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The Use of Gelatin Microparticles to Delay the Release of Readily Water-soluble Materials

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Abstract

The adsorption of D-arabinose onto gelatin microparticles demonstrated a Langmuirian adsorption pattern. Evaluation of the dissolution behaviour of D-arabinose-loaded gelatin microparticles suggested that the saccharide, loaded at a level below the adsorption saturation level, was released uniformly over a 14-h period after the loaded gelatin microparticles had been lyophilized for a second time. When dissolution curves were corrected for the initial burst effect seen after the gelatin microparticles had been loaded at higher levels of D-arabinose and lyophilized, steady-state release rates were also evident over prolonged periods. In addition, it was evident that the D-arabinose was adsorbed onto internal surfaces of the hydrated gelatin matrix. Calculation of this internal surface demonstrated the influence of the concentration of the glutaraldehyde used as a cross-linking agent and this parameter, in turn, influenced both the adsorption maxima and the subsequent equilibrium release rates.

Application of this data base to a highly water-soluble complex polysaccharide antineoplastic agent, which has a higher molecular weight (22·4 kDa vs 150 Da), demonstrated similar behaviour in that a near zero-order release pattern over at least 16 h could be obtained by attention to the conditions under which the gelatin microparticles were made and subsequently loaded before lyophilization.

We have recently isolated and evaluated a complex polysaccharide glycan from Bacillus Calmette-Guérin (BCG) vaccine with potent oncolytic activity (Lou et al 1992, 1994; Lou 1993). This material, termed PS1, has a molecular weight of approximately 22·4 kDa, is highly water soluble and is remarkably heat stable. Because of the solubility in water it was considered to be desirable to delay release of the antineoplastic from an injection site and, preferably, target or direct the drug to the surface of a tumour to potentiate its activity. We therefore developed the use of gelatin microparticles for the delivery of PS1, since gelatin has been demonstrated to target fibronectin-bearing surfaces as BCG organisms target bladder tumours (Ratliff et al 1987; Nakamura 1992; Olson 1992; Lou et al 1995).

Gelatin has been widely evaluated as a drug delivery vehicle (Tanaka et al 1963; Yoshioka et al 1981; Chiao & Price 1989; Tabata & Ikada 1989). Methods of preparation of gelatin particulate systems include simple coacervation (Nixon et al 1968; Nixon & Walker 1971), complex coacervation (Madan et al 1987) or emulsification processes (Tanaka et al 1963). Our laboratory has recently described a precipitation process involving cold water-miscible solvents (Öner & Groves 1993) which produced micron-range gelatin particles. However, since large amounts of water are used to remove the excess cross-linking agent, glutaraldehyde, from these microparticles, it is necessary to load the prepared vehicle by soaking with an aqueous solution of the material under test, followed by lyophilization (Illum et al 1986). While evaluating this process, it became evident that both D-arabinose and PS1 were adsorbed onto the gelatin microparticles, delaying subsequent release in a controlled manner. Proteins may be separated by carbohydrate-loaded columns and this probably involves an adsorption/desorption process. Literature on this subject appears to be sparse, although Beyer (1952) did note the use of gelatin to separate sugars in a general review of chromatography.

Since PS1 itself was not available in large quantities, we initially explored the adsorption properties of saccharides in general by using the monosaccharide D-arabinose as a model drug. This material was selected because PS1 contains arabinose moieties, it is readily water soluble and may also be assayed using a phenol-sulphuric acid procedure (Hodge & Hofreiter 1962).

This paper reports the evaluation of delayed drug-delivery systems using D-arabinose adsorbed onto gelatin microparticles and demonstrates the application of the acquired data to the subsequent formulation of PS1.

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Materials and Methods

Materials

Gelatin (bovine skin, lime cured (type B), Bloom number 225) was obtained from Sigma Chemical Corporation, St Louis, MO. Glutaraldehyde (50% in water), buffered saline (Isoton), sodium metabisulphite and anhydrous ethanol (95% ethanol, isopropanol 5% w/w) were obtained from Fisher Scientific, Itasca, IL. D-Arabinose was from Fluka Inc., Ronkonkoma, NY. PS1 was obtained by hot water extraction from Tice substrain BCG vaccine, lot 105B153C as described by Lou et al (1994).

Preparation of gelatin microparticles

The basic process, described in detail by Öner & Groves (1993), involves adding an aqueous solution of gelatin to an excess of anhydrous ethanol at −15°C, cross-linking and

Table 1. Characteristics of gelatin microparticles.

Lot number	Glutaraldehyde	Size range (μm)				Specific surface area[c] ($cm^2\ g^{-1}$)	Average lot yield[f] percent (%)
		Coulter		Malvern			
		dvn ± s.d.[a]	σ_g[b]	dz ± s.d.[c]	Q ± s.d.[d]		
1–6	4% w/v	1·45 ± 0·82	1·66	2·01 ± 0·98	0·071 ± 0·02	$3{\cdot}466 \times 10^4$	79·5 ± 4·3
7–12	6% w/v	3·25 ± 1·34	2·11	3·47 ± 1·51	0·176 ± 0·12	$1{\cdot}340 \times 10^4$	78·9 ± 3·9
13–18	8% w/v	5·14 ± 0·51	1·70	4·89 ± 1·45	0·423 ± 0·09	$0{\cdot}964 \times 10^4$	79·1 ± 1·7

[a]Mean volume number diameter (dvn) obtained by Coulter Multisizer II. [b]Standard deviation of distribution ($\sigma_g = d_g84/d_g50$ from Coulter). [c]Z-average particle diameter (dz) obtained by Malvern Zetasizer III. [d]Polydispersity index (Q) obtained by Malvern Zetasizer III. [e]External surface area calculated from gelatin particle size and density (based on Coulter Counter measurements and the Hatch-Choate relationship). [f](Weight of microparticles/weight of gelatin in solution)×100.

recovering the microparticles by filtration after extensive washing. After preparation, the microparticles were lyophilized and stored at ~ 4°C.

Analyses

After adjustment of the volume of the collected samples to bring absorbencies within a linear absorbance/concentration range, saccharide and polysaccharide contents were estimated by titration with phenol-sulphuric acid reagent (Hodge & Hofreiter 1962) at 480 nm using a Beckman DU-65 spectrophotometer. The particle size was measured by Coulter Multisizer II, fitted with a 50-μm orifice tube after dispersion in Isoton. Typically, data fitted log-probit plots (Öner & Groves 1993) and specific surface areas were calculated using the Hatch-Choate relationship (Herdan 1960) (Table 1).

Adsorption

D-Arabinose. Gelatin microparticle suspensions (0·05% w/v) were prepared at various concentrations (10^{-3}–10^{-5} M) of D-arabinose in phosphate buffer, pH 7·4, and were shaken horizontally at the rate of 40 strokes min^{-1} in a shaker bath (Precision Scientific) at 25 ± 0·1°C for 2 h. Following centrifugation, the supernatant was analysed by titration with the phenol-sulphuric acid reagent as above and adsorption isotherms constructed point-by-point. Adsorption studies were carried out in triplicate for each type of microparticle.

PS1. For studies of the adsorption profile of PS1, the final gelatin microparticle concentration was 0·1% w/v and the concentration of PS1 was within the range 10^{-4}–10^{-6} M in phosphate buffer (pH 7·4). The suspensions were stored in tightly sealed culture tubes and equilibrated in the shaker bath at 25 ± 0·1°C with continuous shaking overnight. After equilibration, the formulated PS1/gelatin microparticles were separated by centrifugation at 13 000 rev min^{-1}. The supernatant was analysed to assess drug adsorption, as above.

Loading the gelatin microparticles

As noted, the preparation process involves excess volumes of water to wash out the cross-linking agent. Microparticles were therefore loaded with drug after preparation. This was achieved by adding 50 mg dry gelatin microparticles to a solution of D-arabinose or PS1 in 5 mL 1/15 M phosphate buffer, pH 7·4. After mixing and equilibrating for 2 h, the system was again lyophilized.

Dissolution method

Release of either D-arabinose or PS1 loaded onto gelatin microparticles was assessed by dispersing 50 mg of the sample in 5 mL 1/15 M phosphate buffer and transferring to a dialysis tube suspended in 200–500 mL phosphate buffer. The tubing (Spectrum Med. Ind., Los Angeles, CA) had a 12–14 kDa molecular weight cut-off for D-arabinose or a 25 kDa cut-off for PS1. No evidence of hold-up by the membrane could be determined for either compound. Using a magnetic stirrer bar, the dissolution medium was slowly stirred at 25°C. Samples were taken for analysis at appropriate intervals, replacing the volume of the sample with an equivalent volume of medium to keep a constant dissolution volume. Dissolution tests were replicated and samples were analysed in triplicate for each type of microparticle as before.

Results and Discussion

Öner & Groves (1993) noted that the size of the resulting gelatin microparticles increased with the use of higher concentrations of glutaraldehyde. This conclusion was confirmed here although, on a mass basis, the yields of hardened microparticles were essentially similar, irrespective of the preparation conditions (Table 1). The size of the resulting particles was influenced by the temperature of the dehydration process (Fig. 1) tending to optimize at ~ 10°C and below. Scanning electron microscopy of the microparticles demonstrated (Lou 1993) that they consisted of aggregates of small (50–300 nm) particles (Fig. 2). However, preparation of replicate batches (n = 18) of microparticles showed that the factors identified by Öner & Groves (1993) reproducibly controlled the size of the resulting aggregates (Lou 1993). Photon correlation spectroscopy and the Coulter method gave substantially similar sizes (Lou 1993), suggesting that the aggregates were stable following dispersion into an excess of diluent.

Both D-arabinose and PS1 demonstrated classical Langmuir adsorption profiles onto the gelatin microparticles and, in both cases, the adsorption maxima were influenced slightly by the glutaraldehyde used in the preparation of the microparticles (Table 2). Lou (1993) demonstrated that the equilibrium was reached rapidly; the experimental conditions were selected to ensure that the factors were optimal.

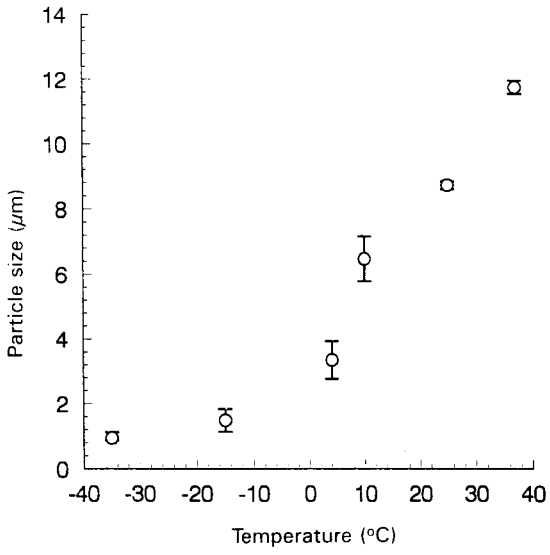

FIG. 1. Effect of precipitation temperature on the size (dvn) of the resulting gelatin microparticle (lime-cured type B, bloom number 225). Bar = s.e., n = 3.

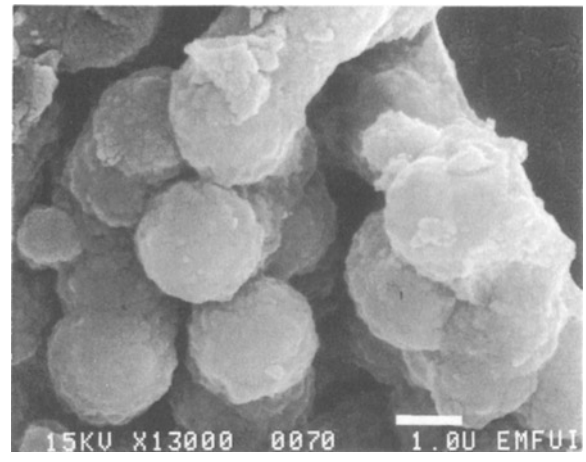

FIG. 2. Scanning electron micrograph of gelatin microparticles cross-linked by 4% glutaraldehyde at −15°C. Individual particles can be seen as aggregates of small primary particles ≈ 0·2 μm diameter. Bar = 1·0 μm.

In its linear form, the Langmuir adsorption isotherm equation becomes:

$$C_{eq}/(x/m) = 1/ab + C_{eq}/a$$

where x is the amount absorbed by mg adsorbent, and C_{eq} is the equilibrium concentration. The constant a is a measure of the capacity of the adsorbent for the adsorbate and b is the affinity constant of the adsorbate for the adsorbent. For D-arabinose with a correlation of 0·97–0·99 for three determinations, $a = 5{\cdot}18 \times 10^{-4}$ M and $b = 1{\cdot}47 \times 10^{4}$ M. For PS1, the data were also fitted to the Langmuir isotherm equation in its linear form (Table 2).

By analogy with glucose, the cross-sectional area of the D-arabinose may be assumed to be approximately 0·55 nm^2 (Sato & Kim 1984). Even at a close-packed saturation of the external surface, the data strongly suggest (Table 2) that a significant proportion of the D-arabinose is adsorbed internally. The effect of the glutaraldehyde on the formation of internal structure in the gelatin microparticles is also clearly demonstrated.

In part, the effect of glutaraldehyde concentration on the internal structure of the microparticles is also evident in the dissolution behaviour of microparticles loaded with D-arabinose (Figs 3, 4). A near zero-order release pattern was obtained with gelatin loaded at a concentration below or close to the measured saturation level (Fig. 3). Generally, the release is similar to a biphasic pattern described by Wagner (1969), with an initial, very rapid, burst effect being evident, followed by a slower release phase. This is often seen with drug loaded onto microparticulate systems (Donbrow 1992) and may be attributed to a number of phenomena occurring sequentially. In the first stage, material that is held loosely on the surface is released rapidly into the surrounding aqueous medium. This release is identifiable with the burst effect seen within the first hour of the dissolution process. After this initial phase, material adsorbed on the external surface is released into the medium followed by material from the interior structure of the bead. The two release processes will appear to proceed at different rates since it will be necessary for the adsorbate to diffuse to the external surface before it can be measured. The two phases can be identified by a simple biexponential equation of the type reviewed by Jalšenjak (1992):

Q = amount remaining in the microparticles at time (t)

$$= Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B are constants and α and β are the respective rate constants. These rate constants are shown in Table 3 for both D-arabinose and PS1. The data demonstrated that, as

Table 2. The effect of glutaraldehyde concentration on the adsorption of D-arabinose and PS1 from the external or internal surfaces of gelatin microparticles.

Glutaraldehyde	External specific surface[a] ($cm^2\ g^{-1} \times 10^4$)	Amount adsorbed in external surface ($mol\ g^{-1} \times 10^{-6}$)		Maximum adsorbed[b] surface[c] ($mol\ g^{-1} \times 10^{-5}$)		Total specific surface ($cm^2\ g^{-1} \times 10^5$)		Amount adsorbed in internal surface ($mol\ g^{-1} \times 10^{-5}$)		Internal specific surface[d] ($cm^2\ g^{-1} \times 10^5$)	
		D-Arabinose	PS1	D-Arabinose	PS1	D-Arabinose	PS1	D-Arabinose	PS1	D-Arabinose	PS1
4%	3·466	10·5	0·763	51·8	0·478	1·713	2·17	50·7	0·402	16·78	1·823
6%	1·340	4·07	0·295	47·4	0·436	1·567	1·98	46·9	0·406	15·54	1·846
8%	0·964	2·93	0·213	39·1	0·403	1·293	1·83	38·8	0·382	12·83	1·734

[a]Calculated from gelatin particle size and density (based on Coulter Counter measurements and the Hatch-Choate relationship). [b]Obtained from Langmuir isotherm. [c]Calculated from Langmuir isotherm and BET equation. [d]Obtained from total surface substrate less that adsorbed on the external surface.

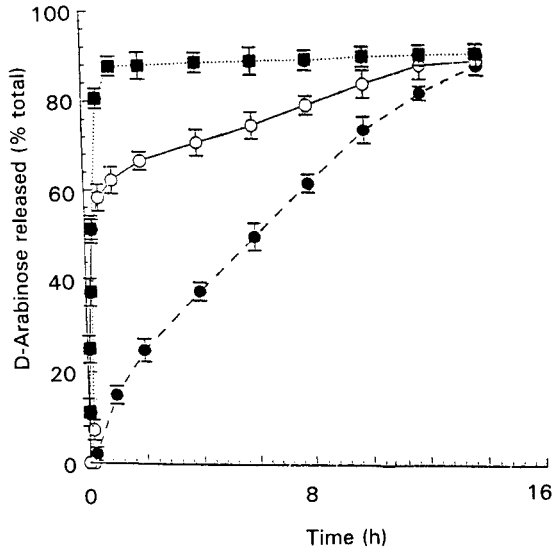

FIG. 3. Effect of D-arabinose loading on the release profile from gelatin microparticles in 1/15 M phosphate buffer (pH 7·4) at 25°C. ●— —● D-Arabinose loading 2·5%, ○—○ 5%, ■···■ 50%. Bar = s.e., n = 6.

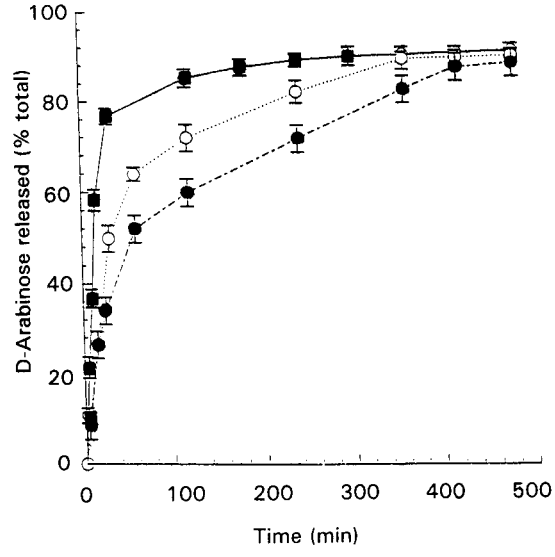

FIG. 4. Effect of glutaraldehyde concentration on release profile of D-arabinose from gelatin microparticles (D-arabinose loading 16%) in 1/15 M phosphate buffer (pH 7·4) at 25°C. ■——■ 4% glutaraldehyde, ○···○ 6%, ●- - -● 8%. Bar = s.e., n = 6.

the loading decreases, the final rate constants (β) increased for the two lower levels of glutaraldehyde used in the cross-linking process, but at 8% glutaraldehyde the release was not affected to any degree. The initial rates are affected since, as the glutaraldehyde level was increased, the initial release rate, α, decreased, and similarly, as the loading was decreased, the release rates were also decreased.

A more direct way to examine the true release rate is to, arbitrarily, subtract the amount due to the initial dump or burst over the first few hours. When this was carried out for subsequent time frames of the experiment, the rates began, in most cases, to settle into a zero-order release pattern (Table 4). Finally, the gelatin microparticles become substantially exhausted, with little D-arabinose remaining to be extracted. In addition, it is also clear that the processes could be seen to be substantially similar irrespective of the drug loading at the lowest (4%) glutaraldehyde levels. Nevertheless, attention must be drawn to the fact that a half-life of approximately 6 h can be achieved with D-arabinose which is otherwise extremely water soluble. Under the optimal conditions of drug loading below the adsorption maximum or saturation level for microparticles prepared with the lowest concentration of glutaraldehyde tested, a near zero-order release pattern over a period of at least 14 h was maintained (Table 4).

Although PS1 has a considerably higher molecular weight than D-arabinose (22·4 kDa vs 150 Da), it also shows an adsorption pattern similar to that observed with the smaller monosaccharide. However, the approximate calculations of surface coverage suggests that some of this adsorbate was also adsorbed on the internal surface of the gelatin particles (Table 2). The burst effect was seen (Fig. 5) but, when corrected for, the subsequent steady-state release rates were generally uniform for the remainder of the dissolution process (Table 4). An increase of glutaraldehyde concentration reduced the burst effect for PS1, but in the terminal phase of the release pattern, large amounts of PS1 are released from gelatin microparticles (Table 4). More PS1 remains associated with the microparticles (cross-linked by a higher concentration of glutaraldehyde) after the initial burst phase, so that there is more PS1 available for release in the terminal phase.

Table 3. The release of D-arabinose and PS1 from gelatin microparticles in 1/15 M phosphate buffer (pH 7·4) as a biexponential system.

Glutaraldehyde (w/v %)	Drug loading (w/w %)		α (initial slope) (s^{-1}) 10^{-4}		β (terminal slope) (s^{-1}) 10^{-5}	
	D-Arabinose	PS1	D-Arabinose (± s.e.)	PS1 (± s.e.)	D-Arabinose (± s.e.)	PS1 (± s.e.)
4	50	16	6·07 (0·07)	4·38 (0·21)	1·55 (0·03)	10·52 (0·16)
6	50	16	4·33 (0·12)	2·87 (0·06)	3·42 (0·17)	8·42 (0·09)
8	50	16	3·54 (0·09)	2·04 (0·13)	4·44 (0·08)	6·75 (0·04)
4	33	11	5·69 (0·13)	3·43 (0·09)	1·67 (0·06)	6·73 (0·12)
6	33	11	3·88 (0·06)	2·33 (0·11)	3·50 (0·15)	5·86 (0·05)
8	33	11	2·79 (0·11)	1·65 (0·09)	4·63 (0·18)	4·56 (0·08)
4	16	6	4·54 (0·10)	2·24 (0·04)	2·95 (0·04)	4·63 (0.14)
6	16	6	2·93 (0·06)	1·69 (0·12)	4·57 (0·07)	4·32 (0·17)
8	16	6	2·20 (0·05)	1·35 (0·08)	4·40 (0·16)	4·28 (0·08)

Table 4. Terminal release rates (into 1/15 M phosphate buffer at pH 7·4 and 25°C) of D-arabinose and PS1 loaded onto gelatin microparticles. The burst effect has been corrected for by removing the mass released over the first 2 h for D-arabinose (4 h for 50% loading) and 5 h for PS1.

D-Arabinose									
				Release rates (mg g^{-1} h^{-1}) over					
Loading (% w/w)	2–4	4–6	6–8	8–10	10–12	12–14	14–16	> 16 (h)	s.e.m. (%)
2·5	2·8	3·1	3·2	3·2	3·0	3·1	2·6	0	6·9
5·0	2·3	2·6	2·4	2·5	2·5	2·4	0	0	3·9
50·0	–	3·8	4·0	3·4	2·6	1·6	0	0	28·6

PS1 (Loading 11%)	Release rates (mg g^{-1} h^{-1}) over					
Gelatin cross-linked with glutaraldehyde (%)	5–6	6–7	7–8	8–9	> 9 (h)	s.e.m. (%)
4	4·2	4·4	4·3	4·0	0	3·5
6	7·3	6·3	7·0	6·4	0	6·2
8	10·2	10·0	9·8	9·0	0	4·7

Returning to the data shown for PS1 released from 4% glutaraldehyde cross-linked gelatin, the plot showing an apparent steady-state release of PS1 over a 16-h period, in fact, has a subdued bi-modal release pattern (Fig. 5). However, the inflection between the two rates occurs at approximately 5 h after the start of the solution process. This is unlikely to be due to any burst effect release from loosely adherent adsorbate. It may represent a transition from the release from the exposed external surface of the aggregated primary particles that constitute the gelatin microparticles to release from the less readily accessible surfaces of the interior of the primary aggregates.

These data confirm that a delayed release formulation of two highly water soluble entities may be achieved if they are capable of being adsorbed onto the matrix or the surface of an insoluble vehicle, in this case glutaraldehyde cross-linked gelatin microparticles, provided the vehicle is then lyophilized for a second time. The lyophilization process accounts for the difference in the rates at which the gelatin microparticles are loaded with drug, and the rates at which the drug is subsequently released. Clearly this is concerned with the rate at which the dried gelatin itself hydrates and expands to allow access of the medium to the interior of the particles. In addition, by careful attention to the conditions under which the gelatin vehicle is initially prepared and loaded with adsorbate, a substantially steady-state release can be obtained over a period of 16 h under the conditions of the experiment.

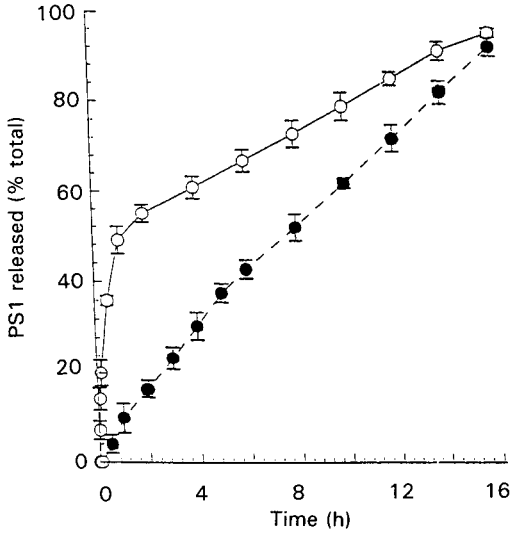

FIG. 5. Effect of PS1 loading on the release profile from gelatin microparticles (cross-linked by 4% glutaraldehyde) in 1/15 M phosphate buffer (pH 7·4) at 25°C. ●– – –● PS1 loading 2·5%, ○—○ 6%. Bar = s.e., n = 6.

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