Antiviral Activity of a Surface-bonded Quaternary Ammonium Chloride

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ABSTRACT

A quaternary ammonium chloride (QAC) covalently bound to alginate beads showed antiviral activity. Bacteriophage T2 and herpes simplex virus type 1 (HSV-1) were used as model viruses in this investigation. The enveloped HSV-1 showed a higher susceptibility to surface-bonded QAC due to hydrophobic interaction. The elution experiments using 1% tryptone solution demonstrated that inactivation, instead of non-specific adsorption, is the main factor for virus titer reduction. Both equilibrium and kinetic studies were conducted in distilled water and protein (BSA, 0.5%) solution to observe the effect of protein molecules on reducing the capacity and rate of virus adsorption/inactivation process. Heating thermolabile proteins at 60°C for 10 hours with various stabilizers has historically been practiced to inactivate any possible viral contaminants. However, the protecting effect of these stabilizers also encourage thermoresistance to the virus. Thermal inactivation and immobilized QAC inactivation procedures were compared using ß-lactamase, a thermolabile enzyme, as a model protein. The activity of ß-lactamase dropped down to 40% of the initial value after heating at 60°C for 10 hours using sucrose as a stabilizer. The virus titer diminished from 10^6 to 10^3 during the first four hours without further reduction. For QAC inactivation, the titer of T2 decreased around one order of magnitude and the recovery of ß-lactamase activity was 70%. Computer simulation results showing the effects of various process variables are also presented.

Quaternary ammonium chlorides (QAC) are cationic surface-active agents with antimicrobial activity (reviewed by Petrocci,1983). The major mode of action of QAC was identified as the effects on cell permeability and cytolytic damage (Hugo, 1967). Klein and Deforest (1983) summarized the viricidal capacities of Zephiran (alkyldimethylbenzylammonium chloride) against various types of viruses. They reported that Zephiran can effectively inactivate lipid-containing viruses, some non-lipid viruses and bacteriophages but is not effective against smaller but non-lipid viruses, such as piconaviruses. QAC display their antimicrobial activity even being immobilized on inert supports because they can act on the membranes of various cells. The effects of surface-bonded organosilicon QAC on bacteria, yeasts, fungi, algaes have been the topic of several papers (Walter,1972, Isquith,1972,1978, Speier,1981, Nakagawa,1983). However, the antiviral activity of these immobilized compounds has not been demonstrated. This is the purpose of this paper.

The presence of low percentage level infectious viral contaminants has been one of the main hurdles for wider application of genetically engineered therapeutic proteins such as blood-clotting factors, interferon, insulin, growth hormones, antibodies, and various clinically significant proteins such as tissue plasminogen activator. Heat treatment is a widely accepted method for sterilizing these complex protein solutions. It is well known that albumin can be pasteurized by heating at 60°C for 10 hours in the presence of certain stabilizers (Gellis *et al.*,1948). Unfortunately, similar attempts to other blood proteins (e.g. clotting factors) resulted in marked reduction or elimination of their functional activities. Schwinn *et al.* (1981) and Fernandes *et al.* (1984) modified the pasteurization procedure using high concentrations (>30% w/v) of polysaccharides as stabilizers. Unfortunately, the protein stabilizing effect of these polysaccharides likewise provides increased thermo-resistance to the viruses (Ng,1985). Heat treatment of lyophilized clotting factor concentrates was also investigated (Rubinstein,1984).

The phenomenon of viral adsorption to various surfaces was

extensively studied from an environmental standpoint (reviewed by Daniels,1980, Gerba,1984) for prevention of various waterborne viral transmissions. The problem of virus removal from complex protein solutions is very different from that of sewage and drinking water treatment processes because most protein molecules compete for the active sites of the adsorbents. Hence, both the adsorption rate and capacity diminish in the presence of protein molecules (Lipson,1984). It is the intention of this paper to demonstrate and to compare the antiviral activity of a surface-bonded QAC in aqueous solutions with and without the presence of proteins. The efficacy of the accepted antiviral thermo-inactivation was compared with the viral inactivation method by the surface-bonded QAC treatment. ß-lactamase was used as a thermolabile model protein (Smith,1969), and bacteriophage T2 and herpes simpex virus type 1 (HSV-1, an enveloped animal virus) were used to test these viral inactivation methods.

MATERIALS AND METHODS

Chemicals 3-(Trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride (Si-QAC), known as Dow Corning 5700 Antimicrobial Agent, was provided by W. Curtis White (Dow Corning Corp., Midland, Mich.). It is a methanolic solution containing 42 wt% of this active ingredient. ß-lactamase was obtained from Sigma Chemical Co (St. Louis, Mo.). Other chemicals were of reagent grade and were purchased from various commercial sources.

Organisms *Escherichia coli* B and bacteriophage T2 are regularly maintained in our laboratories. BSC-1 cells and HSV-1 strain 148 were obtained from Dr. Charles Shipman (Dental School, U. of Michigan, Ann Arbor, Mich.). The cultures are regularly passaged to maintain their viability.

Preparation of Beads Dried alginate/magnetite beads were prepared by a method similar to that described by Burns and Grave (1984). Barium chloride was used as a gel-inducing agent for better stability (Paul and Vignais,1980). The beads were further stabilized by treating with glutaraldehyde in the presence of hexamethylenediamine to avoid the dissolution problem (Murata *et al.*, 1980). Beads with diameters between 0.15 and 0.25 mm were obtained by crushing and then systematically sieving the original spherical beads. Various concentrations of Si-QAC solution were prepared by diluting the 42% active material in distilled water at pH 5. After the beads were added to the Si-QAC solution, the reaction temperature was raised to about 50°C for 10 minutes. Then, the pH was adjusted to 10.5 for another 10 minutes. The beads were then dried in a oven (100°C), rinsed several times with sterile deionized water (pH 7.0) and stored at 4°C.

Cell Culture BSC-1 cells were grown in minimal essential medium (MEM) with Earle salts supplemented with 10% fetal bovine serum (FBS) and 1.1 g/l sodium bicarbonate. Cells were passaged according to conventional procedures by using 0.05% trypsin plus 0.02 wt% ethylenediamineteraacetic acid (EDTA) in a HEPES-buffered balanced salt solution. Tissue culture flasks were incubated at 37°C in a humidified 3% CO_2-97% air atmosphere. Total cell counts were made using a Coulter counter equipped with a 100-µm orifice

and microscopic cell count.

Titration of viruses HSV-1 was assayed by using monolayer cultures of BSC-1 cells grown in 6-well multidishes. The cells were planted $3x10^5$ cells/well in MEM(E) with 10% FBS and 1.1 g/l sodium bicarbonate. After 24 hours the cell sheet was about 75% confluent and was inoculated with 0.2 ml of the virus suspension to be assayed and incubated for 1 hour to permit viral adsorption. The cell sheets were then overlaid with 3 ml medium containing 0.5% methocel and incubated for another 2 days. After aspiration of the overlay, the cells were stained with crystal violet, and macroscopic plaques were enumerated.

The assay procedures for T2 used here were described by Adam (1957) as well as Rovozzo and Burke (1973).

Assays Samples collected in all experiments were cooled and stored at 4°C, then the following analyses were done. The concentration of total protein in the solution was assayed by the Bradford method (Bradford,1978). The concentration of ß-lactamase was assayed according to Sykes and Nordstrom (1972).

Batch Experiments During these experiments, adsorbents and viruses were continuously mixed in Erlenmeyer flasks by a gyratory shaker at 22°C. Reaction mixtures of known composition were made by adding stock solution to 0.01 M Tris/HCl buffer at pH 7.0. All stock chemical solutions were autoclaved and stored at 4°C.

In the equlibrium studies, tests were conducted with various initial concentrations of adsorbents and viruses to determine the amount of viruses adsorbed per unit gram of adsorbent and the virus concentration remaining in the solution at equilibrium. The time required to reach equilibrium was determined by periodically sampling over a 24 hour period. In the kinetic studies, samples were withdrawn at predetermined time intervals and assayed for the virus titers.

RESULTS AND DISCUSSION

Inactivation of T2 phage by QACs in Free Solutions

Susceptibility of bacteriophage T2 to QAC is shown in Table 1. Survivors could not be found in solutions without the bovine serum albumin (BSA). These results demonstrated that bacteriophage T2 can be inactivated by QAC as well as Si-QAC solutions. The presence of protein molecules inhibited the activities of these antimicrobial agents. In fact, BSA even coagulated in the presence of high concentration (>0.05%) of Si-QAC.

Inactivation of Viruses by Surface-Bonded QAC

The attachment of this Si-QAC to surfaces involves a rapid ion-exchange process which coats as a monolayer on the bead surface. Then, the immobilization is further strengthened by the polymerization reactions (Malek and Speier,1982). Table 2 shows the effects of dried alginate beads treated by various Si-QAC concentrations. 0% means untreated beads and were served as controls. When the titer was very low ($4.7x10^2$), viruses were eliminated completely in all cases including the control. This was due to non-specific adsorption. When the titer was raised to $4.0x10^6$, the adsorption capacities of treated beads were distinctly better than the control. For a titer as high as $2.0x10^8$, it seems that beads were nearly saturated with viruses in all cases.

The titer reduction and adsorption capacities of the T2 phage and HSV-1 are compared in Table 3. For similar initial titers (10^6 PFU/ml), the survivor titer of HSV-1 was at least 2 order of magnitude lower than that of T2. For similar equilibrium titer remaining in the solution (10^2 PFU/ml), the adsorption capacity (PFU/ml) of HSV-1 was 2 order of magnitude higher than that of T2. Evidently, HSV-1 is much more susceptible to the surface-bonded QAC than T2. Since HSV-1 is an enveloped virus, the lipid bilayer surrounding the capsid binds strongly to the QAC-treated surface due to additional hydrophobic interaction. It should be noted that the adsorption experiments of T2 were carried out in clean buffer solutions without proteins, while those of HSV-1 were in buffered 1 vol% FBS solution (HSV-1 requires the presence of proteins to avoid natural inactivation).

Viruses can be considered as biocolloids with surface charges that result from ionization of carboxyl and amino groups of

proteins localized on the surface. At a characteristic pH, defined as isoelectric point (PI), the virion can exist in a state of zero charge. Isoelectric point of a virus may vary by the type and the strain of the virus (Zerda,1982). The phage T2 (PI=4.2) posseses a net negative surface charge in solutions of pH 7.0. On the other hand, the QAC treated bead renders a positively-charged surface. This suggests that electrostatic force may play an important role in the adsorption process. However, the electrostatic force may not be the sole mechanism. Besides Brownian motion, the electrical double-layer (Verwey and Overbeck,1948), which is influenced by ionic strength and pH of the medium, may also facilitate the viruse' adsorption to the solid surface. Reduction of this double layer allows the van der waals, hydophobic and affinity interactions (if any) to effect the adsorption of viruses to the immobilized QAC surface. Quantification of these effects is difficult in most complex protein solutions.

Elution Experiments

In addition to reversible adsorption, inactivation or degradation of viruses by various types of surfaces such as metal oxides (Murray and Laband,1979), aluminum metal (Murray,1980), magnetite (Atherton and Bell,1983), clays (Tayler et al.,1980) and soils (Yeager and O'Brien,1979) have been reported. The mechanisms were identified to be either degradation of the capsid and/or the nucleic acid. However, such inactivation may be only specific to certain types of viruses.

Bacteriophage T4 attached on activated carbon can be reversibly eluted by 1% tryptone solution (Cookson and North,1967). In this case, the majority of the adsorbed viruses could not be recovered by the tryptone elution (Table 4). The results suggest that the viruses were eluted out of the surfaces but in an inactivated form. Further verification will be needed using radioactivlly labelled viruses.

Adsorption Isotherms

Removal of T2 onto QAC-treated surfaces with and without the presence of BSA can be correlated using the Freundlich isotherms :

$$q = K C_e^n \qquad (1)$$

where q is the amount of viruses removed and C_e is the virus titer in equilibrium remaining in the solution. K and n are coefficients which can be determined by linear regression. Typical isotherms for removal of viruses by QAC-treated beads are shown in Figure 1. The value of n is close to one in both cases. A significant reduction in the adsorption capacity is observed in 0.5% BSA solution because the BSA molecules interfere the adsorption of the viruses.

In Figure 2 the kinetic study of T2 removal using QAC treated beads is presented. It is obvious that the competitive adsorption between viruses and BSA molecules also reduced the adsorption rate. In both cases viruses were inactivated rapidly at initial 2 hours and titer reduction slowed down after that. This inconsistency with the first-order inactivation model may be due to various interfering mechanisms such as displacement, molecular orientation, multilayer effects, surface heterogenity, and virion clumping.

Adsorption/Inactivation of T2 Phage in a ß-Lactamase Solution

The denaturation or unfolding of a protein leads to loss of its functional activity. The activation energy of the protein unfolding process can be increased in the presence of sucrose (Lee and Timasheff,1981). The activity of ß-lactamase, a model protein, dropped down to 40% of the initial value after heating the mixture at 60°C for 10 hours using sucrose (0.8 g/ml) stabilization (Figure 3). The total amount of soluble proteins decreased because of coagulation. The decline of ß-lactamase activity agreed with that of the total protein. These experimental results compared favorably with various sucrose stabilization studies of thermolabile proteins using blood clotting factors (Schwinn *et al.*,1981, Fernandes *et al.*,1984). It was assumed in those studies that the treatment can render the protein solutions free of hepatitis infection. However, Figure 3 shows the T2 titer also diminished from 10^6 to 10^3 during the first four hours without further reduction.

Inactivation of phage T2 in ß-lactamase solution by QAC-treated beads is shown in Figure 4. The initial T2 titer was 3.0×10^6 PFU/ml. 0.8 g of beads were mixed with 10 ml of ß-lactamase solution. 15% of the viruses survived this treatment. The amount of total protein in the solution was 80% of the initial value after the adsorption process, while the recovery of

ß-lactamase activity was at least 70%. It was the purpose of this experiment to demonstrate that QAC-treated beads can effectively remove viruses from a protein solution without significantly losing the activity of the protein. Optimal adsorption condition and mode of operation ought to be determined by studying the interactive effects of pH, ionic strength, and temperature of the solution, with the specific types of virus and protein of interest.

Simulation Studies of Virus Removal Using Adsorption Column

A fixed-bed adsorption has several advantages over batch and continuous stirred tank reactor (CSTR) because the rates of adsorption depend on the concentration of viruses in solution. This point is especially important for virus removal because of the low concentration of viral contaminants. The design of a fixed-bed adsorption column involves estimation of shape of the breakthrough curve and the appearance of the breakpoint. Computer simulation studies were done here to demonstrate the performance of an virus adsorber using the surface-bonded QAC beads which have a higher binding affinity for viruses over other proteins.

The diffusion model of the system can be described mathematically by sets of material balance equations together with appropriate boundary and initial conditions :

Equation of Continuity

$$\frac{\partial C_i}{\partial t} + v\frac{\partial C_i}{\partial z} - D_L\frac{\partial^2 C_i}{\partial z^2} + \frac{1-\epsilon}{\epsilon}R_{Ai} = 0 \qquad (2)$$

Adsorption Rate

$$R_{Ai} = \frac{3k_{fi}}{R}(C_i - C_{ri}) \qquad (3)$$

Adsorption Equilibrium

$$q_i = K_i C_{ri}^{n} \qquad (4)$$

Initial Conditions

$$C_i(z,0) = 0, \qquad (5)$$

$$q_i(z,0) = 0. \qquad (6)$$

Boundary Conditions

$$C_i(0,t) = C_{i0}, \qquad (7)$$

$$\frac{\partial C_i(L,t)}{\partial z} = 0. \qquad (8)$$

where C_i, C_{ei}, and C_{i0} are the concentrations of i^{th} adsorbate in the bulk solution, at the interface, and of the influent, v is the linear velocity, L is the bed length. Linear adsorption isotherms (n=1) are assumed for both virus and total protein. The equilibrium constants K were obtained from batch experiments. It was also assumed that the complex proteins can be considered as a single component, no radial concentration gradient, and diffusion coefficients, fluid viscosity and density remained constant.

D_L is the longitudinal dispersion coefficient of viruses and can be determined by the empirical correlation given by Chung and Wen (1968) as a function of Reynolds number (Re), density and viscosity of the fluid.

$$\frac{\rho D_L}{\mu} = \frac{Re}{0.2 + 0.11 Re^{0.48}} \tag{9}$$

The mass transfer coefficient k_c is estimated by the correlation of dimensionless j, or Colburn factor, with Sherwood number (Sh), Schmidt number (Sc), and void fraction as described by Cookson (1970).

$$j = \frac{Sh}{Re\, Sc^{1/3}} = \frac{k_c}{v}\left(\frac{\nu}{D}\right)^{2/3} \tag{10}$$

and

$$j = Be\, Re^{-2/3} \tag{11}$$

These equations were solved numerically using finite difference method. Figure 5(a) and 5(b) show the simulated breakthrough curves of both total protein and HSV-1 respectively. It should be noticed that the dimensionless time scales in these two figures differ by four orders of magnitude. The breakpoint of HSV-1 is the operating endpoint at which the effluent from the adsorption column can no longer meet the desired sterilization criterion. Since the HSV-1 has a much higher affinity to the bead surface, the breakpoint of HSV-1 appears much later than that of the total protein. To optimize the protein recovery, one should improve the design of the bead surface (better selectivity, higher loading capacity), size, and the operating parameters to further

delay the breakpoint of the virus.

The effects of desired sterilization criterion on total protein recovery and the amount of adsorbent required are demonstrated in Figure 6. Stringent sterilization criterion (10^{-3}) can only be achieved with reduced protein recovery based on our current design of the beads.

Conclusions

The surface-bonded QAC can effectively adsorb and inactivate viruses based on our initial experimental results. HSV-1, an enveloped virus, is more susceptible than the non-enveloped bacteriophage T2 to the QAC treatment. However, as a non-specific adsorption process, both the rate and capacity reduced due to the competitive binding of the protein molecules. Thermo-inactivation and surface-bonded QAC treatment were compared in terms of titer reduction and remaining functional activity of the model protein, β-lactamase. Process modeling and computer simulation enable us to predict the breakthrough curves of a virus adsorption column. Choosing a sterilization criterion has to be compromised with reduced protein recovery.

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NOTATION

Be = constant depending on void fraction.

C = fluid phase-concentration, virions/cm^3 or $\mu g/cm^3$.

C_0 = fluid phase inlet concentration, virions/cm^3 or $\mu g/cm^3$.

D = diffusion coefficient, cm^2/sec.

D_L = longitudinal dispersion coefficient, cm^2/sec.

D_p = mean diameter of adsorbent, cm.

j = dimensionless Colburn factor.

K = volume equilibrium constant, cm^3/cm^3.

k_c = mass transfer coefficient, cm/s.

L = length of column, cm.

n = parameter in Freundlich isotherm.

Pe = vL/D_L, Peclet number.

q = solid phage concentration, virions/cm^3 or $\mu g/cm^3$.

R = mean radius of adsorbent, cm.

Re = vD_p/ν , Reynolds number.

R_A = adsorption rate, virions/cm^3/sec or $\mu g/cm^3$/sec.

Sc = ν/D, Schmidt number.

Sh = k_cD_p/D, Sherwood number.

T = vt/L, dimensionless time.

t = time, sec.

U = C/C_0, dimensionless fluid-phase concentration.

v = average linear velocity, cm/sec.

Greek Letters

ϵ = void fraction, cm^3/cm^3.

ρ = fluid bulk density, g/cm^3.

μ = absolute viscosity, g/cm/sec.

ν = kinematic viscosity, cm^2/sec.

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Table1. Antiviral Activity of QAC against T2 phage.

Disinfectant	Solution	Initial (PFU/ml)	Survivors (PFU/ml)	Reduction (%)
QAC*	D.W.#	7.4×10^5	0	100
QAC	0.5% BSA¶	7.4×10^5	7.0×10^2	99.9
Si-QAC†	D.W.	2.0×10^4	0	100
Si-QAC	D.W.	1.5×10^3	0	100

* 0.5% hexadecyltrimethyl ammonium chloride.
† 0.5% Dow Corning 5700 antimicrobial agent.
# distilled water buffered by 0.01M Tris/HCl, pH 7.0.
¶ bovine serium albumin buffered by 0.01M Tris/HCl, pH 7.0.

Table 2. Effects of dried alginate beads treated by various concentrations of Si-QAC.

Initial (PFU/ml)	Survivors (PFU/ml) 10%	1%	0.1%	0.01%	0%
4.7×10^2	0	0	0	0	0
4.0×10^4	0	0	2.0×10^1	2.0×10^1	1.4×10
2.0×10^8	1.5×10^7	1.3×10^7	1.6×10^7	1.3×10^7	2.8×10^7

bead preparation : 2g of dried alginate beads in 20 ml of Si-QAC solution.

inactivation reaction : 2g of treated beads in 10 ml of 0.01M Tris/HCl buffer solution, pH 7.0.

Table 3. Comparison of titer reduction and adsorption capacity between HSV-1 and T2 phage using surface-bonded QAC.

Virus	Initial (PFU/ml)	Survivors (PFU/ml)	Titer in Solution (PFU/ml)	Viruses Adsorbed (PFU/g)
T2*	2.12×10^6	1.10×10^4	1.50×10^2	3.70×10^5
HSV-1#	2.03×10^6	6.67×10^2	6.67×10^2	2.03×10^7

* distilled water buffered by 0.01M Tris/HCl, pH 7.0.
\# 1% FBS buffered by 0.01M HEPES, pH 7.0.

Table 4. Elution of phage T2 after the adsorption/inactivation process using 1 % tryptone solution.

Initial (PFU/ml)	Survivors (PFU/ml)	After elution (PFU/ml)	Inactivated T2/Total titer reduction (%)
2.1×10^6	7.0×10^3	1.1×10^4	99.5
1.8×10^5	2.0×10^2	3.9×10^3	97.8
2.0×10^4	4.2×10^1	1.9×10^2	99.0
1.9×10^3	0	0	100

inactivation reactions : 0.5 g of Si-QAC treated beads in 5 ml 0.01 M Tris/HCl buffer solution, pH 7.0.

Table 5. Physical parameters used for simulation studies

	HSV-1	FBS
Adsorbates parameters :		
Equilibrium constant K (ml solution/ml adsorbent)	5.0×10^{4}	5.25
Diffusion coefficient D (cm^2/sec)	6.0×10^{-7}	8.0×10^{-8}
Influent titer (PFU/ml) or Concentration (µg/ml)	1.0×10^{5}	500
Column parameters :		
column diameter (cm) : 3		
column porosity (-) : 0.5		
bead diameter (cm) : 0.02		
bead density (g/ml) : 2.11		
Fluid parameters :		
viscosity (centipoise) : 1.20		
density (g/ml) : 1.01		
flow rate (ml/min) : 1.0		

Figure 1.
Equilibrium isotherms of phage T2
inactivation using surface-bonded QAC

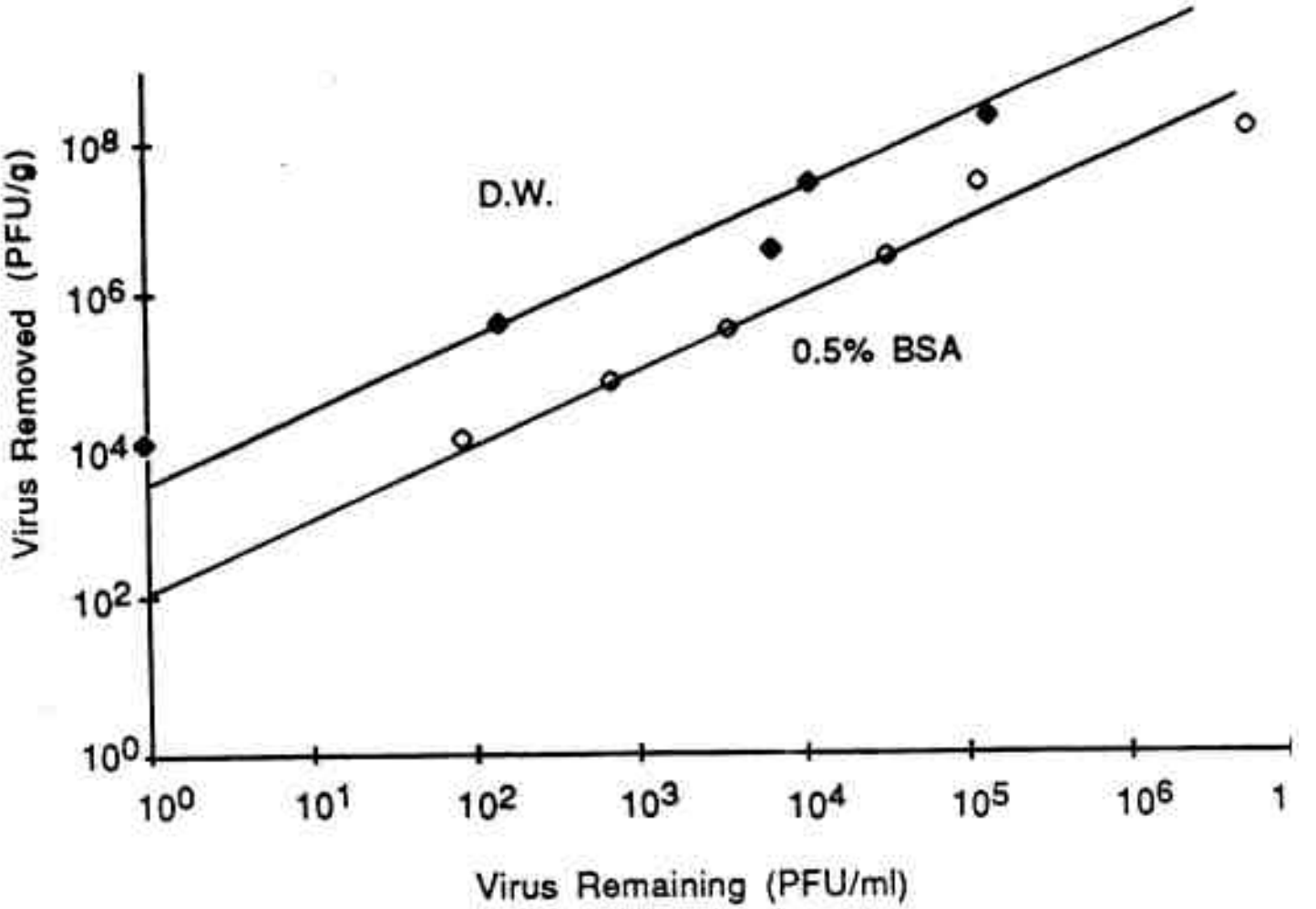
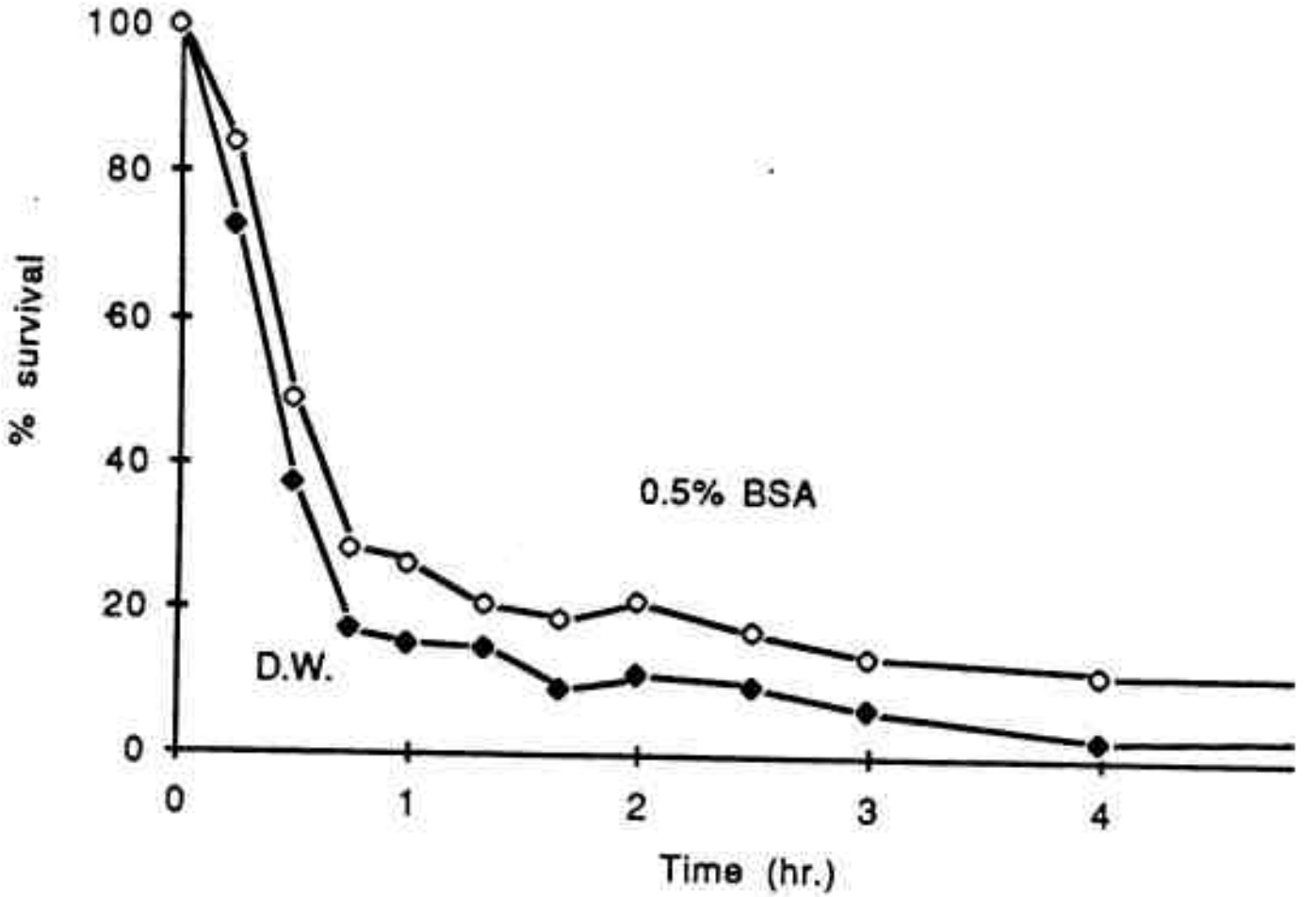
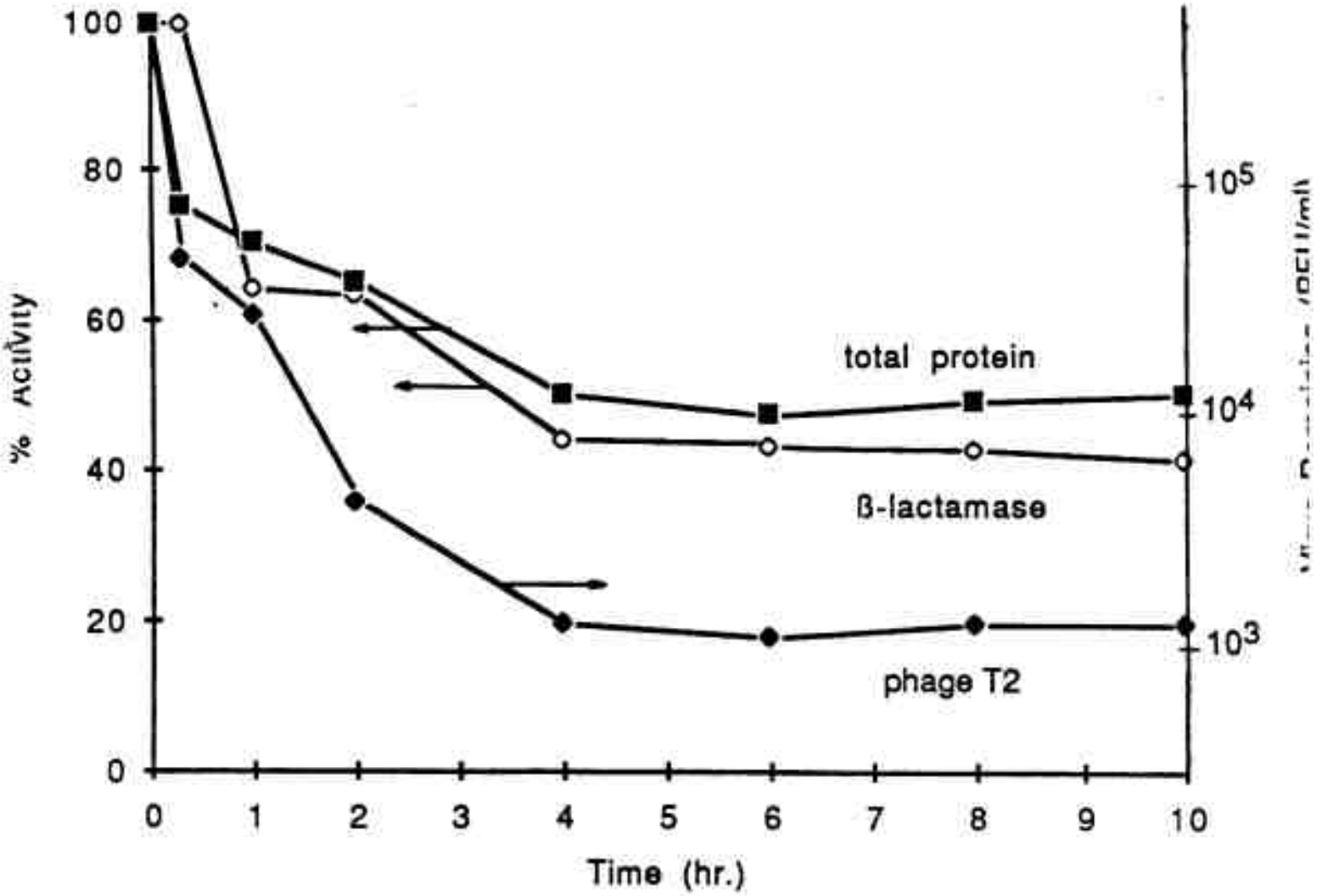


Figure 2.
Kinetic study of phage T2 removal using surface-bonded QAC



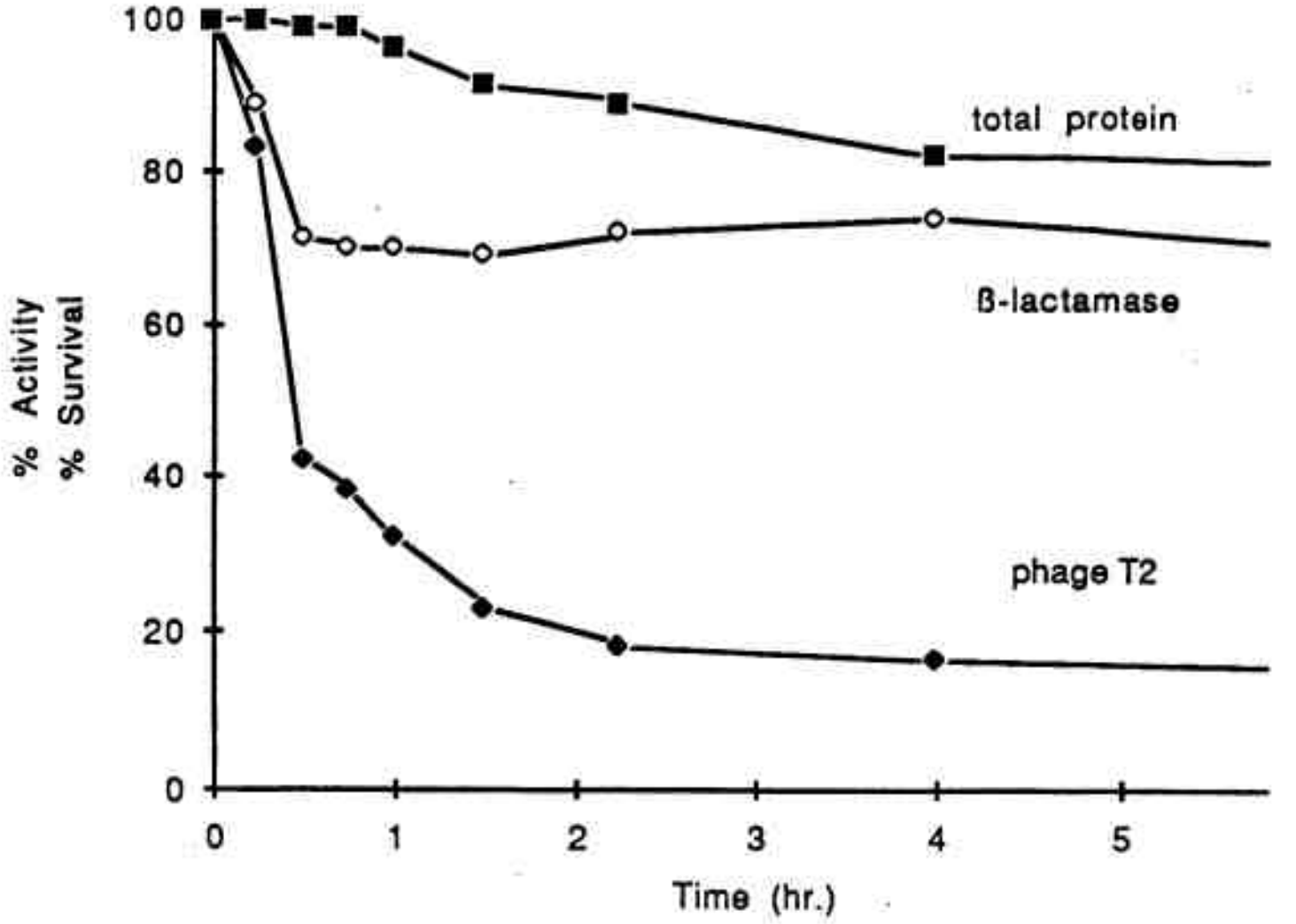
* initial T2 titer : 5.5×10^6 PFU/ml

Figure 3.
Thermal inactivation of phage T2 in ß-lactamase solution using sucrose as stabilizer



* initial T2 titer : 5.0 x 10^6 PFU/ml
total protein : 0.5 mg/ml
ß-lactamase : 885 IU/ml

Figure 4.
Removal of phage T2 from ß-lactamase solution using surface-bonded QAC



* initial T2 titer : 3.1 x 10^6 PFU/ml
total protein : 0.5 mg/ml
ß-lactamase : 885 IU/ml

Figure 5.
Simulated adsorption breakthrough curves of total protein and HSV-1.

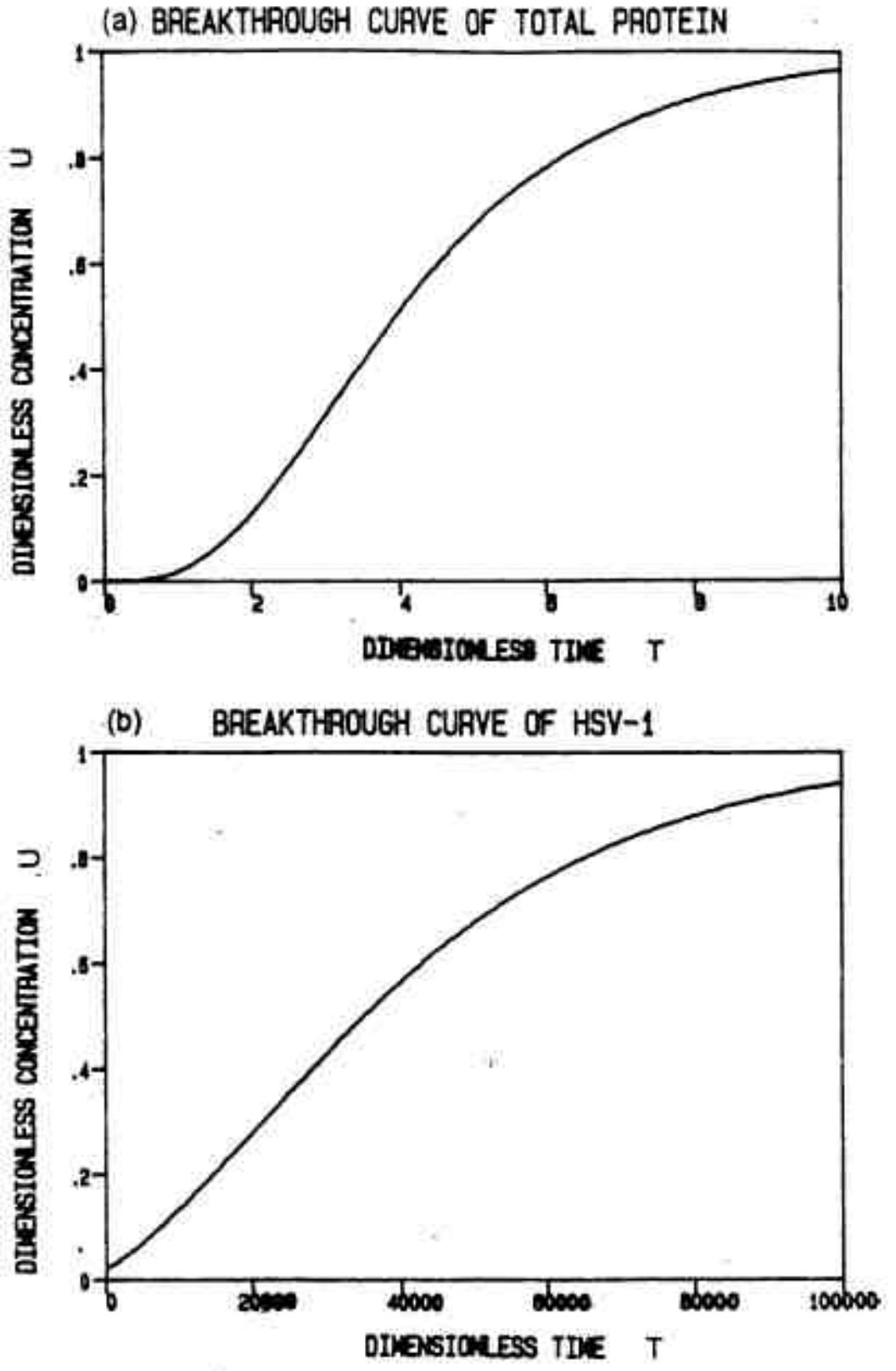


Figure 6.
The effect of sterilization criteria on the protein recovery and the required amount of adsorbent.