Rhinology, 18, 119–133, 1980

The effects of preservatives on the ciliary beat frequency of chicken embryo tracheas

H. J. M. van de Donk, I. P. Muller-Plantema. J. Zuidema and F. W. H. M. Merkus, Amsterdam, The Netherlands

SUMMARY

The effects of preservatives on the ciliary beat frequency of chicken embryo tracheas are determined. Polar compounds like benzalkonium chloride in commonly used concentrations, decrease the frequency less than 30% after a 20 minutes' exposure. The effect is not reversible after rinsing with Locke-Ringer solution.

Lipophilic compounds however, like chlorbutol, cause an arrest of the ciliary movement within 10 minutes. The effect, different from the polar compounds, is reversible; but only after a limited exposure-time. Mercuric compounds, like thiomersal, decrease the frequency non-reversibly 30 to 90% after a 20 minutes' exposure. EDTA decreases the frequency 40 to 50%, independent of the exposure-time and in a reversible way.

The combination of benzalkonium chloride 0.01% and EDTA 0.05% is recommended to preserve nasal drops.

INTRODUCTION

Nasal drops should neither be a medium for bacterial growth, nor decrease the mucociliary clearance. Bacterial growth may lead to (1) instability of the dosage form, (2) decrease of the drug concentration and (3) infection in the patient. Therefore, it is important that nasal drops contain preservatives.

The immotile cilia syndrome causes often rhinitis and sinusitis (Afzelius, 1979). Although the effects of a temporary impediment of the ciliary movement are unknown, nasal drops should not disturb substantially the nasal clearance.

The influence of some preservatives on ciliary movement has been investigated by Gallay (1960) and Perrault et al. (1978). Generally it is stated that preservatives can diminish the ciliary movement in the nasal cavity and trachea. Our investigations were performed to study the effects of preservatives on the ciliary beat frequency, with respect to the influence of concentration of the preservative, exposure time and the reversibility of the effects.

From the results of this study a proposition will be made for a preservative which

can be used in nasal drops and which has to be preferred, regarding its effects on mucociliary clearance.

MATERIALS AND METHODS

Pharmacopoeial or analytical grade chemicals were used without further purification.

Most experiments were performed in Locke-Ringer solution (LR) (pH = 7.4). Some experiments were performed in Locke-Ringer solution without $CaCl_2$ (LR-Ca). The ciliary frequency was assessed by a photo-electric registration device (Van de Donk et al., 1980). The effects of each concentration was assessed on six different chicken embryo tracheas.

Tabel 1. Effects of preservatives, in commonly used concentrations, on ciliary movement.

Compound	(1) Freq. t=0.33 h	(2) Freq. t=1 h	(3) $t_{50\%}$ (h)	(4) $t_{0\%}$ (h)	(5) R	Fig.
Lipophilic:						
chlorbutol 0.5%	0%	0%	0.04	0.08	+	1a, 1b
chlorocresol 0.05%	0%	0%	0.01	0.02	+	1c
methyl-p-hydroxybenzoate 0.15%	0%	0%	0.06	0.33	+	1d
propyl-p-hydroxybenzoate 0.02%	0%	0%	0.10	0.33	+	1d
methyl-p-hydroxybenzoate 0.15%+ propyl-p-hydroxybenzoate 0.02%	0%	0%	0.06	0.33	+	1d
Polar:						
benzalkonium chloride 0.01%	100%	65%	1.13	>2	−	2a, 2f
benzalkonium chloride 0.01%+ EDTA 0.05%	75%	58%	1.12	>2	−	2b
benzalkonium chloride 0.01%+ EDTA 0.1%	50%	26%	0.33	1.33	−	2c
domiphen bromide 0.01%	87%	62%	1.12	>2	−	2d, 2f
chlorhexidine gluconate 0.01%	72%	4%	0.48	1.33	−−	2e, 2f
Mercuric:						
thiomersal 0.01%	9%	0%	0.19	0.50	−−	3a, 3c
thiomersal 0.005%	30%	0%	0.24	0.67	−−	3a, 3c
phenylmercuric borate 0.002%	62%	0%	0.47	1.0	−−	3b, 3c
Others:						
EDTA 0.1%	61%	49%	0.86	>2	++	4a, 4c
EDTA 0.025 to 0.1% (in LR-Ca)	62%	58%	>2	>2	++	4b, 4c

(1) Percentage of frequency of the reference after a contact of 20 min.
(2) Percentage of the frequency of the reference after a contact of 1 h.
(3) Exposure time in hours after which the frequency was 50% of the reference frequency.
(4) Exposure time in hours after which motility lacked completely.
(5) Reversibility: ++ complete; + dependent on exposure time; − nihil; −− decreasing effect continues after rinsing with LR.

RESULTS

The effects of preservatives on the frequency of the ciliary beat after a contact of 20 min, respectively 1 h are demonstrated by Table 1. The initial frequency was defined as 100%. The time necessary to diminish the frequency 50%, compared to the reference and the duration of motion are shown as well. In the fifth column an indication about the reversibility of their effects is given. Detailed data are shown in the Figures 1-4. S.E.M. values are indicated by vertical bars.

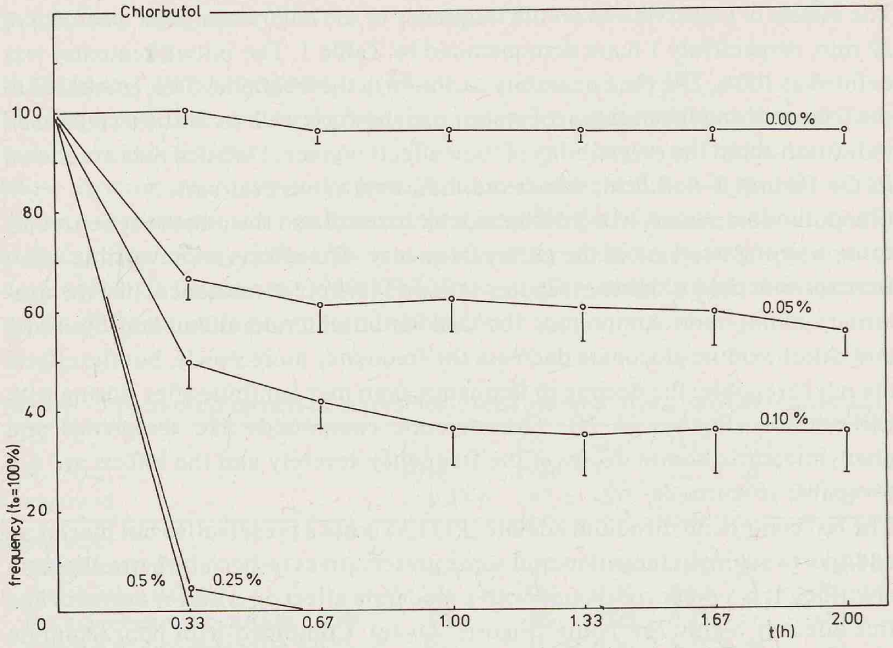
Lipophilic compounds like chlorbutol, chlorocresol and the p-hydroxybenzoates cause a strong decrease of the ciliary frequency. The effects are reversible when the exposure time is limited (Figures 1a-1d). More polar substances like the quaternary ammonium compounds (benzalkonium chloride, domiphen bromide) and chlorhexidine gluconate decrease the frequency more slowly, but the effects are not reversible: the decline in frequency even may continue after rinsing with LR solution (Figures 2a-2f). The mercuric compounds like thiomersal and phenylmercuric borate decrease the frequency severely and the effects are not reversible (Figures 3a-3c).

The last compound disodium edetate (EDTA) is not a preservative but merely an additive to augment the potency of some preservatives (especially benzalkonium chloride). It is a polar compound with a moderate effect on ciliary movement and this effect is highly reversible (Figures 4a-4c). Combined with benzalkonium chloride it accelerates the onset of action on the ciliary movement (Figures 2a-2c) as can be seen after a contact of 20 min.

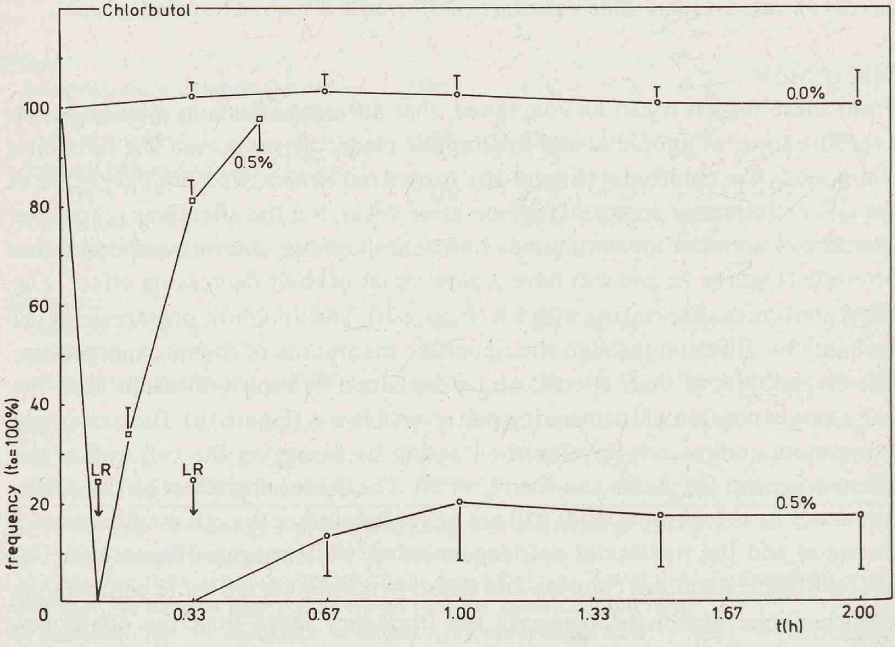
DISCUSSION

From these results it can be concluded, that different effects in relation to the exposure time, of lipophilic and hydrophilic preservatives occur. The lipophilic compounds like chlorbutol (Figure 1b), turned out to provoke a rapid decrease of the ciliary frequency, in normal preservative doses, but the effect was reversible. Quaternary ammonium compounds like benzalkonium chloride and domiphen bromide (Figures 2a and 2d) have a slow onset of their decreasing effect. The effect continues after rinsing with LR (Figure 2f). The lipophilic preservatives act probably by diffusion through the lipophilic membrane of the micro-organism. The reversibility of their effects can be explained by return-diffusion from the cell; a long exposition will damage in a non reversible way (Figure 1b). The quaternary ammonium compounds develop their action by damaging the cell wall of the micro-organism (Richards and Cavill, 1976). The decreasing effect on the ciliary frequency by these compounds will not be reversed since the cell membranes are damaged and the process of cell degeneration will continue (Figure 2f). The effects of benzalkonium chloride and domiphen bromide are quite comparable. Chlorhexidine gluconate decreases the frequency more than the quaternary ammonium compounds (Figure 2e). Both concentrations of phenylmercuric bo-

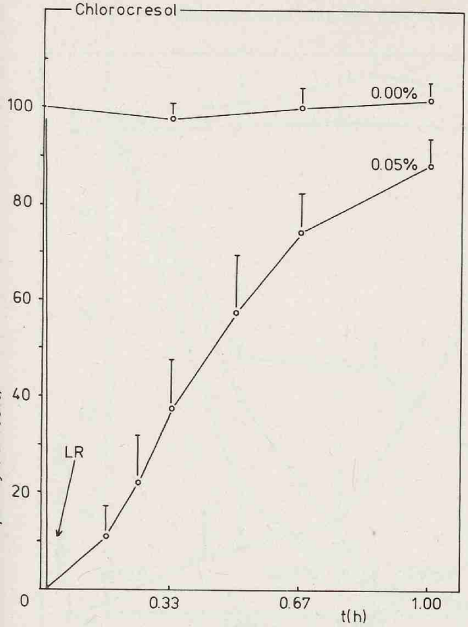
Figure 1. Time versus frequency plot.



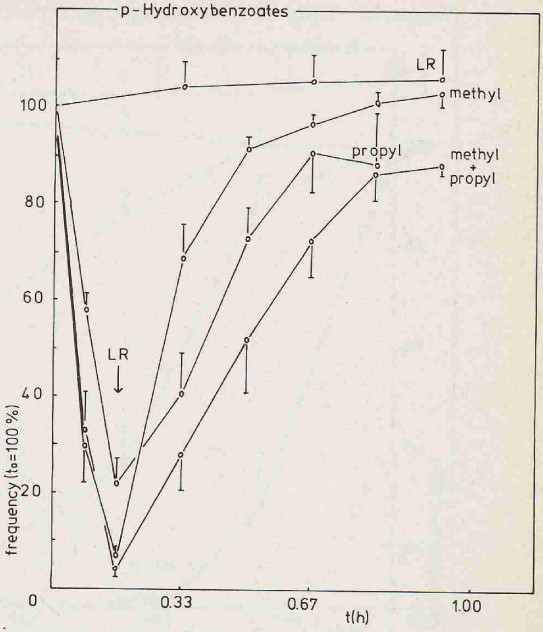
a. Chlorbutol 0.00%, 0.05%, 0.1%, 0.25% and 0.5%.



b. Chlorbutol 0.00%, 0.5% washed after 5 min. and 0.5% washed after 20 min. with LR.



. Chlorocresol 0.00% and 0.05% washed after min. with LR.

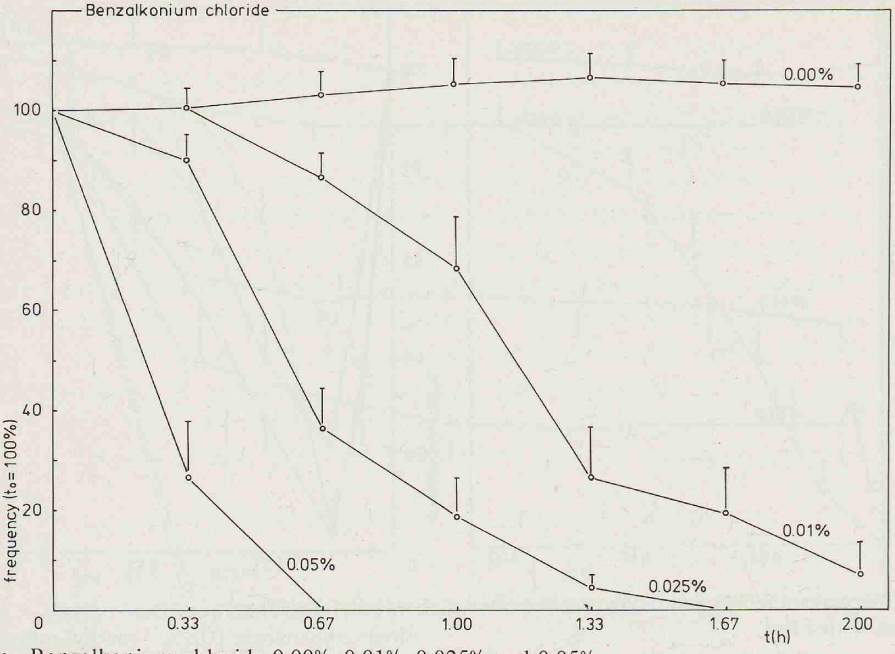


d. Methyl-p-hydroxybenzoate 0.15%, propyl-p-hydroxybenzoate 0.02%, methyl-p-hydroxybenzoate 0.15%+propyl-p-hydroxybenzoate 0.02%, all washed after 10 min. with LR, and as a reference LR.

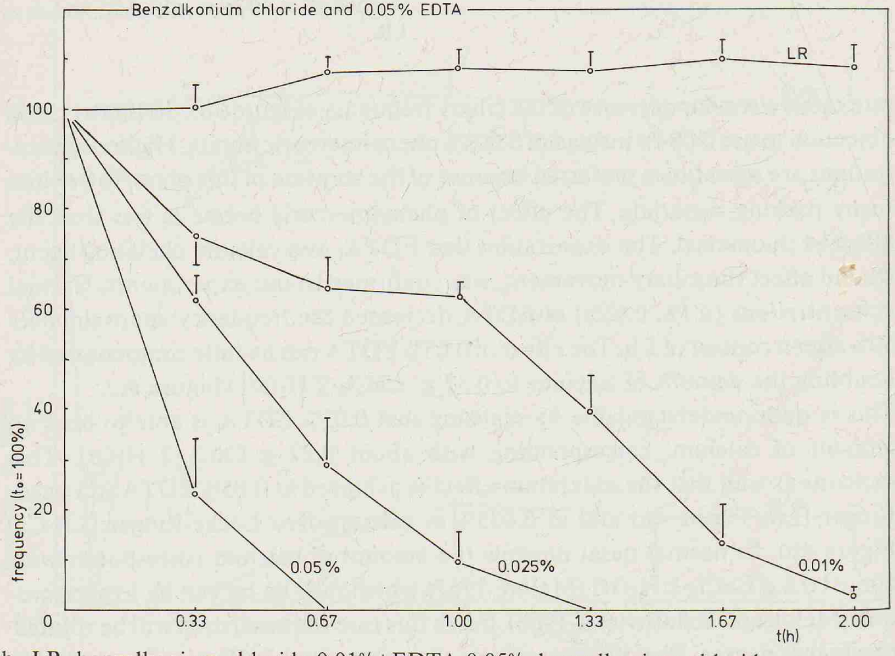
rate cause a similar decrease of the ciliary frequency (Figure 3b). So there is little objection to use 0.004% instead of 0.002% phenylmercuric borate. Higher concentrations are sometimes preferred because of the sorption of this preservative into many packing materials. The effect of phenylmercuric borate is less than the effect of thiomersal. The expectation that EDTA, as a calcium chelating agent, should affect the ciliary movement, was confirmed in our experiments. Normal concentrations (0.1%, 0.05%) of EDTA decreased the frequency approximately 50% after a contact of 1 h. The effect of 0.05% EDTA can be fully compensated by doubling the amount of calcium to 0.32 g $CaCl_2 \cdot 2\ H_2O$/l (Figure 4c).

This is quite understandable by realising that 0.05% EDTA is able to bind an amount of calcium, corresponding with about 0.22 g $CaCl_2 \cdot 2\ H_2O$/l. This explains as well that the maximum effect is achieved at 0.05% EDTA in Locke-Ringer (LR, Figure 4a) and at 0.025% in calcium free Locke-Ringer (LR-Ca, Figure 4b). In normal nasal mucous the amount of calcium corresponds with almost 0.8 g $CaCl_2 \cdot 2\ H_2O$/l (Melon, 1967) which may be halved by hypersecretion (Melon and Schoffeniels, 1966), but in this case the nasal drop will be diluted to a higher degree. The different effects of rinsing with LR-Ca (no effect) and LR

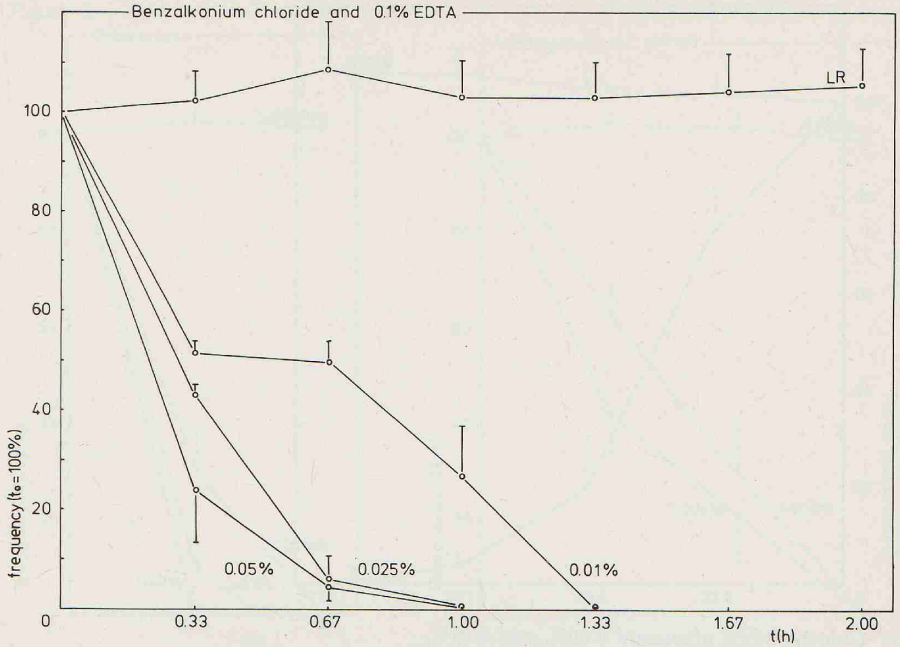
Figure 2. Time versus frequency plot.



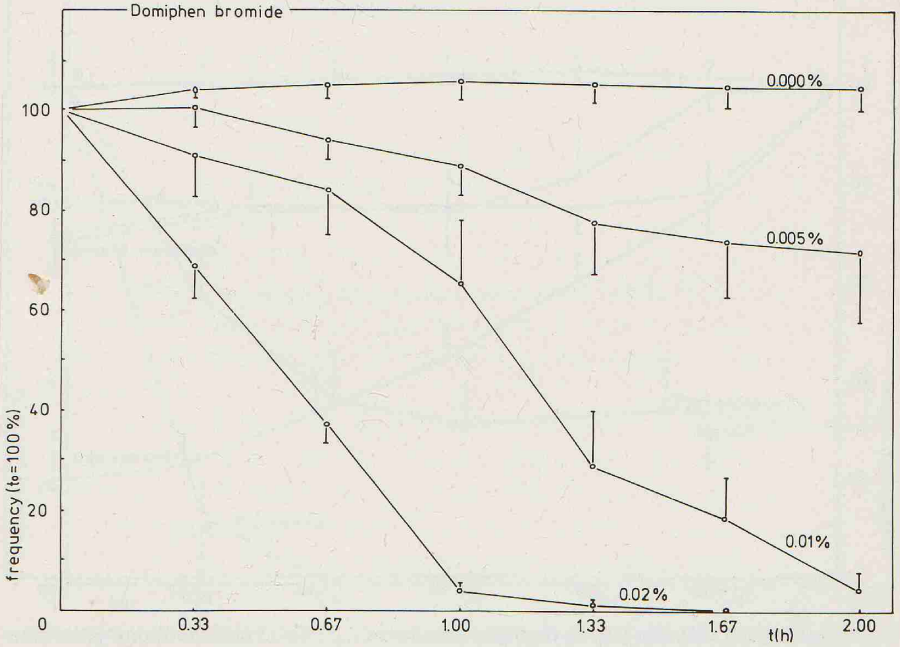
a. Benzalkonium chloride 0.00%, 0.01%, 0.025% and 0.05%.



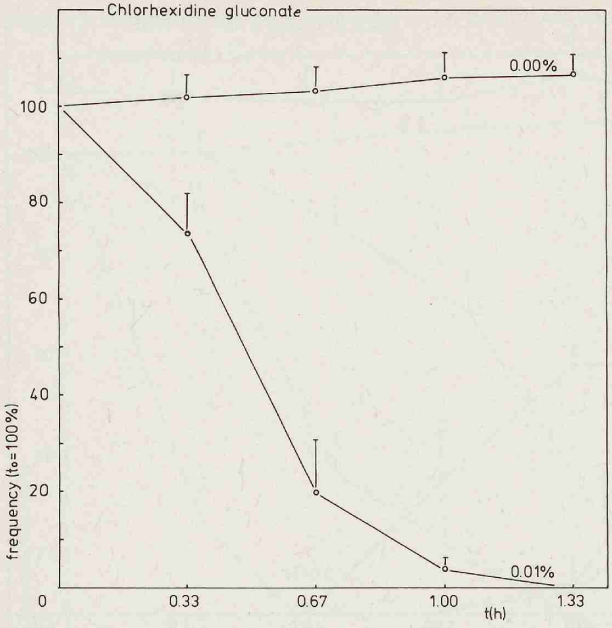
b. LR, benzalkonium chloride 0.01%+EDTA 0.05%, benzalkonium chloride 0.025%+EDTA 0.05% and benzalkonium chloride 0.05%+EDTA 0.05%.



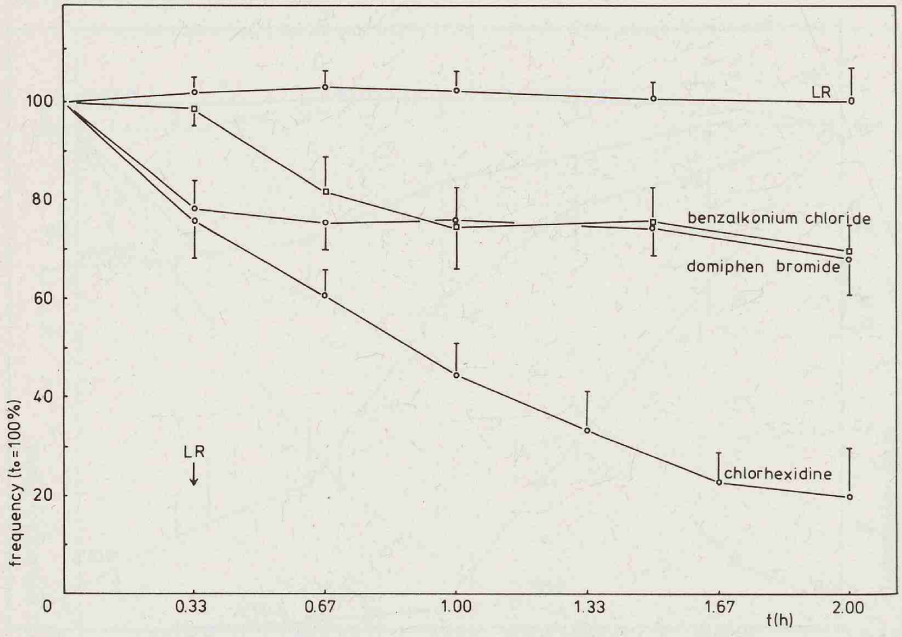
c. LR, benzalkonium chloride 0.01%+EDTA 0.1%, benzalkonium chloride 0.025%+EDTA 0.1% and benzalkonium chloride 0.05%+EDTA 0.1%.



d. Domiphen bromide 0.000%, 0.005%, 0.01% and 0.02%.

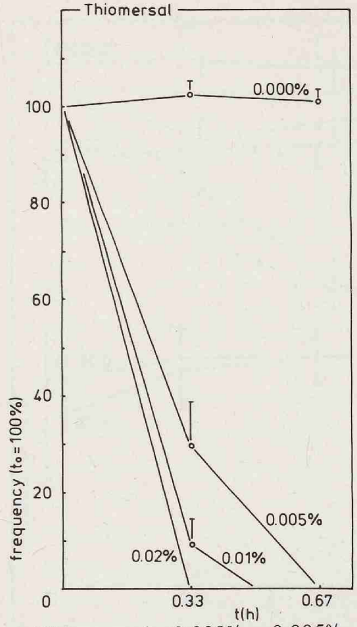


e. Chlorhexidine gluconate 0.00%, and 0.01%.

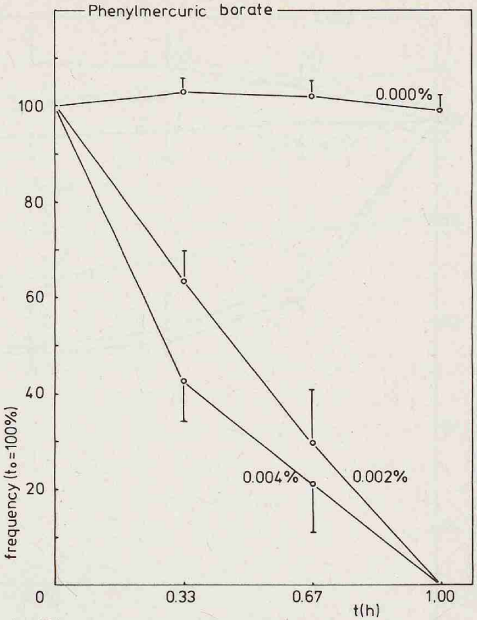


f. Benzalkonium chloride 0.01%, domiphen bromide 0.01% and chlorhedixine gluconate 0.01%, all washed after 20 min. with LR, and as a reference LR.

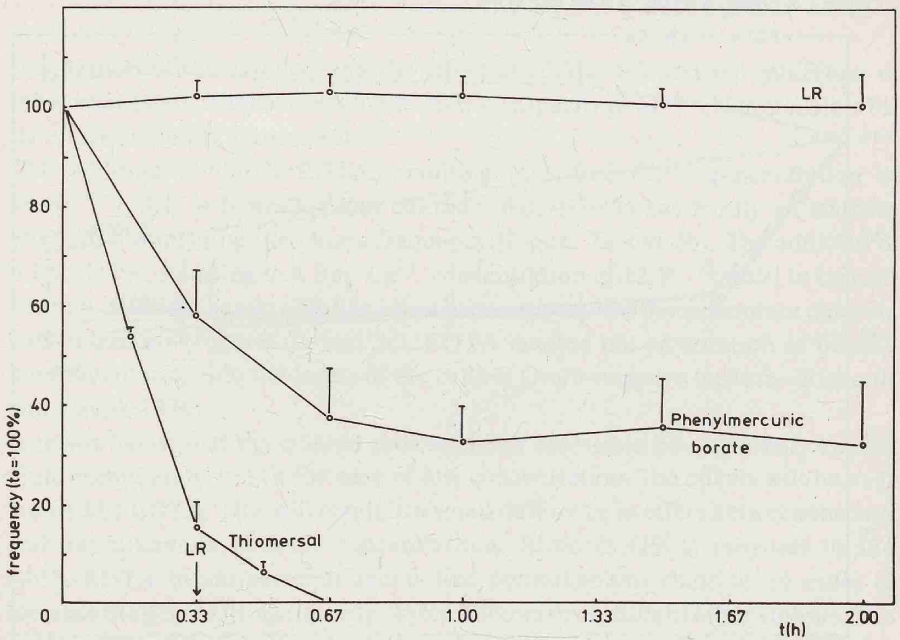
Figure 3. Time versus frequency plot.



a. Thiomersal 0.000%, 0.005%, 0.01% and 0.02%.

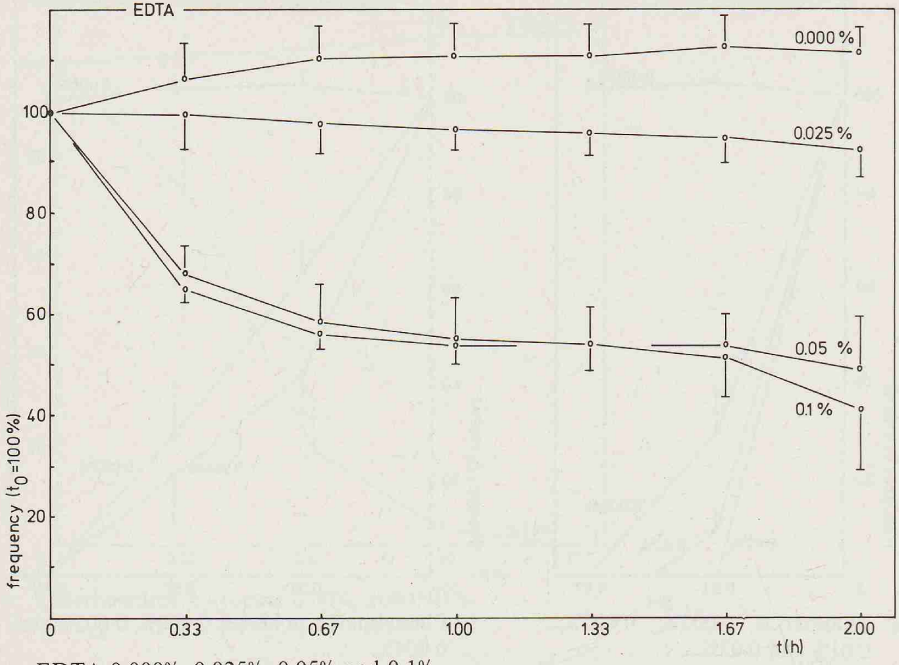


b. Phenylmercuric borate 0.000%, 0.002% and 0.004%.

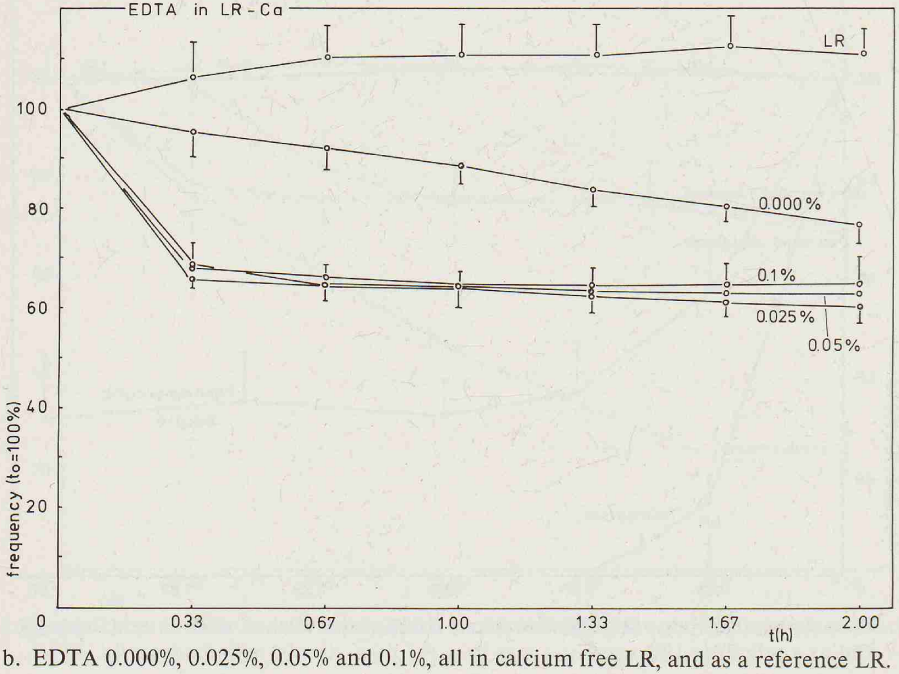


c. Phenylmercuric borate 0.002%, thiomersal 0.01%, both washed after 20 min. with LR and as a reference LR.

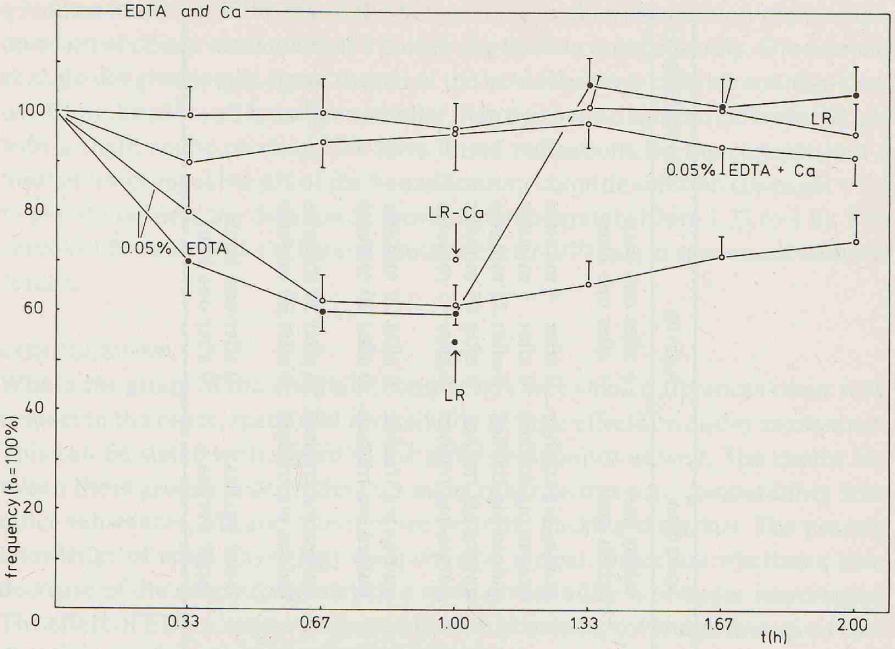
Figure 4. Time versus frequency plot.



a. EDTA 0.000%, 0.025%, 0.05% and 0.1%.



b. EDTA 0.000%, 0.025%, 0.05% and 0.1%, all in calcium free LR, and as a reference LR.



c. EDTA 0.05%+0.16 g $CaCl_2 \cdot 2\,H_2O$/l, EDTA 0.05% washed after 1 h with calcium free LR (o), EDTA 0.05% washed after 1 h with LR (•), and as a reference LR.

(restoration of the activity) and the effect of adding calcium (no inhibition of movement) indicate that the calcium binding capacity of EDTA is responsible for its effect on ciliary movement.

The addition of 0.05% EDTA, resulting in a free Ca^{2+} concentration of 54.10^{-12} mol/l, to benzalkonium chloride (0.01–0.05%) has hardly an additive effect in diminishing the ciliary frequency (Figure 2a and 2b). The addition of 0.1% EDTA, resulting in a free Ca^{2+} concentration of 12.10^{-12} mol/l to benzalkonium chloride, has an additive effect especially on low benzalkonium chloride concentrations (Figures 2a and 2c). EDTA favours the penetration of benzalkonium chloride into the layers of the cells of Gram-negative bacteria (Richards and Cavill, 1976).

It seems likely, that the ciliated cells are fairly accessible for quaternary ammonium compounds, but in the case of low concentrations the effects will be augmented by EDTA. This will result in a small difference in effect between the several benzalkonium chloride concentrations. Richards (1971) proposes to add 0.05% EDTA to antibacterial agents like benzalkonium chloride, in order to increase the germ-killing capacity. Table 2 shows the results of other authors. The results of Greenwood et al. (1946) are in contradiction with the results of Gallay (1960) but in agreement with the results reported in this paper. Gallay found that

Table 2. Comparison of the results from other "in vitro" studies.

Author	Compound	Specimen	Activity	Reversibility (1)
Greenwood, G. et al. (1946)	benzalkonium chloride 0.1%, NaCl 0.9%	rabbit nose	+ after one hour	
	benzalkonium chloride 0.05%, NaCl 0.9%	rabbit nose	+ after one hour	
Gallay, C. (1960)	methyl-p-hydroxybenzoate 0.1%, pH = 7, glucose 5%	guinea pig trachea	0 after 4 minutes	
	benzalkonium chloride 0.025%, pH = 6.7., glucose 5%	guinea pig trachea	0 after 5 minutes	
	benzalkonium chloride 0.0125%, pH = 6.7, glucose 5%	guinea pig trachea	0 after 13 minutes	
	benzalkonium chloride 0.0125%, pH = 7, glucose 5%	guinea pig trachea	0 after 45 minutes	
	phenylmercuric borate 0.025%, pH = 7, glucose 5%	guinea pig trachea	0 after 45 minutes	
Perrault, G. et al. (1978)	chlorbutol 0.5%, pH = 7.7	guinea pig trachea	0 after 20 minutes	+
	methyl-p-hydroxybenzoate 0.05%, pH = 7.7	guinea pig trachea	0 after 20 minutes	+
	methyl-p-hydroxybenzoate 0.025% + propyl-hydroxybenzoate 0.015%, pH = 7.7	guinea pig trachea	0 after 20 minutes	+
	thiomersal 0.004%, pH = 7.7	guinea pig trachea	0 after 20 minutes	0
Mostow, S. R. et al. (1979)	methyl-p-hydroxybenzoate 0.2%, pH = 6.5–7	ferret trachea	0 after one hour	0
	methyl-p-hydroxybenzoate 0.1%, pH = 6.5–7	ferret trachea	0 after one hour	+

(1) + reversible, 0 not reversible.

a modest lowering of the pH of the benzalkonium chloride solution reduces the duration of ciliary movement of a guinea pig trachea quite severely. Greenwood et al. do not give details about the pH of the benzalkonium chloride solution they used, but the pH must have been smaller than 6 as benzalkonium chloride is a salt with a slight acidic reaction. We have found indications for the concept that a limited lowering of the pH of the benzalkonium chloride solution (from pH=7.4 to pH=6) reduces the duration of movement moderately (from 1.33 to 1 h). The results of Perrault et al. (1978) and Mostow et al. (1979) are in agreement with our results.

CONCLUSIONS

Within the group of the lipophilic compounds only small differences occur with respect to the onset, speed and reversibility of their effects on ciliary movement. This can be stated with regard to the polar compounds as well. The choice between these groups is dependent on many other factors e.g.: compatibility with other substances, pH and interference with the packing materials. The present knowledge of nasal physiology does not give a clear indication whether a slow decrease of the ciliary frequency or a rapid reversibility is of major importance. The effect of EDTA can be neglected in both physiological and pathological conditions in the human nose as a result of the high calcium concentration "in situ". The effects of preservatives in the human nose will be less dramatic than in an "in vitro" situation, because of the dilution by the nasal mucus and elimination by the nasal clearance. The latter however, will be of a smaller extension for the lipophilic compounds as they diminish the ciliary movement very rapidly.
Until more information is available regarding the influence of nasal medications in different pathological situations, we would prefer 0.01% benzalkonium chloride with 0.05% EDTA. It is a potent preservative and has only moderate influence on the cilia.

RÉSUMÉ

Les effets de conservateurs sur la fréquence du battement ciliaire de l'embryon de poulet sont déterminés.
Des substances polaires, comme le chlorure de benzalkonium, aux concentrations usuelles, diminuent la fréquence avec moins que 30% après une exposition de 20 minutes.
L'effet n'est pas réversible après rinçage avec une solution de Locke-Ringer. Des substances lipophiles par contre, comme le chlorobutanol, produisent un arrêt du mouvement ciliaire en moins que 10 minutes. L'effet est réversible, autrement qu'avec les substances polaires, mais seulement après un temps d'exposition limiteé.
Des composés de mercure, comme mercurothiolate, diminuent la fréquence avec

30 à 90% après une exposition de 20 minutes, de façon irréversible.
L'édétate de sodium diminue la fréquence avec 40 à 50%, dans l'indépendence du temps d'exposition et de façon réversible. La combinaison de chlorure de benzalkonium 0,01% et d'édétate de sodium 0,05% est recommandée pour la conservation des gouttes nasales.

ZUSAMMENFASSUNG

Es wird die Wirkung von Konservierungsmitteln auf die tracheale Flimmerfrequenz des Hühnerembryos ermitteld.
Poläre Stoffe, wie Benzalkoniumchlorid, in den gebräuchlichen Konzentrationen, vermindern nach einer Einwirkungsdauer von 20 Minuten, die Frequenz um weniger als 30%. Diese Wirkung ist nicht durch Waschen mit Locke-Ringer-Lösung aufzuheben. Fettlösliche Stoffe jedoch, wie Chlorbutanol, verursachen innerhalb von 10 Minuten einen Stillstand der Flimmerbewegung. Diese Reaktion kann, anders als bei den polären Verbindungen, nach einer kurzen Einwirkungszeit, rückgänging gemacht werden.
Quecksilberverbindungen, wie Thiomersal, vermindern, nach einer Einwirkungsdauer von 20 Minuten, die Frequenz um 30 bis 90%. In diesem Fall kann die Wirkung nicht rückgängig gemacht werden.
EDTA verursacht eine Verminderung der Frequenz von 40 bis 50%, die unabhängig von der Einwirkungsdauer und leicht rückgängig zu machen ist.
Für die Konservierung von Nasentropfen ist die Kombination Benzalkoniumchlorid 0,01% und EDTA 0,05% zu empfehlen.

ACKNOWLEDGEMENT

The authors like to thank mrs. F. Gerkens and mrs. J. Stapel for their technical assistance and mrs. B. Eckmann for typing the manuscript.

REFERENCES

1. Afzelius, B. A., 1979: The immotile-cilia syndrome and other ciliary diseases. Int. Rev. Exp. Path. 19, 1-43.
2. Van de Donk, H. J. M., Zuidema, J., Merkus, F. W. H. M., 1980: The influence of the pH and osmotic pressure upon tracheal ciliary beat frequency as determined with a photo-electric device. Rhinol. 18, 93-104.
3. Gallay, C., 1960: Essays physiologiques des gouttes nasales. Pharm. Acta Helv. 35, 358-374.
4. Greenwood, G., Pittenger, R. E., Constant, G. A. and Ivy, A. C., 1946: Effect of zephiran chloride, tyrothricin, penicillin and streptomycin on ciliary action. Arch. Otolaryngol. 43, 623-628.
5. Melon, J. and Schoffeniels, E., 1966: Modification de la composition chimique de la secrétion nasale au cours de la réaction anaphylactique locale. Acta Allergologica, 21, 497-506.
6. Melon, J., 1967: Contribution à l'étude de l'activité secrétoire de la muqueuse nasale. Thesis University of Liège.

7. Mostow, S. R., Dreisin, R. B., Manawadu, B. R., La Force, F. M., 1979: Adverse effects of lidocaine and methylparaben on tracheal ciliary activity. Laryngoscope 89, 1697–1701.
8. Perrault, G., Dreyfuss, M. F. and Saunié, R., 1978: Étude de la fonction ciliaire. Application aux essais de tolérance des gouttes nasales. Sci. Techn. Pharm. 7, 383–388.
9. Richards, R. M. E., 1971: Inactivation of resistant Pseudomonas aeruginosa by antibacterial combinations. J. Pharm. Pharmacol. 23, suppl., 136s–140s.
10. Richards, R. M. E. and Cavill, R. H., 1976: Electron microscope study of effect of benzalkonium chloride and edetate disodium on cell envelope of Pseudomonas aeruginosa. J. Pharm. Sci., 65, 76–80.

H. J. M. van de Donk,
Faculty of Pharmacy and Faculty of Medicine
Department of Biopharmaceutics
Plantage Muidergracht 14
1018 TV Amsterdam, The Netherlands