM Ca²⁺, with xylose uptake slowly declining at higher concentrations of Ca²⁺ (Fig. 1).

These results show that Ca²⁺ significantly increased the uptake across the buccal mucosa of only those sugars transported by the glucose-carrier system in the small intestine, suggesting that Ca²⁺ may either directly or indirectly cause stimulation of a glucose-carrier system in the human buccal mucosa, possibly acting by stimulation of Na⁺ flux. In addition, the lack of action of Ca²⁺ on fructose transport through the buccal mucosa may indicate a separate, Na⁺-independent carrier system for fructose in the mouth as in the intestine.

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Quantitative Studies of Pinocytic Uptake of ¹²⁵I-Labelled Polyvinylpyrrolidone by Pig Aortic Smooth-Muscle Cells in Culture

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Among the few published quantitative studies of pinocytosis by non-macrophage mammalian cells in culture are those of Williams et al. (1975), which describe a method to measure the rate of pinocytic uptake of ¹²⁵I-labelled polyvinylpyrrolidone by the rat visceral yolk sac in vitro. This compound is a useful marker for pinocytosis, because it is not susceptible to lysosomal degradation and its mean mol. wt. of 30000–40000 prevents

![Fig. 1. Effect of Ca²⁺ concentration on the uptake of 5 mM D-xylose across the human buccal mucosa](image-url)
it from permeating cellular membranes. Its rate of pinocytic uptake was expressed as the volume of culture medium whose $^{125}$I-labelled polyvinylpyrrolidone content was taken up per unit time per unit mass of cellular protein and was defined to be the endocytic index for this compound. An adaptation of this method is reported here for the cultured aortic smooth-muscle cell, a cell type whose secondary lysosomes become progressively engorged during the development of atherosclerosis (Shio et al., 1974).

Cells were obtained by outgrowth from cultured explants of tunica media from the pig thoracic aorta (Ross, 1971) and grown in 35 mm-diameter dishes. The medium consisted of 4 vol. of medium 199 with Earle's balanced salts mixed with 1 vol. of foetal bovine serum and contained l-glutamine (2 mM), penicillin G (200 i.u./ml), streptomycin sulphate (200 µg/ml) and kanamycin sulphate (200 µg/ml). Cultures were incubated at 37°C in humidified air + CO$_2$ (95:5) and experiments were performed with third to sixth subcultures.

**Accumulation of $^{125}$I-labelled polyvinylpyrrolidone**

After incubation at 37°C with 2 ml of medium containing $^{125}$I-labelled polyvinylpyrrolidone (The Radiochemical Centre, Amersham, Bucks., U.K.) the dishes were washed six times with ice-cold buffer (Hanks' balanced salt solution) and the cells detached by trypsin treatment. After centrifugation at 4°C for 10 min at 250g, they were washed in buffer, recentrifuged, sonicated in water and assayed for radioactivity by gamma spectroscopy and for protein by the method of Lowry et al. (1951).

During incubation with $^{125}$I-labelled polyvinylpyrrolidone (30 µg/ml), cellular accumulation was directly proportional to the time of incubation over 48.5 h, and there was little, if any, initial adsorption to the washed cell surface (Fig. 1a). The rate of accumulation as measured by the slope of the regression line, was 2.45 ng/h per mg of cellular protein (95% confidence limits 2.12, 2.78). Fig. 1(b) shows that accumulation of $^{125}$I-labelled

![Fig. 1. Accumulation of $^{125}$I-labelled polyvinylpyrrolidone by smooth-muscle cells in culture](image)

Each point corresponds to the cells isolated from a single dish after incubation with culture medium containing (a) $^{125}$I-labelled polyvinylpyrrolidone (30 µg/ml) for various times or (b) various concentrations of $^{125}$I-labelled polyvinylpyrrolidone for 48 h.
polyvinylpyrrolidone over 48 h was directly proportional to its concentration in the medium and indicates that within the range 1–100 \( \mu \text{g/ml} \) it neither stimulated nor inhibited pinocytosis.

**Release of \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone**

Four empty dishes and 20 dishes containing cells were incubated with 2 ml of medium containing \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone (100 \( \mu \text{g/ml} \)). After 48 h the medium was removed and the dishes were washed six times with non-radioactive medium. The cells from four dishes were detached by trypsin treatment at 37°C for 30 min, and the other dishes were re-incubated with 2 ml of non-radioactive medium. The released cells were centrifuged at 4°C as described above and the supernatants and cell pellets, after washing and sonication, were assayed for radioactivity. After various times the media from the re-incubated dishes (both empty and containing cells) were removed and assayed for radioactivity after centrifugation at 4°C to remove any detached cells. The cells in the dishes were assayed for radioactivity as described above.

Fig. 2 shows that during reincubation in non-radioactive medium there was an initial rapid release, followed by a slower progressive release, of \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone into the cell-free medium. There are several potential sources of this released material.

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**Fig. 2. Release of \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone during reincubation of smooth-muscle cells with non-radioactive medium**

Both empty dishes and dishes containing cells were incubated with \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone (100 \( \mu \text{g/ml} \)) for 48 h, washed six times and reincubated with non-radioactive medium. The mass of \( ^{125} \text{I} \)-labelled polyvinylpyrrolidone associated at various times with the cells (●), cell-free medium (▲), supernatants from trypsin-treated, centrifuged cells (○) and the medium from empty dishes (△) are shown (see the text). Each point and error bar corresponds to the mean value ± s.d. from four dishes.
(1) Leakage from extracellular spaces inaccessible to the washing procedure. The supernatant from the centrifuged cells which had been trypsin treated immediately after the washing procedure would have contained all the radioactivity from these spaces and also any that may have been released by exocytosis, cell lysis or desorption from the dish or cell surfaces during trypsin treatment (Fig. 2). However, the maximum possible amount that may have originated from the spaces could not have exceeded $30 \pm 5.4\%$ (mean $\pm$ S.D. calculated from two groups of four dishes) of the total released into the medium during the 48 h re-incubation.

(2) Desorption from the dish surface. This must account for a large proportion of the release, because after 48 h the radioactivity in the reincubation medium from four empty dishes was $48 \pm 13\%$ (mean $\pm$ S.D.) of that in the medium from four dishes containing cells (Fig. 2).

(3) Cell lysis.

(4) Desorption from the cell surface.

(5) Exocytosis of previously pinocytosed material.

The rate of release of cell-associated $^{125}$I-labelled polyvinylpyrrolidone (by exocytosis or possibly desorption) may not be determined from the rate of appearance of radioactivity in the medium because of the unknown contributions of sources (1), (2) and (3). Therefore changes in cellular content of label must be assessed.

A linear-regression analysis of the $^{125}$I-labelled polyvinylpyrrolidone associated with the total cells isolated from each dish revealed no significant decrease ($0.10 > P > 0.05$) over the 48 h re-incubation period (Fig. 2). This absence of statistical significance does not of course eliminate the possibility of a limited release after uptake. The 95% confidence interval for the percentage change in cell-associated label extended from a decrease of about 50% to an increase of a few per cent. Therefore if release does occur, it is unlikely over a 48 h period to exceed 50% of that initially associated with the cells.

Uptake of $^{125}$I-labelled polyvinylpyrrolidone

The rate of cellular uptake of $^{125}$I-labelled polyvinylpyrrolidone will be equal to its combined rates of accumulation and release. Therefore the release experiment indicates that the rate of uptake is between 1 and about 1.5 times the rate of accumulation, assuming that the rate of release during incubation with radioactive medium is the same as or less than that during reincubation in non-radioactive medium. Therefore, from the results of the time-course experiment in which cells were incubated with 30 pg of this substance/ml, the rate of uptake was calculated to lie between 2.4 and 3.7 ng/h per mg. This rate may be alternatively expressed as the endocytic index for $^{125}$I-labelled polyvinylpyrrolidone (see above), whose value would lie between 82 and 123 n1/h per mg.

Certain conditions of relevance to the pathogenesis of atherosclerosis may modify the endocytic index of cultured arterial smooth-muscle cells for $^{125}$I-labelled polyvinylpyrrolidone.

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Vol. 5