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Preservative Efficacy Tests in Formulated Nasal Products: Reproducibility and Factors Affecting Preservative Activity

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Abstract

Preservative efficacy tests were performed in triplicate on each of three batches of three formulated nasal spray preparations to assess the inter- and intra-batch variation in preservative performance which typically results from these procedures, and to assess the relative importance of factors influencing preservative performance in nasal products.

Tests were conducted using procedures conforming, as far as possible, to both the European and the US pharmacopoeias and the results interpreted using the performance criteria of both. Despite the adoption of practices designed to maximize reproducibility, a marked variation in the degree of microbial inactivation was observed, both within and between batches of product.

A preservative system comprising benzalkonium chloride and phenylethyl alcohol was found to be far superior to combinations of either benzalkonium chloride plus disodium edetate or potassium sorbate plus disodium edetate, both of which failed to satisfy the EP performance criteria on a number of occasions. Proposals are made for the adoption of inactivation criteria which incorporate realistic error limits reflecting the inherent problems of reproducibility of the viable counting procedures involved.

Over the course of the last 30 years very few new antimicrobial preservatives have been introduced for use in parenteral and ophthalmic products or in products applied to mucous membranes. Toxicity considerations have restricted the application of those preservatives which have been developed during this period to the protection of topical products, and the same considerations have caused a marked reduction in the use of some long-established agents, e.g. organomercurials, in parenterals and ophthalmics. It is, therefore, becoming increasingly common for formulated medicines to be protected against microbial spoilage with a combination of preservatives rather than a single agent (Denyer & King 1988). Frequently the reason for this change is because a single agent cannot provide a sufficiently broad spectrum of antimicrobial activity. There is, however, also the need to achieve acceptable levels of product protection from the limited choice of available agents by capitalizing on potential synergistic interactions between preservatives whilst at the same time minimizing the risk of adverse reactions by avoiding the use of unnecessarily high concentrations (Hodges & Denyer 1995).

In striving to limit preservative concentrations without compromising product safety it is important to recognize, and investigate the many factors which influence preservative performance (Denyer & Wallhäuser 1990) and to maximize the reliability and repeatability of preservative efficacy tests in order to improve their ability to predict product vulnerability to spoilage and enhance the usefulness of the test results in the pre-formulation process (Davison 1988; Baird 1995; Hodges & Denyer 1995).

Because preservative efficacy tests are normally employed to provide a qualitative pass or fail result, it is important for the formulator to have a good appreciation of the precision achievable, particularly when assessing results from a product in which preservative efficacy is at the borderline of acceptability. Although pharmacopoeial tests have been in use since 1973 and there are numerous publications which analyse and assess their virtues and shortcomings (Allwood 1986; Cooper 1989; Spooner & Davison 1993), there appears to be very little published information about the level of inter- and intra-batch reproducibility that might be achieved using pharmacopoeial methods; this work was intended to address this deficiency.

It is proposed that nasal products are, for the purposes of preservative performance criteria, to be regarded as topicals (Anon 1993), but the selection of a preservative system in such products requires particular care because application to mucous membranes might carry a greater risk of sensitization or adverse reaction than application to the skin. Nasal products, therefore, represent a product category where preservative performance needs to be properly optimized and assessed; this study evaluates the preservative activity in three such products each protected by a different system.

Materials and Methods

Products examined

Three nasal sprays used for the treatment of perennial or seasonal allergic rhinitis were studied. Flixonase (Allen & Hanbury) contains fluticasone propionate as the active agent, benzalkonium chloride 0·02% (w/v) with phenylethyl alcohol 0·25% (w/v) as preservatives and dextrose, carmellose and polysorbate 80 as excipients; it is sold in a 25-mL brown glass bottle and the three batches examined had a mean measured pH of 5·9. Rhinocort Aqua (Astra) contains budesonide as the active agent, 0·12% (w/v) potassium sorbate with 0·01% (w/v) disodium edetate as preservative and glucose, carmellose sodium, polysorbate 80 and hydrochloric acid as excipients; it is sold in a 6-mL brown glass bottle and the three batches examined had a measured mean pH of 4·1. Rynacrom (Fisons)

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contains 4% (w/v) sodium cromoglycate as the active agent and 0·01% (w/v) benzalkonium chloride with disodium edetate as preservatives; it is sold in a 22-mL white plastic bottle and the three batches examined had a mean measured pH of 5·2

Preservative testing

Three different batches of each of the three products were tested on each of three occasions. The testing procedure was designed to satisfy, as far as possible, the requirements of both the European Pharmacopoeia (1994) and the United States Pharmacopeia (1995). Thus, freshly prepared suspensions of the ATCC-recommended strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Aspergillus niger* and *Candida albicans* in sodium chloride-peptone water were standardized to a concentration of approximately 1×10^8 cells mL^{-1} using a turbidity calibration graph. Accurately weighed quantities of these suspensions were added to the products in their original containers in order to give the USP-specified 200-fold dilutions and initial concentrations of approximately 5×10^5 mL^{-1}. There is a requirement that *E. coli* should be used as a supplementary test organism when the EP preservative efficacy test is applied to oral products, and *E. coli* was used in this present study: to satisfy the USP requirement; to provide a fuller assessment of the antimicrobial spectra of the preservative systems; and because the potential hazards presented by enteric pathogens in oral products also exist to a lesser, but significant extent, in nasal products.

Samples (1·00 mL) were removed at time zero (nominal), and at 2, 7, 14, 21 and 28 days during storage at a temperature of 20–25°C protected from light. The samples were subjected to decimal dilutions in letheen broth (Difco), and 0·20-mL volumes of appropriate dilutions were surface-spread on triplicate tryptone soya agar plates incubated at 35°C for 48 h or on Sabouraud-dextrose agar plates incubated at 25°C for 48–72 h for the bacteria and fungi, respectively. The A. niger plates were examined at intervals during the incubation period to avoid recording artificially low counts resulting from confluence of colonies.

Controls confirmed that the addition of 9·00 mL letheen broth diluent to 1·00 mL product eliminated the activity of all three preservative systems when the mixture was inoculated with low concentrations of the most sensitive of the test organisms (*P. aeruginosa*).

Colonies were enumerated, and the extent of inactivation calculated as the $\log_{10}$ reduction in viable count in the inoculated product using the control count of the inoculum suspension as the baseline. Any test in which the time zero count exceeded the expected value by more than 15% was repeated.

All testing was performed by a single, experienced operator whose reproducibility of viable counting was confirmed, all the tests were performed within a three-month period using the same batches of media and diluents throughout and the three products were tested simultaneously not sequentially. All batches of the three products were within their expiry dates throughout the testing period and were stored according to the manufacturer's instructions both before, and during, testing.

Results

It was possible to reduce the large volume of data generated in this work by eliminating from Tables 1–3 and Fig. 1 all data derived from Flixonase, because this product permitted no survivors of any bacterial species or *C. albicans* at 48 h, nor any survivors of *A. niger* at 14 days; all data obtained with *P. aeruginosa* because this organism invariably showed no survivors at 48 h; all data for *S. aureus* and *E. coli* after 7 days because, invariably, no survivors were recorded; and part of

Table 1. Triplicate determinations of *S. aureus* and *E. coli* inactivation ($\log_{10}$ reduction in viable count mL^{-1} with time (days)) in three batches of rhinocort and rynacrom.

	Day			Day		
	0	2	7	0	2	7
		Rhinocort			Rynacrom	
Batch A						
S. aureus	0·05	0·07	> 4·06	0·10	3·81	> 4·11
	0·04	0·49	> 4·19	0·03	> 4·20	> 4·20
	0·04	1·54	> 4·26	0·03	> 4·28	> 4·28
E. coli	0·16	2·44	> 4·43	0·14	2·77	> 4·43
	0·19	1·86	> 4·60	0·18	0·65	2·18
	0·08	1·97	> 4·49	0·05	0·94	4·50
Batch B						
S. aureus	0·01	0·51	> 4·19	−0·07	3·60	> 4·30
	− 0·02	0·34	> 4·16	0·00	3·03	> 4·21
	0·05	1·40	> 4·41	0·07	> 4·43	> 4·43
E. coli	0·08	1·07	> 4·49	0·15	1·31	2·95
	− 0·01	1·60	> 4·61	0·09	0·47	2·09
	0·06	1·17	> 4·46	0·09	1·52	> 4·49
Batch C						
S. aureus	0·09	0·52	> 4·14	0·14	> 4·19	> 4·19
	− 0·05	0·15	> 4·20	−0·04	3·23	> 4·31
	0·03	0·58	> 4·27	0·06	> 4·30	> 4·30
E. coli	0·01	0·92	4·20	0·02	1·08	3·42
	− 0·01	1·59	> 4·36	−0·04	0·55	4·07
	0·29	1·19	> 4·60	0·09	1·71	> 4·66

Table 2. Triplicate determinations of *A. niger* and *C. albicans* inactivation (log_{10} reduction in viable count mL^{-1} with time) in three batches of rhinocort and rynacrom.

	Day				Day			
	0	14	21	28	0	14	21	28
	Rhinocort				Rynacrom			
Batch A								
A. niger	0·19	1·81	1·89	2·89	− 0·03	2·50	3·48	> 4·18
	0·19	1·25	1·71	2·60	0·19	3·03	3·78	4·00
	0·09	1·54	1·75	4·27	− 0·01	3·53	3·63	4·23
C. albicans	0·11	0·71	4·01	> 4·31	0·05	0·43	0·52	0·73
	0·13	0·29	1·56	3·98	0·09	0·39	0·65	1·15
	0·14	> 4·59	> 4·59	> 4·59	− 0·04	0·19	0·34	0·72
Batch B								
A. niger	− 0·02	1·33	1·67	2·47	0·06	2·32	2·90	4·67
	0·07	0·63	1·24	1·87	0·00	3·34	4·04	3·86
	0·03	1·71	2·42	3·68	0·04	4·39	3·27	3·24
C. albicans	0·09	0·65	3·25	> 4·51	0·07	0·37	0·30	0·76
	0·07	0·36	> 4·46	> 4·46	0·03	0·23	0·46	1·00
	0·09	> 4·60	> 4·60	> 4·60	0·26	0·41	0·67	0·90
Batch C								
A. niger	− 0·04	1·40	2·29	2·64	0·04	2·90	3·30	4·38
	0·19	1·84	2·43	3·47	0·15	4·31	4·31	> 4·31
	0·19	1·34	1·74	2·55	0·23	2·92	3·62	3·20
C. albicans	0·09	0·76	4·18	> 4·48	0·11	0·37	0·49	0·64
	0·12	1·84	> 4·35	> 4·35	− 0·06	0·33	0·67	1·06
	− 0·04	1·73	> 4·49	> 4·49	0·02	0·51	0·64	1·04

the data at 48 h and 7 days for *C. albicans* and *A. niger* because these time points are not considered in the EP or USP performance criteria.

For a product to be considered effectively preserved the USP requires that the concentration of viable bacteria should be reduced one thousand fold within 14 days of inoculation and the concentration of yeasts and moulds should remain at or below the initial level during the first 14 days.

The survival of *S. aureus* and *E. coli* is shown in Table 1, and that of *A. niger* and *C. albicans* in Table 2 for all tests performed on rhinocort and rynacrom. It is evident from these data and the facts stated above that all batches of the three products satisfied these performance criteria on each occasion. The USP further requires that the concentration of each test organism remains at or below these designated levels during the remainder of the test period, and this requirement, also, was satisfied in every instance but one, where a small numerical increase was observed in the *A. niger* viable count in rynacrom between days 14 and 28.

All three products invariably rendered the bacteria undetectable at 14 days, and bacterial survivors in Flixonase were rarely detectable even in the time zero sample. Three batches of Flixonase each examined on three occasions and inoculated with three bacterial species resulted in a total of 27 time-zero observations for this product, and survivors were detected in only three of these despite the control experiments demon-

Table 3. Frequency of failure to achieve European Pharmacopoeia performance criteria for each batch in relation to the number of observations.

		Bacteria		Yeast and mould		Overall test fails	
No of observations		9*		6*		3	
Product	Batch	Performance criteria					
		A	B	A	B	A	B
Rhinocort	A	5	0	5	2	3	2
	B	6	0	5	3	3	3
	C	6	0	6	1	3	1
Mean % failure**		63	0	89	33	100	67
Flixonase	A	0	0	0	0	0	0
	B	0	0	0	0	0	0
	C	0	0	0	0	0	0
Mean % failure		0	0	0	0	0	0
Rynacrom	A	2	0	3	3	3	3
	B	3	0	3	3	3	3
	C	3	0	3	3	3	3
Mean % failure		30	0	50	50	100	100

*Three species of bacteria and two yeasts/moulds with triplicate tests give nine and six observations, respectively. **No product ever failed against *P. aeruginosa*.

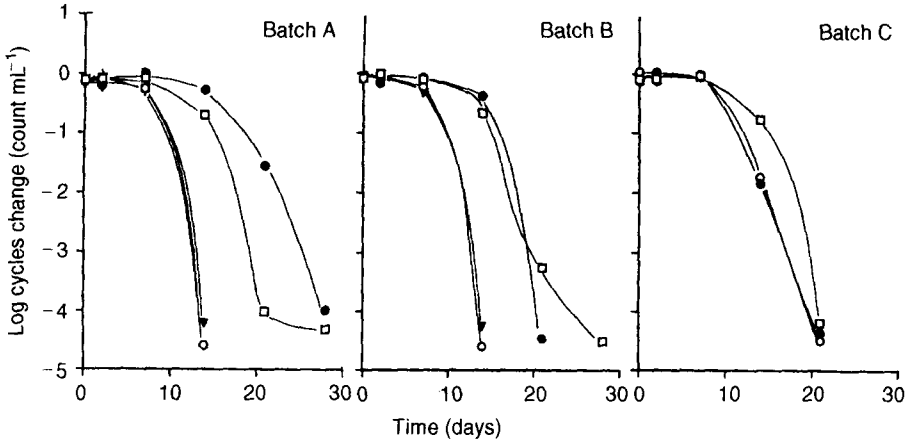

FIG. 1. Replicate survivor plots for *C. albicans* in three batches of rhinocort. Symbols represent replicate tests.

strating that the letheen broth diluent effectively eliminated the antibacterial activity of the preservative in that product. This indicates that the preservative activity is such that even the transient exposure of the bacteria between inoculation and the removal of the (nominal) time zero sample was sufficient to produce irreversible fatal damage to the cells.

If nasal products are to be regarded as topicals (Anon 1993) the performance criteria recommended by the European Pharmacopoeia (the A criteria) are two decimal reductions in bacterial count within 48 h and three decimal reductions in seven days, combined with two decimal reductions in yeasts and moulds within 14 days. For both classes of organism there should be no subsequent increase in count above these levels during the remainder of the 28 day testing period. Flixonase invariably satisfied all the EP A performance criteria but rhinocort and rynacrom displayed certain weaknesses.

All products showed satisfactory performance against *P. aeruginosa*, and inadequate antibacterial activity was seen with *S. aureus* and *E. coli* only. In no case were surviving bacteria detected in rhinocort after 7 days so the preservative deficiencies in this product were restricted to an inadequate bacterial killing rate over the first 48 h. Although the rhinocort preservative system was more effective against *E. coli* than *S. aureus*, as shown by the consistently higher decimal reductions in viable count, it nevertheless failed to produce an adequate level of inactivation against these two species to satisfy the A criteria on every occasion except one (Table 1).

In contrast, the rynacrom preservative was more active against *S. aureus* than *E. coli* and on every one of the nine occasions on which it was inoculated with *S. aureus* a satisfactory performance was observed at both 48 h and seven days. It failed the A criteria eight times out of nine and three times out of nine at the 48 h and seven day points respectively against *E. coli* (Table 1), however.

Seven days after inoculation the preservatives in both rhinocort and rynacrom had invariably achieved greater inactivation of *A. niger* than of *C. albicans* (data not shown) and this was also true for rynacrom at 14 days (Table 2), but not always for rhinocort at this time. Indeed, the activity of rynacrom against *A. niger* was sufficient to ensure that it never failed to satisfy the EP criteria against *A. niger* and its failures on nine occasions out of nine were always because of inadequate activity against *C. albicans*. In contrast, rhinocort failed the A criteria on almost every occasion against both organisms.

Table 3 summarizes the performance of the various batches of the three products in terms of failures to meet the EP performance criteria in respect of: bacteria, yeasts and moulds, and overall. The EP describes a second set of criteria that must be satisfied in justified cases where the A criteria cannot be attained. The B criteria require the preservative to achieve three decimal reductions in the bacterial count and one decimal reduction in the yeast and mould count by the 14th day. It is clear that the preservative deficiencies in rhinocort and rynacrom resulted in these products failing, on some occasions, even to achieve these minimal performance targets.

The pattern of results for one of the three replicates of rhinocort Batch A inoculated with *C. albicans* was markedly different from the other two and this combination was, therefore, examined on two further occasions. Similarly, rhinocort Batch B was examined a total of four times not three. It is clear from the results (Fig. 1) that this organism does not exhibit first order inactivation kinetics by this preservative but displays a marked shoulder on the survivor plot so that there is no appreciable reduction in viable count over the first seven days but there is rapid inactivation thereafter. Both the duration of the initial lag and the subsequent rate of decline varied from one replicate test to another, however.

Discussion

All three products contain two ingredients with antimicrobial activity, most probably because each of the four agents employed is considered to have gaps in its antimicrobial spectrum which necessitate its combination with others (Wallhäuser 1984). Benzalkonium chloride is vulnerable to failure in the control of *Pseudomonas* species, and potassium sorbate is regarded primarily as an antifungal agent with deficiencies in its activity against bacteria, whereas the converse is true for phenylethyl alcohol. Disodium edetate is only weakly active when used alone and this activity is largely confined to Gram-negative species, thus it is better regarded as a potentiator rather than a preservative in its own right. Disodium edetate nevertheless has considerable value as a component of preservative systems, and Hart (1984) lists 32

preservatives, including benzalkonium chloride and potassium sorbate, which it is known to potentiate. Synergy has also been reported between phenylethyl alcohol and other preservatives, including benzalkonium chloride (Allwood & Baird 1994).

The combination of benzalkonium chloride and EDTA is extensively employed as a preservative system in products applied to sensitive tissues; e.g., of the 58 proprietary ophthalmic products currently available on the UK market 47 contain benzalkonium chloride, and of these, 17 also contain disodium edetate (Anon 1996). There has been some controversy in recent years about the potential toxicity of benzalkonium chloride. Concern has been expressed that it might be responsible for adverse effects on mucociliary function after exposure to decongestant products (Berg et al 1994; Graf et al 1995), although studies using the frog-palate preparation, which resembles the nasal mucosa in retaining an intact mucus layer, have shown no effect on ciliary structure or mucus transport in concentrations up to 0·05% w/v (Braga et al 1992). No adverse effects on nasal ciliated epithelium were, furthermore, seen in monkeys or rats which received steroid-containing nasal sprays preserved with benzalkonium chloride for 28 days (Ainge et al 1994). When assessing such conflicting indications of potential toxicity, it is, however, essential not to overlook the obligate requirement for adequate preservation of the product, and a reduction in preservative concentration prompted by toxicity considerations is only of value if it does not create an even greater risk to the consumer as a result of product spoilage by pathogenic organisms.

Of the many factors known to influence preservative activity in pharmaceutical products (Denyer & Wallhäuser 1990) the presence of non-ionic surfactants, sorption on to plastic containers and sensitivity to product pH are the three most pertinent to the present study. Benzalkonium chloride, phenylethyl alcohol and potassium sorbate are all known to be inactivated to varying extents by non-ionic surfactants (Wade & Weller 1994) and this might be considered as a possible contributory factor to the failure of rhinocort, which contains polysorbate 80, to meet the EP preservative efficacy test performance criteria. Both components of the Flixonase preservative system are, however, also vulnerable to non-ionic surfactants. Sorption on to plastics, particularly polypropylene and polyethylene, has been shown to cause significant losses of benzalkonium chloride and other preservatives from solution (Autian 1968). The poorer performance of the benzalkonium chloride-EDTA combination compared with the benzalkonium chloride-phenylethyl alcohol system is, therefore, likely to be primarily because of the higher benzalkonium chloride concentration in Flixonase, but sorption by the plastic of the rynacrom container is a possible contributing factor. Preservative activity of weak acids resides primarily, but not exclusively, in the undissociated fraction of the molecules and this fraction is directly dependent upon pH. The pK_a of sorbic acid is 4·76, so products with pH less than this will contain the majority of their sorbate molecules in the undissociated state and thus have maximum preservative activity. The recorded pH of rhinocort was 4·1, at which value approximately 80% of the sorbate molecules are not ionized, and the data of Eklund (1983) suggest that the growth-inhibitory action of the sorbate would not be appreciably enhanced by a further reduction in pH.

Davison (1988) commented that the recommendations of the 1980 BP for preservative performance in topical products were neither attainable nor reproducible, and he recorded a variation of up to three log cycles in the viable count reduction of *C. albicans* and *A. niger* between duplicate tests performed on the same sample of a cetomacrogol cream. The data of Fig. 1 clearly illustrate the potential for such variations to occur in other products, particularly when the inactivation kinetics comprise an initial lag followed by a period of rapid viability loss.

Although the variability recorded between the replicate determinations on the three batches of rhinocort inoculated with *C. albicans* appears to be substantial, such marked variation is not confined to this product-organism combination. For example, the three replicate determinations of *A. niger* survival in rynacrom batch B show log reductions at 14 days which vary from 2·32 to 4·39, and it is possible that variations of similar magnitude could have been recorded elsewhere if samples had been taken at more frequent time intervals. The extent of variation is less apparent if the organisms are all killed quickly, as in this study, when all the bacteria were killed by day 7; this leaves only the day-2 data by which to assess variability. If these for rhinocort are examined with the rynacrom data for *A. niger* and *C. albicans* at day 21 (selected because the organisms still had survivors in every instance so there was not an artificial limit on the extent of the log reduction imposed by complete inactivation), marked variations between replicates are apparent. Indeed, the extent of this is such that analyses of variance show no evidence (at $P = 0·05$) that the apparent differences between batches are real, except in the case of the three rhinocort batches inoculated with *E. coli*, where the mean $\log_{10}$ reductions of 2·09, 1·28 and 1·23 were different ($P = 0·025$).

Possible explanations for the observed variation in results include inadequate mixing of the inoculum and variation in fill volume of the product containers. Inadequate mixing of the inoculum was, however, considered improbable because the low product viscosities enabled easy mixing, and variation in fill volume was unlikely to be of sufficient magnitude to account for the observed differences. The most likely possibility was considered to be variation in the temperature of storage of the inoculated products. The storage temperature used conformed to the USP and EP requirements (between 20 and 25°C) but was not constant within this range. It is known that variation in temperature might influence the activity of antimicrobial chemicals (Denyer & Wallhäuser 1990) and greater reproducibility might be achieved by specifying a single storage temperature rather than a range for the performance of the test.

The data obtained in this study were produced using a single experienced operator whose counting technique was shown to be good. Throughout the work triplicate Petri dishes were used for viable counts rather than the duplicates recommended in the EP, so these, together with the use of the same batches of culture media and diluents, were steps which would be expected to enhance reproducibility. It is, nevertheless, clear from Tables 1–3 that in certain situations marked differences arose between replicate determinations, and in the experience of the authors, such variation is not unusual in preservative efficacy tests. There appears to be very little published information to indicate the magnitude of the variability that might be expected in test data, but there are statements in official compendia which indicate how different two viable counts

must be before the difference is to be considered significant. In the context of validation of counting methods, the EP states that if a limit of 100 organisms is specified in a monograph the maximum limit of acceptance should be 500 organisms. Furthermore, in this context of preservative efficacy testing, it was proposed in Pharmeuropa (Anon 1993) that "no increase in viable count" should be taken to mean not more than half a log scale higher than the last value for which a criterion is given. In view of these statements and the variability observed in some aspects of this study it is clear that a measure of reproducibility is likely to be of major benefit in the interpretation of preservative efficacy data and the authors suggest that this should become an integral part of the test.

In conclusion, therefore, it is evident that marked differences in the performance of different preservative systems can arise even when the preservatives are employed at concentrations within the normal ranges (Wade & Weller 1994), and that products which are on the borderline of acceptability in terms of preservation might perform satisfactorily on one occasion and fail on another. Such a situation would dictate that single point acceptance criteria are inadequate. In recognition of this, it is suggested that performance criteria are adopted together with realistic limits of error which adequately reflect the precision of the viable counting procedures involved.

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