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Composite affinity sorbents and their cleaning in place

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ABSTRACT

Making large-scale affinity sorbents that are reusable under acceptable hygienic conditions implies specific treatments for cleaning in place with known aqueous solutions of chemical agents. However, common agents such as sodium hydroxide are frequently considered too drastic for the stability of macromolecular biologically active immobilized ligands. According to a large series of trials, it was found that only a mixture of sodium hydroxide and ethanol was actually effective in sterilizing a sorbent in a single step. When hydroxide or an ethanol–acetic acid mixture were used alone, they were not totally efficient in the inactivation of sporulated *Bacillus subtilis*. Conversely, they were efficient when used sequentially. All these solutions were able to remove pyrogens from chromatographic sorbents. As the sterilizing solutions contained a certain amount of ethanol, the most suitable chromatographic affinity sorbents had to be based on an incompressible matrix. When washing an affinity silica sorbent that had proteins as ligands with solutions such as sodium hydroxide, ethanol–acetic acid or ethanol–sodium hydroxide, it was found that certain sorbents were able to tolerate the treatments without a noticeable decrease in their biochemical activity.

INTRODUCTION

Scaling up affinity chromatographic systems implies repeated utilization of sorbents for the sake of economical exploitation of the technique. For the separation of therapeutic components, the sorbents must be washed periodically either to remove adsorbed undesirable substances (*e.g.*, pyrogens, pigments, lipids or protein aggregates) or for sterilization purposes. These treatments are well known in ion-exchange chromatography or in gel filtration but they still give problems in affinity chromatography, in particular when the ligand is not extremely stable. The proteins used as ligands (lectins, antibodies, etc.) may be denatured during the regeneration cycle and sterilization operations.

In this paper, we report preliminary results concerning the cleaning in place of composite silica–dextran supports bearing biologically active proteins. These supports, specially designed for preparative applications, are obtained by coating the

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available surface of porous silica with dextran molecules[1,2]. Once the complex has been stabilized by cross-linking, the affinity ligands are immobilized on the dextran part by classical methods[3-5]. We have found that these affinity sorbents are particularly resistant to acidic, alkaline or hydroorganic media. First, the silica is well proteced by the dextran layer against aggressive alkaline media. Second, the silica or the silica-based composite does not shrink in organic media, in contrast to clascical organic supports and particularly agarose-based supports.

During this study, we examined the depyrogenation and sterilization effect of three aqueous or hydroalcoholic solutions, and then studied the chromatographic behaviour of affinity supports bearing a proteic ligand in these solutions.

EXPERIMENTAL

Chemicals and biological materials

Silica porous microbeads were obtained from our production unit or purchased from Merck (Chelles, France) or Amicon (Epernon, France). Dextran cationic derivatives were obtained from Pfeiffer and Langen (Dormagen, F.R.G.), human IgG from Sigma (La Verpillère, France) and protein A from Fermentech (Edinburgh, U.K.). All other chemicals and biochemicals were purchased from Aldrich (Strasbourg, France) and Sigma.

Microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Escherichia coli* were obtained from Institut Mérieux (Lyon, France) and limulus test from Hemachem (St. Louis, MO, U.S.A.).

Coating of silica with dextran

Dry silica beads with a porous volume of 1 cm^3/g and a mean pore size of 1200 Å were coated with dextran according to a previously described method[1,2]. Dextran had a molecular weight between 70 and 500 kDa and possessed a small amount of cationic groups (about 0.5–1%).

Briefly, the coating was done as follows: 25 g of dextran derivative were dissolved in 150 ml of 0.2 *M* carbonate buffer (pH 10.5), then 5 ml of butanediol diglycidyl ether were added with stirring, followed by 100 g of dry silica beads (40–100 μm). The suspension was then filtered under vacuum to remove the excess of the dextran solution. The gel was placed in a ventilated oven and kept at 80°C for 24 h, and the dry product obtained was repeatedly washed with water and acidic and alkaline solutions and finally stored in the presence of 1 *M* sodium chloride. Dextran-coated silica was ready for use as an affinity sorbent after activation of the dextran hydroxyl groups and ligand immobilization.

Immobilization of a protein as a ligand: IgG, concanavalin A and protein A

Dextran-coated silica was first activated with *p*-nitrophenyl chloroformate according to Wilchek's method[5,6] and then the proteins were immobilized in the presence of 0.2 *M* carbonate buffer (pH 8–9). The protein concentration before immobilization was 3 mg/ml for protein A and 10 mg/ml for human IgG and concanavalin A. The amount of immobilized protein was 2.5 mg/ml of sorbent for protein A, 7.5 mg/ml for immunoglobulins G and 8 mg/ml for concanavalin A. The sorbents obtained were stored in aqueous suspensions in the presence of 1 *M* sodium chloride and 0.02% sodium azide as a bacteriostatic agent.

Treatments for cleaning in place (sterilization and pyrogen removal)

Three separate treatments were performed to check their efficiency against four standard strains: 0.1–1 *M* sodium hydroxide, 60% ethanol–0.5 *M* acetic acid and ethanol (up to 60%)–0.2 *M* sodium hydroxide.

The strains used were *Bacillus subtilis* (sporulated form) ATCC 6633, *Candida albicans* 562, *Escherichia coli* ATCC 10536 and *Staphylococcus aureus* ATCC 14154.

The washing efficiency of the various treatments was determined by mixing a volume of chromatographic sorbent with $6 \cdot 10^6$–$7 \cdot 10^6$ microorganisms/ml and then mixed with three volumes of sterilizing solution. After incubation (up to 24 h at 25°C), the amount of remaining microorganisms was detected by standard culture. Briefly, after sterilization the supernatant was neutralized and diluted 5-fold with phosphate-buffered saline (PBS). Solutions (0.5 ml) to be tested were serially diluted into BTS (tryptase-soya sterile broth in a ratio of 1:10) in triplicate per dilution. The tubes were incubated at 33°C for 14 days. Observations were effected every 24 h. The determination of the number of microorganisms per millilitre was calculated according to Spearman-Karber[10].

Experiments were also done on-column; contamination was induced by injecting into the column inlet the strain suspension (see above) and then the decontaminating solution was perfused from the top to the bottom of the column for 4 h. The column was neutralized by washing with five volumes of PBS and then the gel was treated as indicated above.

The solutions used for microorganism sterilization were also studied to determine their efficiency in pyrogen removal. These operations were effected on-column. To remove the pyrogens from contaminated sorbent, the column filled with the sorbent was washed with the same solutions as used for the microorganism decontamination and the amount of endotoxin (pyrogens) was determined at the column outlet using the standard limulus lysate test.

Stability studies on affinity sorbents

The decontaminating solutions used for sterilization and pyrogen removal were specifically studied in association with immobilized protein A, immunoglubulins G and concanavalin A on dextran-coated silica. The sorption capacity of the affinity sorbents was determined after repeated cleaning cycles alternated with separation cycles.

In the case of protein A–dextran-coated silica, the column was repeatedly used for the separation of human IgG. The sorption was effected in physiological buffer (PBS) and the elution using 1 *M* acetic acid. Between runs, four volumes of sterilizing solution were passed through the column at room temperature. The linear flow-rate was 15 cm/h and the time of contact with the cleaning solution was about 45 min per run.

The column of immobilized IgG was used for the separation of rabbit anti-human IgG antibodies. An excess of total rabbit antiserum was injected into the column previously equilibrated with PBS. After washing, the antibodies were desorbed using 0.2 *M* glycine–HCl buffer (pH 2.8). Between runs, four column volumes of sterilizing solution were passed through the column at room temperature for about 15 min (linear flow-rate 15 cm/h).

Concanavalin A–dextran-coated silica was used for the separation of egg

albumin in 0.05 *M* Tris–HCl buffer (pH 7.4) containing 0.15 *M* sodium chloride and 1 m*M* calcium and manganese chloride. The elution was performed by adding 0.3 *M* α-methyl mannoside to the adsorption buffer. Between runs, four volumes of sterilizing solution were passed through the column at room temperature for about 45 min (linear flow-rate 15 cm/h).

RESULTS AND DISCUSSION

Sorbent synthesis

Coating porous silica beads with dextran was necessary to eliminate the well known non-specific protein adsorption. Indeed, silica has a very acidic character and most proteins are strongly adsorbed and frequently denatured.

Complete coating with a polysaccharide avoided direct contact between the protein and the silica acidic groups; this is clearly evidenced by the progressive decrease in the adsorption of cationic proteins such as cytochrome *c* (see Fig. 1). An increase in the dextran content lowered to zero the interaction between cytochrome *c* and the silica gel. The dextran layer also contributes to making the silica more resistant to strongly alkaline media and, further, makes easy the activation of polysaccharide-based supports involving the clasical reagents used in affinity chromatography. Although a large amount of dextran is beneficial to the elimination of non-specific adsorptions, it also diminishes the support porosity. The value of V_e/V_t (ratio of the elution volume for bovine serum albumin to the total column volume) decreased when the amount of dextran was increased. This decrease is linked with progressive reduction of the pore

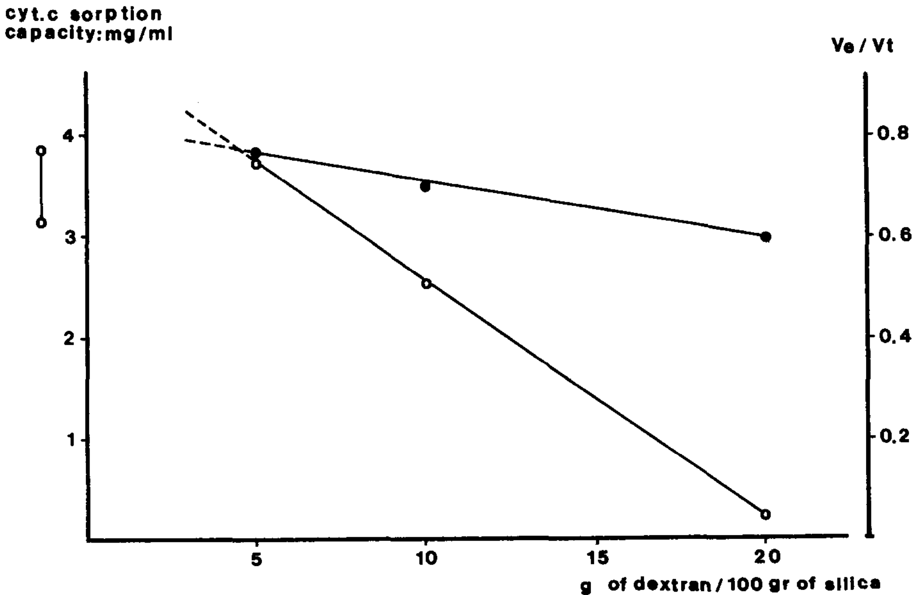

Fig. 1. Influence of the amount of dextran used for coating silica (○) on the adsorption of cytochrome *c* (non-specific adsorption) and (●) on the porosity (V_e/V_t) for bovine serum albumin. V_e = elution volume determined by gel chromatography in a physiological buffer; V_t = total volume of the column.

size inside the silica network. The degree of cross-linking (different amounts of cross-linking agent) did not significantly modify the behavior of the silica–dextran composite towards cytochrome *c* and bovine serum albumin.

The degree of activation of a silica–dextran composite (containing about 10 g of dextran for 100 g of silica) in the *p*-nitrophenyl chloroformate (*p*-NPC) method was directly proportional to the amount of the activating reagent added to the reaction mixture. The amount of *p*-NPC regularly used in our laboratory for activating these supports prior to protein immobilization is 25 mg/g of dry silica, which provides an active support containing about 20–30 μmol/ml of *p*-nitrophenol. Under these conditions, the amount of immobilized proteins (protein A, immunoglobulins G and concanavalin A) were 2.5, 7.5 and 8.0 mg per ml of support, respectively. Their sorption capacities towards human immunoglobulins G, anti-human IgG antibodies and egg albumin were 15–20, 2.5–3.5 and 3–4 mg/ml, respectively.

Sterilization and pyrogen removal

It was found that in the sodium hydroxide treatment of chromatographic supports previously contaminated by different microorganisms, the inactivation of the latter was dependent on the nature of the strains used. *Escherichia coli* was the most sensitive to alkaline media (Fig. 2), being totally inactivatad by sodium hydroxide at concentrations as low as 0.05 *M*. *Candida albicans* and *Staphylococcus aureus* were also sensitive to sodium hydroxide treatment but their total inactivation was observed only when the alkali concentration was 0.1 *M* or higher. However, sodium hydroxide at any concentration (and at temperatures of 20–25°C for 3 h) was not found to be very efficient in the inactivation of the sporulate form of *Bacillus subtilis* (Figs. 2 and 3), even when the sodium hydroxide treatment was extended to 24 h. In all instances, the

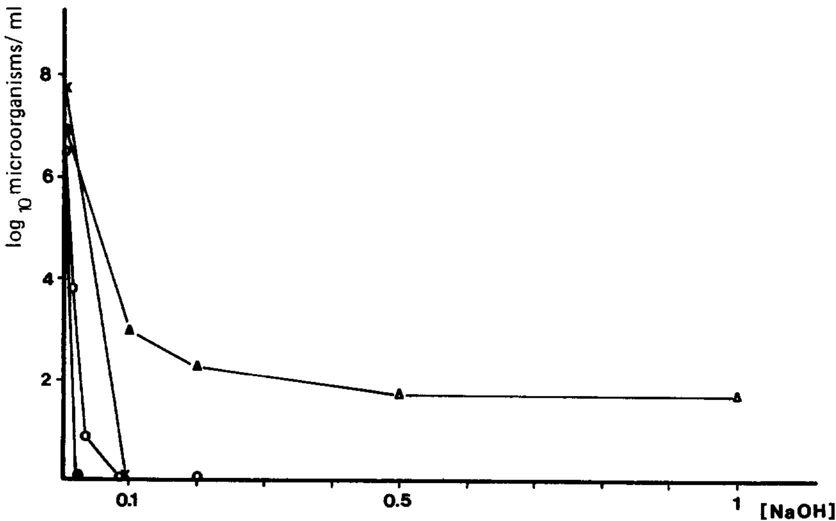

Fig. 2. Inactivation of different microorganisms using various concentrations (0–1 *M*) of sodium hydroxide. Experiments were effected at room temperature for a contact time of 3 h. ○ = *S. aureus*; ● = *E. coli*; × = *C. albicans*; ▲ = *B. subtilis*.

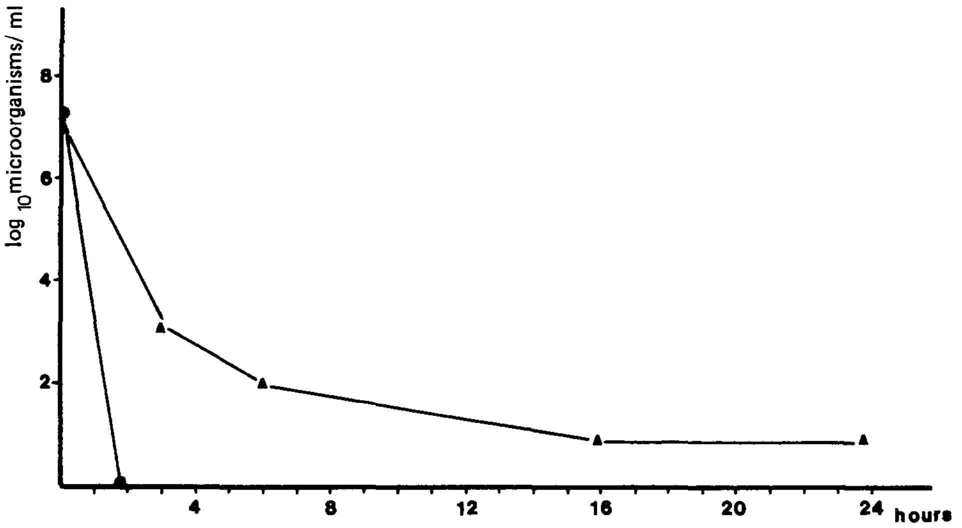

Fig. 3. Inactivation of different microorganisms by means of 0.2 *M* sodium hydroxide solution. Experiments were performed at room temperature. ▲ = *B. subtilis*; ● = *S. aureus*, *E. coli* and *C. albicans*.

diminution of the amount of *Bacillus subtilis* was about 5 logs, which means that in the studied case, from an initial concentration of about 10^7 microorganisms/ml, about 100 microorganisms/ml (on average) were still present at the end of the treatment.

The treatment of contaminated supports with an aqueous mixture of 60% ethanol and 0.5 *M* acetic acid elicited extreme sensitivity from all the strains studied, except *Bacillus subtilis*. Within 1 h of ethanol–acetic acid treatment, all *E. coli*, *C.*

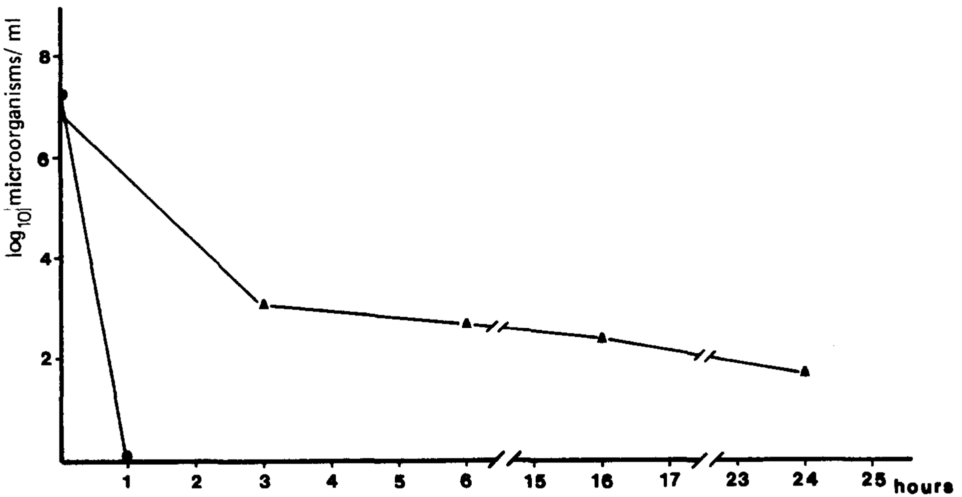

Fig. 4. Inactivation of different microorganisms by means of 60% ethanol–0.5 *M* acetic acid. Experiments were performed at room temperature. Symbols as in Fig. 3.

albicans and *S. aureus* microorganisms were inactivated (Fig. 4); the number of *B. subtilis* decreased greatly during the first hour of treatment (from 10 millions to 1000 germs) and then very slowly in the subsequent hours. After 24 h of treatment, a significant level of contamination (180 microorganisms/ml) still persisted. The strong stability of *B. subtilis* towards the treatment with sodium hydroxide and ethanol–acetic acid represents a fundamental problem in the sterilization of the chromatographic support.

These results are in accordance with those reported by Whitehouse and Clegg[7], who found a strong resistance of *B. subtilis* toward treatments with sodium hydroxide. It was demonstrated that at 22°C, 1 *M* sodium hydroxide decreased the initial amount of *B. subtilis* by about 4 logs in 12 h, whereas under the same conditions it took 25, 49 and about 70 h to obtain similar results with 0.6, 0.4 and 0.2 *M* sodium hydroxide, respectively.

On the basis of the above results, we tried to check the efficiency of two methods: (i) with 0.2 *M* sodium hydroxide treatment followed by treatment with ethanol–acetic acid and (ii) a unique treatment involving mixtures of ethanol (20, 40 and 60% concentration) and 0.2 *M* sodium hydroxide. Fig. 5 shows that alternate treatments for 1.5 h each with sodium hydroxide and acetic acid-containing ethanol totally eliminated *B. subtilis*. The results obtained with these experiments led us to consider a treatment with a solution composed of 0.2 *M* sodium hydroxide and ethanol at concentrations between 20 and 60% for 3 h. Under these conditions, the ethanol concentration played the most important role: when it was 50 and 60%, the inactivation of sporulated *B. subtilis* was complete. On decreasing this concentration to 20%, the efficiency of this mixture is sterilizing contaminated solutions was very good for *E. coli*, *C. albicans* and *S. aureus*, but *B. subtilis* was not totally destroyed (Fig. 6).

From the above-mentioned results, it can be ascertained that, if total elimination of microorganisms in a chromatographic support is to be achieved, it is necessary to

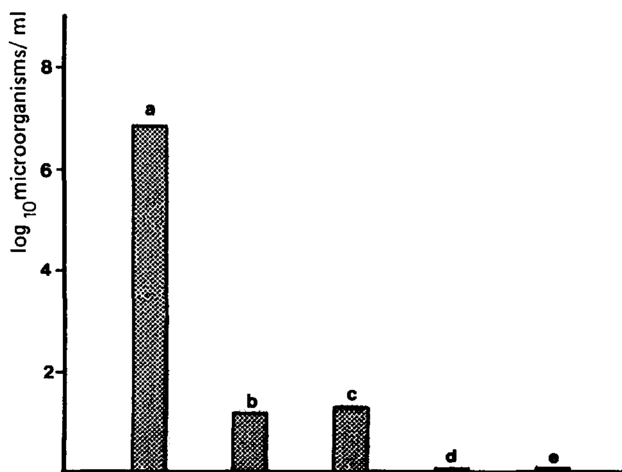

Fig. 5. Inactivation of sporulated *Bacillus subtilis*, (b) with 0.2 *M* sodium hydroxide, (c) with 60% ethanol–0.5 *M* acetic acid, and with successive inactivations (d) using solution b followed by the solution c or (e) using solution c followed by solution b. (a) Microorganism content before inactivation. All experiments were done at room temperature; the inactivation time was 3 h.

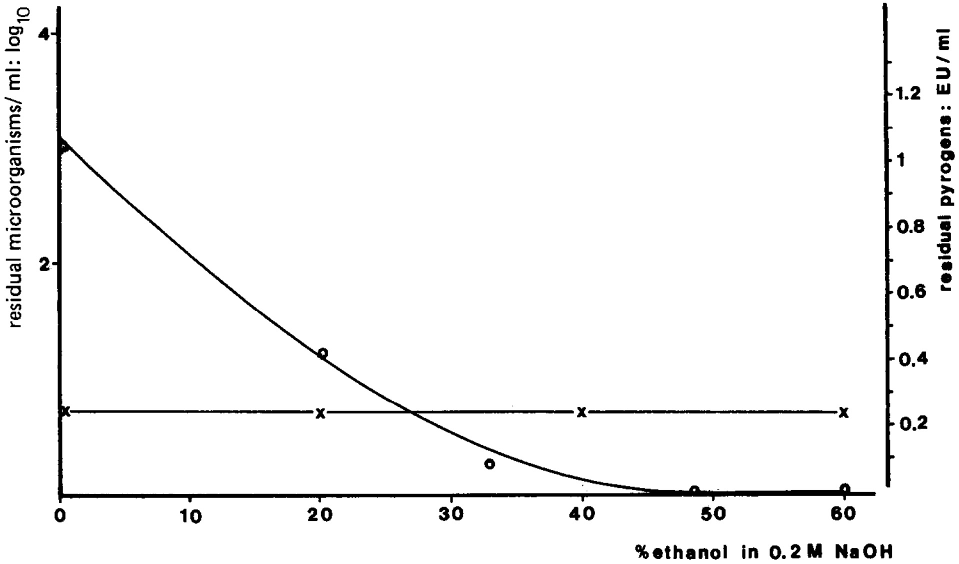

Fig. 6. Effect of solutions of 0.2 *M* sodium hydroxide containing different amounts of ethanol on sterilization (○) and pyrogen removal (×). The strain studied was *Bacillus subtilis* seeded at a concentration of $6.8 \cdot 10^6$ microorganisms/ml of sorbent. The time of contact with sterilizing solutions was 3 h for microorganism inactivation and about 30 min for pyrogen removal. The initial pyrogen content was about 100 EU/ml. All experiments were performed at room temperature.

resort either to alternate treatment with 0.2 *M* sodium hydroxide and ethanol–acetic acid or to a low-concentration alkaline treatment in the presence of ethanol. In this situation, two general problems could occur for affinity sorbents at the practical level: (i) with such treatments in the presence of ethanol, soft or semi-rigid gels may shrink to various degrees, thus making the operation on-column very difficult; and (ii) the stability of the biologically active ligand attached to the sorbents may be affected. To avoid at least the first problem, rigid sorbents (dextran-coated silica) were selected in order to support treatments with high concentrations of ethanol without shrinking and channelling.

Concerning the pyrogen removal problem, it is known that washing chromatographic supports with 0.1 or 0.2 *M* sodium hydroxide permits any pyrogens to be removed. The mechanism of pyrogen removal is based on good solubility of bacterial lipopolysaccharides in alkaline media and on the possible modification of their chemical structure[8], reducing the pyrogenicity level substantially. For completing our study on chromatographic support sterilization, the efficiency of pyrogen elimination by using these sterilizing solutions was checked.

As shown in Table I and Fig. 6, effectively all the washing solutions tested eliminated pyrogen completely. Whatever the initial degree of contamination was, the pyrogen content, after 2–3 column volumes of washing solution, fell below the level of acceptability or limit of the detection by the limulus test [0.12 Endotoxin unit (EU)/ml].

We conclude that the proposed treatments for sterilizing supports also permit pyrogens to be eliminated completely.

TABLE I

PYROGEN REMOVAL FROM A DEXTRAN–SILICA SORBENT WITH DIFFERENT SOLUTIONS

Solution	*Initial pyrogen content (EU/ml)*	*Final pyrogen content (EU/ml)*
0.2 *M* NaOH	100	<0.12
Ethanol–acetic acid[a]	600	<0.12
Ethanol–NaOH (a)[b]	100	<0.24
Ethanol–NaOH (b)[c]	100	<0.24
Ethanol–NaOH (c)[d]	100	<0.12

[a] 60% Ethanol–0.5 *M* acetic acid (final concentrations).
[b] 60% Ethanol–0.2 *M* sodium hydroxide (final concentrations).
[c] 40% Ethanol–0.2 *M* sodium hydroxide (final concentrations).
[d] 20% Ethanol–0.2 *M* sodium hydroxide (final concentrations).

Study of affinity support stability in sterilizing media

Protein A immobilized on the dextran-coated silica was resistant to repeated treatment with the 60% ethanol–0.5 *M* acetic acid mixture; its chromatographic behaviour remained almost unchanged and its sorption capacity for human IgG and the chromatographic pattern did not vary significantly (Table II). Alkali treatment without ethanol moderately affected its sorption capacity for human IgG. There was on average a 12% decrease in its sorption capacity in the two first treatments and then the capacity remained constant.

The treatment with ethanol–sodium hydroxide mixture was also well supported by immobilized protein A with an overall decrease in the sorption capacity of about 7%. Hence this biospecific sorbent showed a remarkable stability which is probably related with the intrinsic properties of protein A.

When immobilized concanavalin A was treated with ethanol–acetic acid, a 25% decrease in the sorption capacity was observed. Treatment with 0.2 *M* sodium hydroxide solution denatured concanavalin A very quickly: after four cycles, the sorption capacity for ovalbumin had decreased to zero. The treatment of immobilized concanavalin A with ethanol–sodium hydroxide resulted in total disruption of the sorbent binding capacity. This was probably due both to the higher sensitivity of this protein in comparison with protein A and to the impossibility of stabilizing it by addition of divalent ions. It should be noted in fact that concanavalin A is tetrameric whereas protein A is a single chain.

Immobilized immunoglobulins G did not seem to lose their capacity to interact with specific antibodies after repeated treatment with ethanol–acetic acid. This may be explain by the stability of these macromolecules in acidic medium (elution in affinity chromatography is often realized at pH between 2.5 and 3) and by their stability in ethanol. It should be recalled that ethanol is classically used for purifying IgG from human plasma according to the Cohn's *et al.* method[9].

In strongly alkaline medium, the denaturation of immobilized human IgG was also very limited; the support sorption capacity diminished by about 2% after the first treatment in 0.2 *M* sodium hydroxide solution and then remained constant. However,

TABLE II

EFFECT OF STERILIZING SOLUTIONS ON THE SORPTION CAPACITY OF AFFINITY SORBENTS

Solution	*Cycle No.*	*Sorbent capacity (mg affinant/ml sorbent)*		
		Immobilized protein A[a]	*Immobilized h-IgG*[b]	*Immobilized Con A*[c]
0.2 *M* Sodium hydroxide	0	21.15	3.47	3.30
	1	14.20	3.43	1.47
	2	18.77	3.68	0.71
	3	18.66	3.70	0.58
	4	18.98	3.80	0.5
	5	19.45	3.70	0.0
60% Ethanol–0.5 *M* acetic acid	0	18.36	3.48	3.34
	1	18.79	3.23	2.50
	2	17.87	3.34	2.69
	3	18.44	3.40	2.70
	4	16.90	3.60	2.23
	5	17.17	3.35	2.48
60% Ethanol–0.2 *M* sodium hydroxide	0	20.05	4.11	2.51
	1	19.85	3.90	0.48
	2	19.75	3.30	0.0
	3	20.05	3.20	0.0
	4	19.05	3.30	0.0
	5	19.06	3.50	0.0

[a] Specific sorption determined using human IgG.
[b] Specific sorption determined using rabbit anti-human IgG antibodies.
[c] Specific sorption determined using egg albumin.

after 24 h of contact in the same solution, the sorption capacity for these antibodies diminished by about 25%.

The properties of immobilized immunoglobulins after repeated cycles with 60% ethanol–0.2 *M* sodium hydroxide were almost unchanged. The diminution of the antigen–antibody association was only about 15% after five treatments but it was about 70% after a 24-h incubation.

The results obtained with immobilized immunoglobulins G do not necessarily mean that all the imombilized antibodies, independent of their species, class or specificity, remain stable towards these treatments. In our experimental model, immobilized IgGs were used as antigens. The solution to the problem of the stability of immobilized antibodies, and particularly monoclonal antibodies (mAbs), is still to be studied in detail. It seems likely, however, that the monoclonal antibody stability cannot be generalized for all classes and species or specificities.

In preliminary work, it was found in fact that an immobilized rat mAb specific for mouse IgG_1 was partially damaged by the 60% ethanol–0.5 *M* acetic acid mixture.

The behaviour of the affinity support towards the studied sterilizing solutions seemed dependent on the nature of the protein. The ethanol–acetic acid mixture seemed to be the least denaturing treatment. The sorption capacity of protein A for human IgG diminished slightly; the capacity of immobilized IgG to adsorb specific antibodies and the concanavalin A binding to ovalbumin decreased by less than 20%

after repeated treatments. The latter adsorbent is doubtless the most sensitive but its sensitivity may be decreased considerably when glucose is added to the sterilizing solution at a concentration of 0.3–0.5 *M*. Preliminary tests have actually shown that the sorption capacity of concanavalin A was partially protected when 0.3 *M* glucose or 0.3 *M* α-methyl mannoside was added to the sterilizing mixtures (data not shown).

CONCLUSION

The problem of support sterilization in affinity chromatography when ligands are biologically active is a vast topic which is just being explored. This type of sorbent is, as explained by its nature, particularly sensitive to contamination and easily inactivated. In this situation, a decontamination procedure must be efficient, rapid and non-denaturing for the ligand. Such decontamination treatments are necessary when these sorbents are used to purify products for diagnostic or therapeutic purposes and when, for economic reasons, these expensive supports must last for a long time.

The results obtained showed that all the sterilizing treatments devised for chromatographic supports are not totally efficient in any instance. Only alternate treatments with sodium hydroxide and ethanol–acetic acid mixture and alkali treatments in the presence of ethanol showed total efficiency. These two possibilities are applicable only on rigid supports, such as those based on a silica matrix, which are being increasingly used in preparative and industrial applications. However, sterilization methods should be set up according to the stability of the immobilized macromolecule while bearing in mind that *a priori* a single treatment with ethanol–sodium hydroxide is the simplest, the most practical and often the most efficient.

The study reported here represents a novel and almost unexplored approach to the sterilization of supports bearing biologically active macromolecules. The extension of these sterilization methods to other microorganisms is doubtless desirable; the search for the best compromise between the concentration of the sterilizing agent and the denaturation of macromolecular ligands should also be investigated in detail case by case.

Finally, another field to be investigated is the protection of macromolecular ligands against denaturation during sterilization. When we consider the encouraging results obtained on the protection of concanavalin A in the presence of glucose, this line of research may be very promising. Further studies are being carried out in our laboratories and will be reported elsewhere.

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