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The parabens: Bacterial adaptation and preservative capacity

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Synopsis

The initial rates of kill of *Escherichia coli* by methyl, ethyl and propyl parabens are similar at equal saturation fraction in a buffered salts-glucose medium. This is as expected from the Ferguson principle of equal activity of homologs, but the survival curves soon diverge strongly, apparently because the rate of adaptation increases markedly with increasing molecular weight; *E. coli* adapts readily to propyl paraben, hardly at all to methyl. As a result, the parabens clearly rank methyl > ethyl > propyl > butyl > benzyl in their antimicrobial capacities at equal saturation fraction. The same ranking holds when equal weight concentrations are challenged with other bacteria in oil/water emulsions and in a shampoo base. Performance in these systems is discussed in terms of apparent solubilities. Mixtures of parabens are not superior except when multiple saturation is possible. In overall performance, only methyl paraben appears to be a good preservative.

INTRODUCTION

In two previous papers (1,2) we showed on theoretical grounds that the choice among methyl, ethyl and propyl parabens of a preservative for any cosmetic or pharmaceutical product could be based on solubility measurements. The paraben least soluble in the product should be the one most effective in both microbial kill rate and inoculum capacity in comparison with an equal weight of the more soluble homologs or with any equal weight combination of any two of the three parabens. This was an extension of W. P. Evans' argument (3) that as preservatives for simple emulsions, paraben mixtures can never be more effective than an equal weight of the less soluble one if the Ferguson principle (4) is applicable. According to the Ferguson principle, the members of many homologous series of biologically active materials are equally potent at their respective saturation concentrations or at any given fraction of saturation. Allawala and Riegelman (5) have shown that this principle applies quite uniformly to their measurements of the efficacy of a variety of phenols representing several distinct homologous series. If it applies to the parabens, then in water, propyl paraben at its saturation concentration of 0.3 grams per liter should be as potent as ethyl paraben at its solubility limit of one gram per liter or methyl paraben at two grams per liter. This prediction is in accord with numerous reports of minimum inhibitory concentrations (MIC's) in aqueous broth (see, for example, Reference 6); for a wide variety of bacteria

the MIC's increase in the order: butyl < propyl < ethyl < methyl and the ratios, as would be predicted, are roughly the ratios of the solubilities.

In cosmetic ingredients such as vegetable oils, the solubility order is the reverse of that in water; methyl paraben is least soluble and therefore should be the most efficient preservative for oil-rich emulsions. Evans (3) showed that for simple oil/water mixtures the best preservative may be propyl paraben at low oil/water ratios or methyl paraben at high oil/water ratios but that methyl/propyl mixtures are less efficient in both cases.

There seems to be a contradiction here between theory and practice. Parabens are almost always used in combinations in preserving cosmetics. A search of the literature, however, yielded no data unequivocally showing synergism in either aqueous broths or complex products.

In our own experimental work we first attempted to demonstrate the applicability of the Ferguson principle to the parabens in simple, well-defined aqueous solutions as a step toward resolving the question of the utility of mixtures and also to support our theoretical proposal that single parabens be selected according to solubility. The earliest of these experiments (7) showed that the parabens do not follow the Ferguson principle to a useful extent; at saturation their antimicrobial potencies are not equal. In fact, they drop sharply in the order: methyl > ethyl > propyl > butyl (and benzyl paraben, not a member of the homologous series, is less potent yet).

The ranking of the parabens is evident from the way the survival curves of *E. coli* change as the inoculation level and saturation fraction are varied. At levels of 10^3 per ml or less the curves are roughly log-linear with about the same slope for methyl, ethyl and propyl parabens at saturation; the bacterial population is extinguished in a day or two and no survivors are detected thereafter for as long as three weeks.

The Ferguson principle is clearly applicable under these conditions. With methyl paraben at saturation the survival curve remains log-linear to extinction with the same slope, as the inoculation level is increased to over 10^7 per ml; as its saturation fraction is decreased the rate of kill decreases but kill is persistent and appears to be complete in all cases until the saturation fraction is reduced to less than one-half, where the initial slope of the survival curve approaches zero. With propyl paraben the initial kill rate at saturation remains the same as the inoculation level is increased but at levels of about 10^5 per ml the survival curve becomes concave up within hours of inoculation and in some cases it passes through a deep minimum followed shortly by regrowth at about the same rate as in the unpreserved control. At still higher inoculation levels the minimum is shallow and occurs so early that the initial killing phase (if it occurs at all) is not detected and only a lag relative to the unpreserved control is noticed. The performance of ethyl paraben is intermediate but qualitatively more similar to that of propyl paraben: the transition from persistent kill to the kill-minimum-regrowth pattern occurs but it takes place at higher inoculation levels and lower saturation fractions than with propyl paraben.

We found the same paraben ranking in experiments with *Pseudomonas aeruginosa* ATCC #9721 but with this organism the superiority of methyl paraben is much more striking; at saturation it extinguishes inoculations as high as 10^7 per ml in less than one day while the ethyl and propyl esters cause only transient reductions in survivor counts at inoculations as low as 10^4 per ml.

Because of its strong dependence on both the inoculum size and the solubility of the paraben we first thought that regrowth might be due to depletion of the preservative because of its partitioning into the cytoplasm of both the declining number of survivors and the growing volume of dead bacteria. We had quantitatively predicted such an effect from the reported bulk/cytoplasm partition coefficient (see the Discussion) on the assumption that the rates of growth and reproduction of the survivors is unaffected by the presence of the antimicrobial. We found, however, that the concentration of preservative (methyl and propyl parabens) in the bulk phase does not change detectably by analysis of the supernatant (UV spectrophotometry and high pressure liquid chromatography) after removing the bacteria by centrifugation from samples taken frequently over the entire course of the kill-minimum-regrowth sequence. Adaptation was confirmed as the causative mechanism by using the survivors of the regrowth process in 90% saturated propyl paraben as inoculum into a fresh propyl paraben solution; they grew out promptly while a naive inoculum reenacted the kill-minimum-regrowth sequence.

In later experiments we found that the survivors of a single exposure to propyl paraben retained their immunity completely after forty one days of repeated culturing in the absence of the preservative; to this extent the adaptation is permanent and, as such, it may help explain why extraordinarily refractory strains are occasionally encountered in cosmetic manufacture.

Butyl paraben at high saturation fraction in water initially kills *E. coli* (but not *Pseudomonas aeruginosa*) much more rapidly than the lower esters. An inoculum of 10^3 to 10^5 appears to have been extinguished completely after only an hour or so of exposure to a 90% saturated solution and for several tens of hours no survivors are recovered but as with propyl paraben this may be followed by explosive regrowth. In this case, however, survivors transferred to fresh butyl paraben solution did not fare much better than the naive culture. Because its performance was poor for practical purposes against *E. coli* and even poorer against other bacteria as reported in this paper, we did not pursue further the interesting matter of its distinctive, non-Ferguson behavior.

Finally, we found benzyl paraben at near saturation in water so feebly antimicrobial even against *S. aureus,* that we omitted it from consideration as a useful preservative after only a few further trials.

In this paper we report on some additional experiments in water and on more recent work in prototype products designed to simulate a wide range of real cosmetics.

MATERIALS AND METHODS

Both ATCC strains and wild isolates from products or processing equipment were used. The bacteria were grown at room temperature (ca. 23°C) for 48 hours in a nutrient-buffer salts-glucose solution, pH 6.7, adapted from that of Rye and Wiseman (8) shown in Table I. For convenience, it was prepared as a stock solution at twenty times the concentrations shown.

The fungi were grown on Sabouraud Dextrose Agar (BBL) for seven days. The spores were harvested and suspended in saline.

Table I
Nutrient-Buffer Solution, pH 6.7

NH_4Cl	0.05M
$MgCl_2$	0.0005M
Na_2SO_4	0.0005M
Na_2HPO_4	0.05M
KH_2PO_4	0.05M
Glucose	1 g/l

The compositions of the prototype products, a mineral oil emulsion, a vegetable oil emulsion and a shampoo, are given in Tables II, III and IV. They were prepared from ordinary cosmetic raw ingredients without special efforts to avoid contamination. Usually, a one-kilogram batch was prepared without preservative, withholding a few per cent of the water. The desired amount of preservative was weighed into a 100-g

Table II
Mineral Oil Emulsion

Ingredient	Per kg
Light mineral oil	200 g
Oleyl alcohol, 10 mole ethoxylate	30 g
Nutrient-Buffer Stock Solution[1]	5.0 ml
Preservative	q.s.
Water	to 1 kg

[1]20 times concentrations in Table I.

Table III
Peanut Oil Emulsion

Ingredient	Per kg
Peanut Oil (Planters', 100%)	200 g
Stearyl alcohol, 2 mole ethoxylate	15 g
Stearic acid, 40 mole ethoxylate	20 g
Nutrient-Buffer Stock Solution[1]	5.0 ml
Preservative	q.s.
Water	to 1 kg

[1]20 times concentrations in Table I.

Table IV
Shampoo

Ingredient	Per kg
Sodium lauryl sulfate, 100%	75 g
Sodium lauryl ether (2 mole) sulfate, 30%	100 g
Lauroyl diethanolamide	35 g
Linoleoyl diethanolamide	10 g
Sodium chloride	2.0 g
Orthophosphoric acid, 85%	3.0 g
Nutrient-Buffer Stock Solution[1]	5.0 ml
Preservative	q.s.
Water	to 1 kg

[1]20 times concentrations in Table I.

sub-batch in an eight-ounce screw-cap jar. The preservative was dissolved by heating for several hours at 60°C with occasional mixing. After cooling to room temperature, the pH was adjusted with 4N HCl or NaOH and water was added to 100.0 g.

Emulsions prepared in this fashion are of poor stability but when higher levels of emulsifiers were used to improve the quality of the base formulas, the addition of each paraben had a specific degrading effect, in some cases causing phase inversion. Since it would have been pointless to compare, say, methyl and ethyl parabens in an oil-in-water system with propyl and butyl parabens in water-in-oil, we accepted uniformly poor stability as the lesser evil.

The concentration (basis water content) of nutrient salts and glucose in the prototype products is about one-eighth of that in the aqueous broths. The intent here is to swamp out the possibly distorting effects of chance nutrification and the nutrient differences inherent in the three product formulas.

It was not possible to measure inoculum growth in unpreserved control systems because these were invariably found grossly contaminated with stray microbes but the rapid growth to about 10^7/g of recognizable inoculum bacteria and the persistence of mold spores in poorly preserved systems left no doubt that these prototype products, like their real cosmetic product counterparts will support damaging growth of the challenge organisms.

Systems challenged with bacteria at 10^5/g or mold spores at 10^3/g were incubated at room temperature. Aliquots were diluted in one tenth strength Nutrient Broth (BBL), dispersed in Nutrient Agar (BBL) and incubated for three days at room temperature before counting. All challenged systems were sampled about one hour after inoculation, on day 1, 2, 3 or 4 and on days, 7, 14 and 21. Sampling was terminated on or after day 7 only if two successive counts clearly showed persistence or growth of bacteria.

RESULTS

Water nutrified with mineral salts and glucose, buffered at pH 6.7 and saturated with methyl paraben successfully resisted challenge by two fungi and by thirteen gram-negative bacterial strains including the most resistant wild isolates in our collection. In the same medium saturated with ethyl paraben, five of the thirteen bacteria grew out; propyl paraben failed against ten of them and butyl paraben failed against all but one bacterium and one mold. Table V shows these results in the form of the kill time which we define throughout this report as the earliest time in the sampling schedule at which the count of survivors was less than 10/g (no survivors detected) and remained so until 21 days after inoculation. These data clearly rank the parabens: methyl > ethyl > propyl > butyl. (They also imply a ranking of the challenge organisms in terms of their ability to resist attack by the parabens, and they are listed in Table V in this fashion.) Several of the entries in Table V are "G(A)" indicating growth after adaptation. In these cases 95% or more of the inoculum died in the first few days but the survivors grew to the limit of the nutrient system.

In Table VI we show the kill time of *P. aeruginosa* ATCC #9721 in saturated aqueous paraben solutions at various pH's. In this experiment there is less discrimination among the parabens, but the indication remains that the efficacy ranking is not strongly pH dependent; from low to high pH, methyl or ethyl paraben is the most potent, butyl paraben is least.

Table V
Kill Time of Parabens at Saturation in Nutrient-Buffer Solution pH 6.7

Microbe	Origin	Code	Kill time, days*			
			Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben
Serratia marcescens	Wild	ED-2	7	G	G	G
Pseudomonas aeruginosa	Wild	MEM	1	G	G	G
Pseudomonas aeruginosa	Wild	BB-1A	1	G(A)	G	G
Enterobacter hafnia	Wild	LSC	1	G(A)	G	G
Serratia liquifaciens	Wild	T-1	1	G(A)	G	G(A)
Pseudomonas cepacia	Wild	RS	7	7	G	G
Pseudomonas aeruginosa	Wild	SM-5	4	14	G	G
Pseudomonas aeruginosa	ATCC	#9721	4	14	G	G
Serratia rubidaea	Wild	CW-1	4	4	G	G
Pseudomonas putida	Wild	SM-6	1	1	G	G
Enterobacter cloacae	Wild	PLS-2	1	1	1	G(A)
Escherichia coli	ATCC	#25922	1	1	1	G(A)
Enterobacter hafnia	Wild	SG	4	14	14	14
Aspergillus niger	ATCC	#16404	4	4	4	>21
Penicillium species	Wild		7	1	7	1

*G indicates heavy growth; G(A) indicates growth preceded by 95% or greater kill.

Table VI
Kill Time of Parabens at Saturation in Nutrient-Buffer Solution at Various pH's Challenged with *Pseudomonas aeruginosa* ATCC #9721

pH	Buffer[1]	Kill time, days			
		Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben
5.4	Malic acid	1	1	1	G(A)
6.7	Phosphate	1	G(A)	G(A)	G(A)
7.7	Tris-Phosphate	1	1	G(A)	G(A)
8.6	Tris-Glycine[2]	1	1	1	1

[1]Apart from the buffer changes and substitution of glycine for NH_4^+, the nutrients are as given in Table 1.
[2]In this solution, glycine is also the source of nitrogen.

Table VII shows the kill time of ED-2, a very resistant isolate identified as *Serratia marcescens,* in neutral mineral oil and peanut oil emulsions and in the shampoo, with and without nutrients with 0.8% nominal paraben level in all cases. In the mineral oil emulsion the methyl, ethyl and propyl parabens readily dissolve to this extent at 60°C but crystallize out in part on standing at room temperature; these systems are at saturation at about 0.6%. Re-precipitation does not occur with butyl paraben in the mineral oil emulsion nor with any of the parabens in the peanut oil emulsion or the shampoo; these systems are at or below saturation.

In the nutrified systems, only methyl paraben kills this organism in the emulsions; in the shampoo even methyl paraben fails to check its growth. In the absence of nutrient the preservatives do better in general; methyl and ethyl parabens are effective in the emulsions but propyl and butyl parabens still fail, and in the shampoo all four parabens fail.

Table VII
Kill Time of 0.8% Paraben in Prototype Products at pH 6.5 Challenged with ED-2[1]

Medium	Paraben	Kill time, days	
		No Nutrient[2]	Nutrified
Mineral Oil Emulsion	Methyl[3]	1	2
	Ethyl[3]	1	G(A)
	Propyl[3]	G(A)	G
	Butyl	G(A)	G
Peanut Oil Emulsion	Methyl	1	2
	Ethyl	7	G
	Propyl	G	G
	Butyl	G	G
Shampoo	Methyl	G	G
	Ethyl	G	G
	Propyl	G	G
	Butyl	G	G

[1]*Serratia marcescens*, wild isolate.
[2]Orthophosphate buffer.
[3]Saturated.

In the mineral oil emulsion at saturation the performance of the parabens is not very different from that in water. In Tables VIII and IX we show kill time data on the first three parabens at saturation in the peanut oil emulsion and in the shampoo. Performance is marginally better in the peanut oil emulsion than in water, but the

Table VIII
Kill Time of Parabens at Saturation in Nutrified Peanut Oil Emulsion, pH = 6.5

Microbe[1]	Kill time, days[2]		
	Methyl Paraben 1.0-1.2%	Ethyl Paraben 0.8-1.0%	Propyl Paraben 1.2-1.6%
A. niger, ATCC 16404	2	2	7
P. aeruginosa, ATCC 9721	1	1	1
EB-1	1	2	1
ED-2	1	G	G

[1]EB-1 and ED-2 are wild strains of *Serratia marcescens*.
[2]Percentages are approximate concentrations.

Table IX
Kill Time of Parabens at Saturation in Nutrified Shampoo, pH 6.5 (ca. 2.5% in all cases)

Microbe[1]	Kill time[2]		
	Methyl Paraben	Ethyl Paraben	Propyl Paraben
A. niger, ATCC 16404	1d	1d	1d
P. aeruginosa, ATCC 9721	1h	1h	1h
EB-1	1h	1h	G(A)
ED-2	1h	1h	G(A)

[1]EB-1 and ED-2 are wild strains of *Serratia marcescens*.
[2]Days or hours as indicated.

ranking methyl > ethyl > propyl is still evident. In the shampoo, kill rates are enhanced relative to saturated water, but propyl paraben, even at a concentration of about 2.5%, ultimately fails against two of the three bacteria.

Binary mixtures of the parabens were examined in the emulsions as shown in Tables X and XI, which show kill times for *P. aeruginosa* and ED-2. In the peanut oil emulsion

Table X
Kill Time of Methyl Paraben and Mixtures in Nutrified Peanut Oil Emulsion, pH ca. 6.7

Paraben System	Kill time, days	
	P. aeruginosa[1]	ED-2
0.8% methyl	1	2
0.4% methyl	G	G
0.4% methyl, 0.4% ethyl	1	G
0.4% methyl, 0.4% propyl	G(A)	G
0.4% methyl, 0.4% butyl	G	G

[1]ATCC 9721

Table XI
Kill Time of Methyl Paraben and Mixtures in Nutrified Mineral Oil Emulsion, pH ca. 6.5

Paraben System	Kill time, days	
	P. aeruginosa[1]	ED-2
0.8% methyl[2]	1	2
0.4% methyl	1	G
0.4% methyl, 0.4% ethyl	1	1
0.4% methyl, 0.4% propyl	1	G

[1]ATCC 9721
[2]Saturated.

methyl paraben suffices at 0.8% but fails against both organisms at 0.4%. Addition of 0.4% of a second paraben gives improvement in the order ethyl > propyl > butyl, but in no case is the more resistant bacterium killed as it is by 0.8% methyl paraben alone.

The mineral oil system is similar except that the methyl/ethyl combination is a bit better than methyl alone. Note that this is not an equal weight comparison because of partial recrystallization of the methyl paraben at 0.8%. If we take the solubilities of both methyl and ethyl paraben as 0.6% in this system, then at 0.4% of each (two-thirds of saturation with each) then the cumulative saturation fraction is about 1.3. In aqueous broths we have found that such multiply saturated systems can be even more lethal than methyl paraben alone at saturation since the saturation scale extends beyond unity.

DISCUSSION

Lang and Rye (9) found that the growth of *E. coli* remains exponential or log-linear in the presence of methyl, ethyl and propyl parabens with decreasing slope up to about half their saturation concentrations. To a good approximation, their data can be summarized as a demonstration that the growth rate constant, k, in $N = N^0e^{kt}$, depends on the paraben saturation fraction as

$$k = k_0(1 - as_i), \quad \text{(I)}$$

where k_0 is the growth rate constant when no antimicrobials are present and s_i is the saturation fraction of the ith paraben.

The Ferguson principle is implied by the absence of the subscript on the dimensionless constant a (which has a value of about 2.0); all three parabens have the same inhibitory effect when their levels are expressed as fraction of saturation.

By independent radiochemical measurements, Lang and Rye also showed that the intracellular paraben concentration, c_i', is approximately the same for all three homologs when their equilibrium levels in the extracellular or bulk phase are expressed as saturation fractions, s_i:

$$c_i' = f_i c_i = f^* c_i/\sigma_i = f^* s_i, \quad \text{(II)}$$

where c_i is the bulk concentration and σ_i is the solubility. The constant f^* like the constant a in Equation I, has the same value for all three homologs (about 7.0 g/l).

In the Lang and Rye study, the applicability of the Ferguson principle is both demonstrated and "explained," where "explanation" follows from the plausible assumption that the parabens are equitoxic at equal intracellular concentrations. The assumption is plausible, in turn, on the further conjecture that the parabens are toxic to microbes because they partition reversibly into the lipid bilayer of the cell membrane and disorder its barrier function and the functions of embedded transport enzymes. A molecule of one homolog ought then to be about as disruptive as that of another.

In retrospect, it is not too surprising that such a structure of assumptions and conjectures failed to support extrapolation. All that remains of the Ferguson principle in the range of paraben concentrations beyond half saturation (the limit of the Lang and Rye study) is an indication that at low levels of inoculation the initial kill rate is given by Equation I. Thereafter, survival and growth are determined by the rate of adaptation which increases markedly with the molecular weight of the paraben.

Solubility in the medium does not serve as the sole index of efficiency as it would if the Ferguson principle were applicable, but it remains a crucial property. Methyl paraben is a potent antimicrobial in water at saturation at 0.2%, but it fails at 0.4% in the emulsions and at 0.8% in the shampoo; it is a good preservative only for products in which it is not too soluble. Propyl paraben is inadequate in water at 0.03% and remains so at 0.8% in the emulsions and even at about 2.5% in the shampoo.

For practical purposes, our earlier solubility-efficacy proposal (1,2) is supplanted by a strong endorsement of methyl paraben as the best member of the series, to be used at the highest practical concentration, with a secondary recommendation of ethyl paraben as a supporting preservative when the amount of methyl paraben that can be used is limited by regulation (0.4% maximum in Brazil, for example) or by solubility at low storage temperatures. Only rarely might it be useful to include propyl paraben as a third preservative.

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