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Effect of Hydroxypropyl-/J-cyclodextrin on the Antimicrobial Action of Preservatives

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Abstract The interaction between hydroxypropyl-/j-cyclodextrin (HP-/j-CyD) and several preservatives with different chemical structures was investigated in aqueous solution. Complex stability constants of the I: I complexes were calculated from differential spectra. Using the serial dilution test the antimicrobial activities of the preservatives and their complexes against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* were tested and MIC values determined. For highly water-soluble substances like thimerosal and bronopol, low or no inactivation was found; the more lipophilic substances, such as the phenolic compounds, showed strong inactivation when used in combination with HP-/j-CyD. The loss in activity by complex formation correlated with the bound fraction, thus suggesting that the appropriate antimicrobial substance for the preservation of cyclodextrin solutions can be selected according to the results of this study.

Cyclodextrins, cyclic molecules of six to eight glucose units, are in widespread use in pharmaceutical fields because of their ability to form inclusion complexes. In particular, the hydroxypropyl derivatives with nearly unlimited solubility in water and low toxicity are used for increasing the solubility and stability of drug molecules (Albers & Millier 1992). Furthermore, bioavailability can be enhanced by drug complexation (Uekama & Otagiri 1987).

Aqueous solutions of cyclodextrins have to be preserved to prevent contamination with microorganisms, particularly if they are used in multi-dose applications.

Loftsson et al (I 992) investigated the interactions between four preservatives and hydroxypropyl-/j-cyclodextrin **(HP-**P-CyD). They observed a reduction of the antimicrobial activity against microorganisms in the presence of HP-P-CyD. Uekama et al (1980) found an increase of minimum inhibitory concentration (MIC) of p-hydroxybenzoic acid esters if they are complexed with et- and P-cyclodextrin. Similar results were confirmed by us (Lehner et al 1993) for HP-/j-CyD complexes of p-hydroxybenzoic acid esters.

In this study, a screening for preservatives with different chemical structures is reported with the aim of finding the most suitable compound for preserving HP-/j-CyD containing solutions. Furthermore, the variations in antimicrobial activity are described by physicochemical parameters as shown for the homologue system of p-hydroxybenzoates.

Materials and Methods

Materials

Hydroxypropyl-/j-cyclodextrin (average degree of substitution 0·49) was obtained from Janssen Pharmaceutics (Beerse, Belgium). p-Chloro-m-cresol, thimerosal and phenylmercury acetate were obtained from Fluka (Buchs, Switzerland), benzyl alcohol from Merck (Darmstadt, Germany). Propylhydroxybenzoate and 2-phenoxyethanol were a gift from Nipa Laboratorien (Norderstedt, Germany), chlorhexidine diacetate was a gift from ICI Pharma (Plankstadt, Germany) and bronopol was purchased from Boots (Nottingham, UK). All substances were used without further purification.

Casein-peptone soymeal-peptone (CASO) agar, CASO broth and thioglycollate broth were purchased from Merck. The solvents were of HPLC grade, all other chemicals were commercially available products of reagent grade.

Staphylococcus aureus (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 10231) were used for the microbiological assays.

HPLC analyses

Quantitative determinations of the preservatives were carried out using a high-performance liquid chromatographic system consisting of a High Precision Pump Model 300 (Gynkotek, Munich, Germany), an Automatic Sampler 360 (Kontron Instruments, Zurich, Switzerland), a Hypersil ODS 5 μm column, a Hypersil SAS 5 μm column (4·6 x 250 mm) (Shandon, Runcorn, UK) and a UV spectrophotometric detector SPD-6A Module (Shimadzu, Kyoto, Japan). Detailed HPLC conditions for analysing the preservatives are given in Table I.

Solubility studies

Solubility measurements were carried out according to the method of Higuchi & Connors (1965). An excess amount of preservative was added to 10 mL phosphate buffer solution pH 7·4 containing 0-17% (w/w) hydroxypropyl-P-cyclodextrin (except for chlorhexidine diacetate where all determinations were made in distilled water because of interactions between chlorhexidine and phosphates). After shaking at 25 °C until equilibration, the suspensions were filtered through a 0·2 μm cellulose acetate membrane filter (Sartorius, Gottingen, Germany). The content of preservative in the diluted samples was analysed by HPLC.

UV measurements

UV differential spectra were performed with an Uvikon 930

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Table I. Conditions for quantitative determinations of different preservatives by HPLC.

Preservative	Mobile phase	Stationary phase	Flow (mL min-1)	Wavelength (nm)
Thimerosal	Acetonitrile--PIC A[1] buffer (34:66), pH 7-4 with phosphoric acid[2]	**RP-18**	2·0	254
p-Chloro-m-cresol	Acetonitrile-water-tetrahydrofuran (38: 57: 5)	**RP-18**	2·0	282
Phenylmercury acetate[3]	Acetonitrile-methanol-0·005 M KH_2PO_4 in water (175: 75: 300) 5 mg L - I mercaptopurine	**RP-18**	2·0	293
Benzyl alcohol	Acetonitrile-water-tetrahydrofuran (12: 83: 5)	**RP-18**	2·0	256
Propyl p-hydroxybenzoate	Acetonitrile-water (40: 60)	RP-18	2·0	256
2-Phenoxyethanol	Acetonitrile-water (24: 76)	**RP-18**	2·0	272
Chlorhexidine diacetate	Acetonitrile-methanol-tetrahydrofuran-water (I7·5:45: 10: 32·5), 0·0035 MTBA[4], pH 3·5 with acetic acid	RP-2,4,6	2·0	260
Bronopol	Acetonitrile-0·005M TBA[4] in water (5:95)	**RP-18**	2·0	240

[1] Millipore, Eschborn, Germany, [2] Thoma & Schubert (1987), [3]Parkin (1986), [4]tetrabutylammoniumhydrogensulphate Fluka, Buchs, Switzerland.

spectrophotometer (Kontron Instruments, Zurich, Switzerland). For measurements, two double cuvettes were used. The front compartment of the reference cuvette contained HP-J3-CyD and the rear compartment, the preservative, both in buffered solution. In the sample cuvette the preservative and HP-J3-CyD were combined in the rear compartment, the front compartment was filled up with buffer solution. The UV spectra were recorded in the range of 200-400 nm.

Microbiological assay

Stock solutions of the preservatives were prepared with the addition of HP-J3-CyD in equimolar concentrations to the preservative; identical concentrations of preservatives without cyclodextrin were prepared as blanks. A mixture of CASO broth in phosphate buffer pH 7·4 (1: 10) was used as solvent (exception: chlorhexidine acetate in CASO broth/water I : 10). To get sterile samples all solutions were filtered through a 0·2 *μm* cellulose acetate filter. In the filtrated solutions the content of active substances was determined by HPLC.

The stock solutions were diluted with the same solvent in steps of I0% maximum. Only the very potent substances like thimerosal were diluted in concentration steps up to 50%.

The following microorganisms were used in the test: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans*. After cultivation in **CASO** broth at 37 °C for 24 h (C. *albicans* 72 h), the suspension was diluted with sterile saline (0·9% NaCl) to result in 10^6 organisms mL - 1 (C. *albicans* 10^4 mL - 1). Aliquots (0· 1 mL) of these inocula were transferred into each 10·0 mL sample. The test was carried out with duplicate samples.

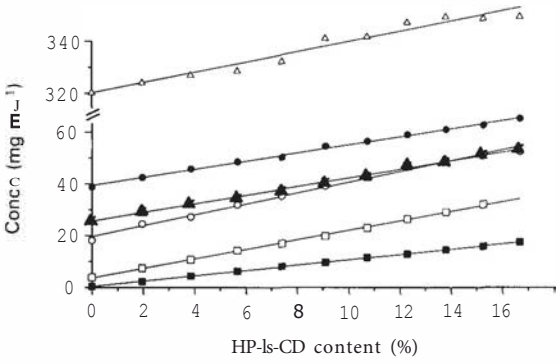
After an incubation time of 72 h at 37 °C the test solutions were diluted with inactivation liquid (CASO broth with I% Tween 80 and 0·3% lecithin; thioglycollate broth with 0· 1% cystin for all mercury-containing preservatives and bronopol) to neutralize the antimicrobial activity of the preservatives. Samples of the diluted test solutions were transferred with a spiral plater (Meintrup-Labortechnik, Liihden, Germany) on CASO agar plates. After incubation the number of colony forming units (cfu) were counted.

Results and Discussion

Solubility studies

Fig. I shows the solubilities of six preservatives and the solubility enhancing effect of HP-J3-CyD in phosphate buffer pH 7·4. According to the classification of Higuchi & Connors (I 965) all solubility isotherms are of linear type (aL type). The correlation coefficients rare estimated to be greater than 0·98 (n = I0). The linear relationship between increase in solubility of the guest molecule and cyclodextrin concentration is well known for the hydroxypropyl derivatives and suggests a complex formation of the added preservative (Millier & Brauns 1985). On the other hand, in the group of mercury salts tested, thimerosal and phenylmercury acetate, no solution isotherm can be determined. The solubility of thimerosal is much too high to reach saturation. The surface of phenylmercury acetate is very lipophilic; the poor wettability may be the reason for its behaviour which results in very confusing solubility data (the experiment was stopped after an equilibration time of 30 days).

Fig. I shows that the solubilities of the measured substances without HP-J3-CyD vary from 0·35 mg mL - 1 (propyl p-hydroxybenzoate) to 320 mg mL - 1 (bronopol) whereas the slopes of the lines are almost parallel. According to these



Fm. I. Effect of HP-/J-CD on solubility of preservatives in phosphate buffer pH 7-4. ▲ bronopol, • benzylalcohol, • 2-phenoxyethanol, o chlorhexidine diacetate, D p-Cl-m-cresol, ▪ p-hydroxybenzoate.

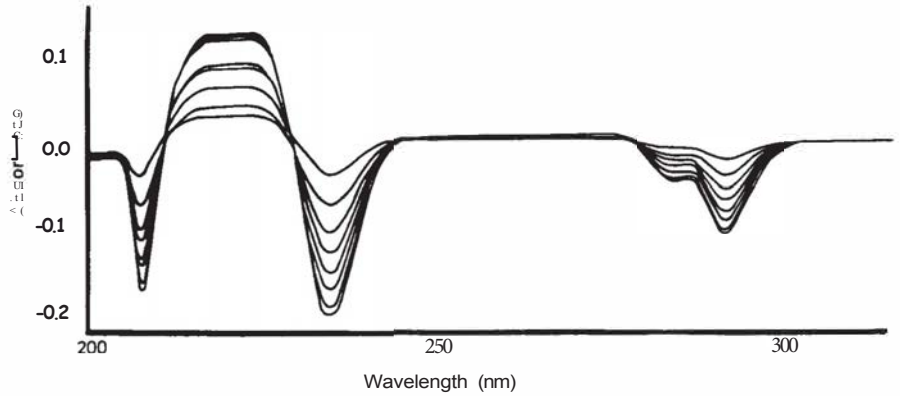


FIG. 2. UV differential spectra of complexes between p-Cl-m-cresol (0·25 mM) and HP-P-CyD (0·3-5·0 mM) in phosphate buffer pH 7·4.

findings the percentage increase in solubility will be higher for substances with lower ground solubility or with higher lipophilicity. Similar results were recorded in the homologue series of p-hydroxybenzoic acid esters {Lehner et al 1993).

The quantitative evaluation of complex stability constants from solubility isotherms has not been carried out because in the case of the two mercury-containing substances no isotherms could be obtained.

UV measurements

The method of Scheraga described by Seydel & Schaper (1979) is proposed for quantitative determination of interactions between active substances and complexing agents by measuring their differential spectra. As an example of this the differential UV spectra of 0·25 mmol L^{-1} p-Cl-rn-cresol in the presence of 0·3-5·0 mmol L_, HP-P-CyD (Fig. 2) are presented. From the plot of the measured absorption differences at 3 different wavelengths against the HP-P-CyD concentration (Fig. 3), the complex constants $K_{1:1} = 1/Kd$ can be ascertained. Calculations were made with the following equation derived from the law of mass action:

$$Li = \frac{Li_{max}}{2} \cdot \frac{(Kd + [P] + [HP\text{-}P\text{-}CyD])}{[P]} - \sqrt{\frac{Li_{max}}{4 \cdot [P]^2} \cdot (Kd + [P] + [HP\text{-}P\text{-}CyD])^2 - \frac{Li_{max}[HP\text{-}p\text{-}CyD]}{[P]}}$$

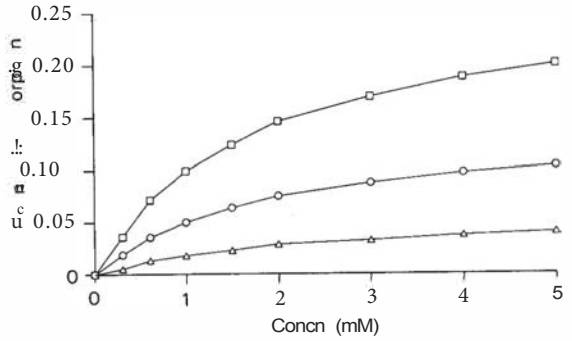


FIG. 3. Absorption shift of p-Cl-m-cresol (0·25 mM) in the presence of HP-f/-CyD (0·3-5·0 mM), measured at 234 , 286 A, 292 o nm.

Table 2. Complex stability constants of complexes between preservatives and HP-f/-CyD in phosphate buffer pH 7·4 at 25 °C.

Preservative	Kd±s.d. (mmol L-1)	Kl,1 (L moI- 1)
Thimerosal	78·74+5·38	12·7
p-Cl-m-Cresol	1·54+0·04	652
Phenylmercury acetate	18·88±2·15	53
Benzyl alcohol	4·66+0·47	215
Propyl p-hydroxybenzoate	0·52+0·02	1916
2-Phenoxyethanol	10·85+0·20	99·5
Chlorhexidine diacetate	2·51+0·16	399
Bronopol	11·57±0·93	86·4

where Li is the difference in absorption and Li_{max} the calculated maximum of absorption at the point of a fully saturated complex. [P] and [HP-P-CyD] are the total concentration of preservative and HP-P-CyD respectively. The Kd and Li_{max} values have been calculated with a computer program which is based on the Marquardt algorithm (Canela 1984). The K_{11} values for the complexes of all preservatives used in this study are calculated in this way. They are listed in Table 2. The unbound fraction of preservative at the concentration of minimum inhibition is calculated from Kd values.

Microbiological studies

Preceding tests have shown that there is no influence of HP-*p-CyD* alone (up to concentrations of 5%) on the growth of the tested microorganisms. But at concentrations of 10% HP-p-CyD the growth of *Ps. aeruginosa* and *E.coli* is clearly inhibited. In contrast to these results, Loftsson et al (1992) found no damage of bacteria below concentrations of 20% HP-P-CyD. They applied a higher number of bacteria (10^5 cfu mL^{-1}) and their experiments were performed with a short incubation time of 24 h. These variations in test design are probably responsible for the different results seen for bacterial growth.

Fig. 4 shows the MIC values of the preservatives and their 1:1 complexes calculated for the four microorganisms. lnocula with low numbers of microorganisms were used (10^4 cfu mL-1 of test solution for bacteria and 10^2 cfu mL-1 for yeasts) in contrast to other methods (preservation efficiency

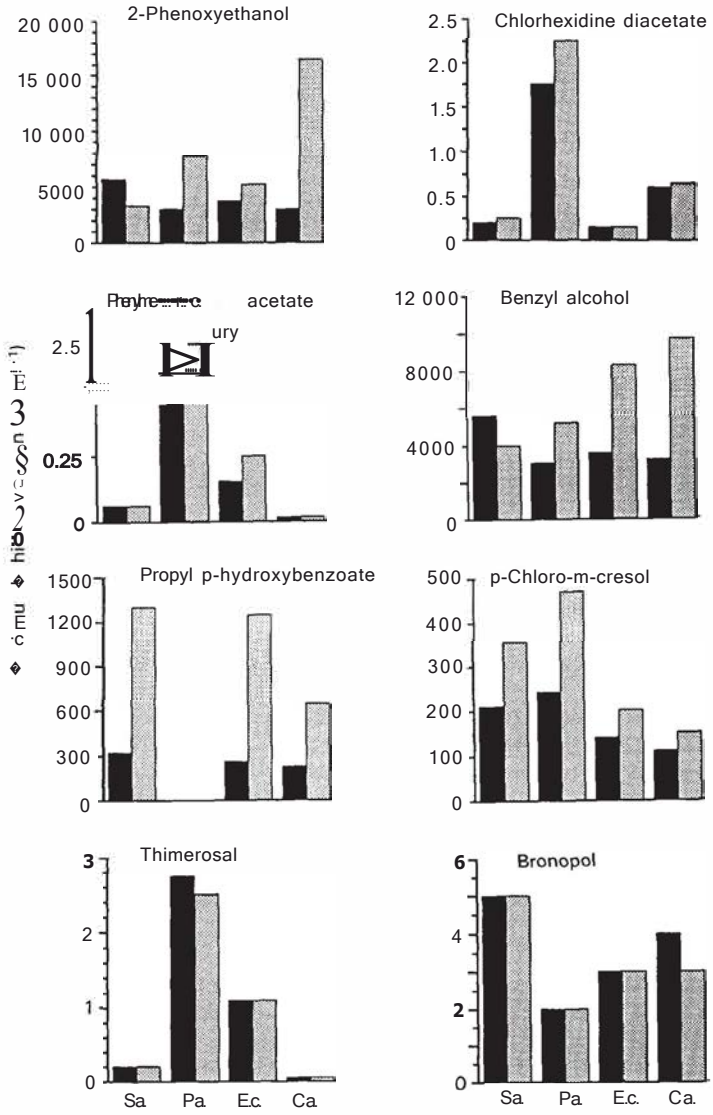


FIG. 4. Minimum inhibitory concentrations of preservatives and their HP-P-CyD-complexes (1:1 mol) against microorganisms after 72 h (S.a. ***St. aureus;*** P a. ***Ps. aeruginosa;*** E.c. ***E. coli;*** C a. C. ***albicans;*** without HP-P-CyD ; preservative with HP-P-CyD).

testing in US Pharmacopeia **XXII,** British Pharmacopoeia 1988, Deutsches Arzneibuch 1992; Horneff et al 1981). The contamination of preserved systems with low numbers of microorganisms is more realistic than an input of 10^5- 10^8 cells mL^{-1} of test solution; furthermore in this study only the relative differences between cyclodextrin containing systems and cyclodextrin-free systems were of interest. Since the MIC values of 10^4 cfu mL^{-1} bacteria are found to be close to the saturation concentration of propyl p-hydroxybenzoate the **MIC** of C. *albicans* is expected to exceed the point of saturation. According to WallhiiuBer (1988) yeasts are much more resistant against p-hydroxybenzoates than bacteria, therefore 10^2 cfu mL^{-1} were used for this test organism. Under these conditions it is possible to determine the **MIC** value of propyl p-hydroxybenzoate for *C. albicans.* For *Ps. aeruginosa* and propyl p-hydroxybenzoate, no **MIC** value could be determined; in the saturated solution of the ester no inhibition of microbial growth occurred. The high resistance of *Ps. aeruginosa* can be explained by its ability to degrade the ester. This has already been described by Beveridge & Hart (1970) for some strains of *Pseudomonas.*

From Fig. 4 it is obvious that the effect of HP-/1-CyD on the antimicrobial activity of preservatives with lower complex stability constants is negligible (except chlorhexidine diacetate) whereas the stronger bound substances, e.g. propyl p-hydroxybenzoate, show considerably increasing **MIC** values. Highly active, hydrophilic substances like thimerosal and bronopol show only slight interactions with HP-/j-CyD. These results are in good agreement with the experiments of Loftsson et al (1992) who published similar

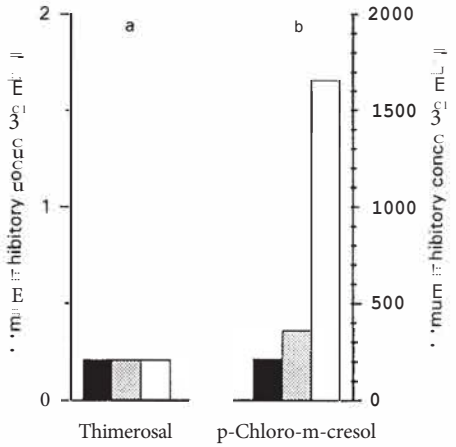


FIG. 5. Minimum inhibitory concentrations of (a) thimerosal and (b) p-Cl-m-cresol without (▪) and after addition of HP-,8-CyD complexes (▨ I: I mol; D 4·5%) tested against ***St. aureus.***

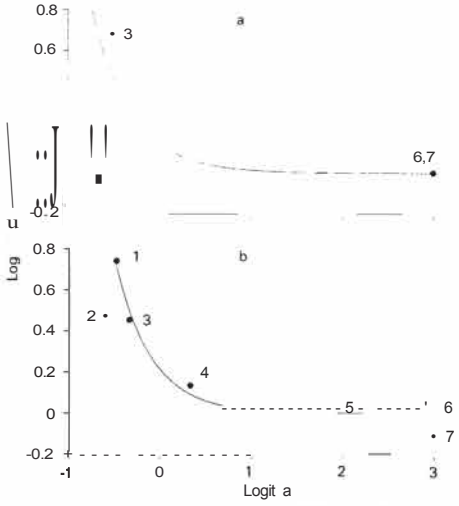


FIG. 6. Correlation between dissociation ofHP-,8-CyD-preservative-complexes (I: I mol) and decrease of the antimicrobial activity against (a) £. ***coli*** and (b) C. ***albicans.*** (I: phenoxyethanol; 2: benzyl alcohol; 3: propyl p-hydroxybenzoate; 4: p-Cl-m-cresol; 5: chlorhexidine diacetate; 6: thimerosal; 7: bronopol).

results for complexed chlorobutanol and benzalkonium chloride.

In the determinations of MIC values, an irregularity for *St. aureus* is observed: the HP-p-CyD complexes of 2-phenoxyethanol and benzyl alcohol are more active than the uncomplexed substances (Fig. 4). The difference cannot be explained by variations in the biological test system, but because *St. aureus* is the only Gram-positive bacterium in this series, it may be possible that this effect is due to specific properties of the cell wall.

To support this hypothesis, the experiment was repeated with a second Gram-positive bacterium, *Micrococcus luteus.* The antimicrobial testing shows the MIC of 2-phenoxyethanol at 5·25 mg mL-1 (uncomplexed) and at 1·30 mg mL-1 for the complex (inoculum with 10^4 cfu mL-1 of *Micrococcus).* These results confirm the assumption of an interaction between components of the Gram-positive cell wall and the cyclodextrin complex.

The experiments for determination of the MIC were performed with an equimolar ratio of HP-P-CyD and preservative to obtain good comparisons among substances with large differences in their biological activities. This means that a very low HP-P-CyD concentration, about COI%, is applied for potent preservatives and concentrations up to 15% for poorly active preservatives.

However, for drug formulations it is very important to know the effect of higher concentrations of HP-P-CyD on preservative inactivation. The results of testing thimerosal and p-Cl-m-cresol in the presence of 4·5% HP-P-CyD against *St. aureus* are presented in Fig. 5. Thimerosal is not inhibited by HP-P-CyD; its action is independent of the cyclodextrin concentration. In contrast to this p-Cl-m-cresol shows an exponential increase of inactivation with rising cyclodextrin concentration. From these results it is evident that more lipophilic substances are not suitable for antimicrobial stabilization of more highly concentrated cyclodextrin solutions.

Structure-activity relationships

For the series of p-hydroxybenzoic acid esters the relationship between inactivation by complexation and physicochemical parameters such as $K_{1,1}$, log P or grade of dissociation, IX, have been demonstrated (Lehner et al 1993). One of the aims of the present study was to investigate if these relationships were also valid for other preservatives.

From estimated $K_{1,1}$ values, the degree of complex dissociation has been calculated. According to the data of ***p-***hydroxybenzoic acid ester, only the free fraction is regarded as being active against the microorganisms. Fig. 6 shows the relationship between IXand inactivation of the tested preservatives calculated for £. *coli* and ***C. albicans.*** The inactivation is expressed by the ratio between MIC values of complexed and of uncomplexed preservative. To normalize data distribution, the values of IXwere logit transformed (logit IX=log [1X/l00-1X]) (substances with IX= 100·0% were not considered). The plots show an exponential relationship between the loss of antimicrobial activity and the free fraction of the preservative. This is important for preserving agents with strong complex formation (low 1X) They are disproportionately inactivated in contrast to agents with only poor complexation. Also, an exponential decrease of the activity is obtained if the amount of HP-P-CyD is increased at a constant concentration of an interacting preservative as shown for p-Cl-m-cresol (Fig. 5). Under these conditions the unbound and thus active fraction of preservative is strongly decreased.

From the results of this study the appropriate substances for the preserving of HP-P-CyD-containing solutions can be selected. Thimerosal, phenylmercury acetate and bronopol are well suited even if higher concentrations of HP-P-CyD are used. The antimicrobial activities of phenolic substances and aliphatic arylsubstituted alcohols are reduced too much and should not be applied in the presence of HP-P-CyD.

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