

The effect of pH on the multiplication of a pseudomonad in chlorhexidine and cetrimide

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SYNOPSIS *Pseudomonas cepacia* (*Ps. multivorans*) can become adapted to a 1 in 30 dilution of Savlon hospital concentrate in distilled water, and can multiply in it.

The organism so adapted did not survive in Savlon of the same strength made with hard tap water (pH 7.2).

This effect was due to the difference of pH between the two solutions.

At the pH of the distilled water solution (pH 6.0) Savlon also showed reduced activity against *Proteus vulgaris*, but activity against *Ps. aeruginosa* was not affected.

An outbreak of wound infection due to *Pseudomonas cepacia* (*Ps. multivorans*) was described by Bassett, Stokes, and Thomas (1970). The organism was isolated from wound swabs from nine patients, and subsequently from bottles of a 1 in 30 dilution of Savlon hospital concentrate (Savlon H.C.). This, the highest concentration that the manufacturers recommend for use, contains chlorhexidine gluconate 0.05% and cetrimide 0.5%. Further investigations disclosed *Ps. cepacia* in a water softener, in samples of de-ionized and distilled water, and in the mains water supplied to the hospital concerned and to the surrounding district.

Because it was possible to select the organism from a number of water samples by the addition of autoclaved Savlon, we expected that the organism would multiply under experimental conditions in 1 in 30 Savlon in tap water. In fact it commonly failed to survive, except when the inoculum was taken from solid medium and consisted of large aggregates of bacteria. When survivors were obtained, they had an adaptive resistance to Savlon which was lost on subculture. No multiplication of *Ps. cepacia* in Savlon was observed until dilutions of the disinfectant in distilled water were used. A 1 in 30 dilution of Savlon H.C. in distilled water allowed the multiplication of the organism in its adapted state.

The effect of the water used as diluent on the activity of Savlon was investigated further.

Methods

CULTURES

Pseudomonas cepacia (no. A46/69) was obtained from the mains water supply of the hospital in which wound infection with this organism had occurred. The organism was maintained as a suspension in tap water; viability was not lost in one year of observation. The viability of the organism in nutrient media was short. Fresh cultures were obtained from the aqueous suspension as required. This is referred to as the 'unadapted' organism.

Ps. cepacia, as above, adapted to 1 in 30 Savlon H.C. in the manner described below, was maintained in 1 in 30 Savlon in distilled water. Because subculture produced reversion to sensitivity, suspensions for use were prepared by centrifugation of the Savlon and resuspension of the organisms in distilled water.

Ps. cepacia no. NCTC10661

Ps. cepacia no. A103/69

Proteus vulgaris no. NCTC4635

Ps. aeruginosa no. NCTC6749

MEDIA

Modified Wright and Mundy's medium (Oxo Ltd) A modification of the medium described by Wright and Mundy (1960) contained acid-hydrolysed casein 11.4 g/l, MgSO₄ 0.1 g/l, KH₂PO₄ 1.5 g/l, Na₂HPO₄ 4.0 g/l, thiamine 0.01 g/l, and nicotinamide 0.01 g/l. Dextrose 10.0 g/l was added.

Asparagine agar

This consisted of 1% L-asparagine either in tap water or in distilled water, solidified with 1% Oxoid no. 1 agar. The tap water and distilled water media were prepared both with and without the addition of 1 part in 30 of Savlon H.C. Further modifications of electrolyte content and of pH were made as described below.

Glucose broth

This was prepared from Oxoid nutrient broth no. 2, by the addition of 0.5% glucose.

Lubrol broth

This was Oxoid nutrient broth no. 2, with 7.5% lubrol W added.

Blood agar

This was 5% horse blood in Hartley's digest agar base.

ADAPTATION OF *Ps. cepacia* TO GROWTH IN SAVLON

Survivors were obtained when large aggregates of *Ps. cepacia* from culture on solid medium were exposed to 1 in 30 Savlon H.C. These surviving organisms were used as an inoculum for 1 in 30 Savlon prepared in a 1% peptone solution in distilled water. The initial viable count was 10 organisms per millilitre. The inoculated solution remained at room temperature in the dark for four weeks, in which time a thousandfold increase in the viable count occurred. In the next four weeks the organisms were deposited by centrifugation and resuspended in fresh 1 in 30 Savlon H.C. in distilled water on three occasions. The viable count rose steadily to reach 4.1×10^7 organisms per ml by the end of this period.

MINIMUM INHIBITORY CONCENTRATION (MIC) TESTS

These were performed in doubling dilutions in 1 ml volumes of glucose broth, with pH adjusted to the appropriate value. Each tube received an inoculum of 0.02 ml of an overnight culture of the test organism, and was then incubated at 30°C for 24 hours. Subcultures were made on blood agar plates. Tests were carried out in duplicate.

CAPACITY TESTS

These were carried out as described by Kelsey and Sykes (1969). The test organisms, except for the Savlon-adapted *Ps. cepacia*, were grown overnight in modified Wright and Mundy's medium after serial subculture in the same medium. The suspensions contained between 1.0×10^8 and 1.0×10^9

organisms per ml and in certain tests, as specified below, the equivalent of 2% dry weight of bakers' yeast was added. The sample 0.02 ml volumes taken from the tests were added to tubes of 10 ml Lubrol broth: these were incubated for 48 hours at 32°C after which all that showed turbidity were subcultured.

The tests on the adapted organism differed in that the suspension was prepared without subculture, as described above, and contained only 1.8×10^8 organisms per ml, without yeast. The sample tubes were incubated at 32°C for 72 hours.

In all tests, the pH values of the suspensions were adjusted to match those of the Savlon dilutions under test.

MEASUREMENT AND ADJUSTMENT OF pH

Measurements of pH were made on a Pye Dynacap pH meter. Adjustments of pH were made with 1/5 N and 1/20 N solutions of HCl or NaOH.

Results

Ps. cepacia, adapted to Savlon as described, grew readily when subcultured on to asparagine agar made with either tap water or distilled water. In the case of the tap water medium the addition of Savlon rendered the medium completely inhibitory, but in the distilled water medium Savlon caused a reduction in the size of colonies, but not in their number.

The addition to Savlon asparagine agar in distilled water of CaCl_2 and MgSO_4 to give concentrations of the cations similar to those in the hard tap water did not make the medium inhibitory to the adapted organism.

EFFECT OF pH

Ps. cepacia grew vigorously in glucose broth adjusted to pH values from 5.4 to 7.8 but grew less well at pH 8.2: the range below 5.4 was not tested.

The pH of 1 in 30 Savlon H.C. in hard tap water was found to be 7.2, while that of similar solutions made in water distilled once in glass were in the range 5.8 to 6.2. When the pH of Savlon asparagine agar in distilled water was raised from its initial value of 5.4 to 7.0 the medium became inhibitory to adapted *Ps. cepacia*: conversely, when the pH of the tap water medium was lowered from 7.0 to 5.4, growth occurred.

Minimum inhibitory concentration tests were carried out at pH 7.2 and at pH 6.0 with Savlon, chlorhexidine, and cetrимide against unadapted *Ps. cepacia*. The results are shown in Table I. The inhibitory power of both constituents of Savlon was reduced at the lower pH, but the concentration of chlorhexidine in 1 in 30 Savlon H.C. (0.05%) was

pH of Test	MIC of		
	Savlon H.C. ¹	Chlorhexidine	Cetrimide
7.2	1 in 480	0.013%	0.13%
6.0	1 in 120	0.05%	> 0.5%

Table I Effect of pH on MIC of disinfectants against unadapted *Ps. cepacia*

¹Savlon H.C. is equivalent to 1.5% chlorhexidine gluconate and 15% cetrimide.

still just sufficient to inhibit the unadapted organism in the test at this pH.

Capacity tests were carried out in the presence of 2% baker's yeast with the unadapted *Ps. cepacia* and 1 in 30 dilutions of Savlon H.C. in tap water and in distilled water. Each of these solutions was divided, and the pH of one half was adjusted so that, for each diluent, there was one Savlon solution at pH 7.2 and another at pH 6.0. The experiment was carried out in quadruplicate. The results are shown in Table II. All these solutions would be considered to have passed the capacity test, but there was a highly significant difference between the numbers of tubes showing growth in the tests at pH 7.2 and at pH 6.0 ($\chi^2 20.88$; $P < 0.001$). There was no significant difference between the numbers of tubes showing growth in tests in tap water and in distilled water at each pH.

The capacity tests in which the adapted organism was used, without yeast, showed the expected result (Table II). Growth occurred in all tubes from Savlon at pH 6.0, but in none of those from Savlon at pH 7.2. In the case of the unadapted organism without yeast, Savlon H.C. passed the test at 1 in 250 dilutions

at pH 7.2, but failed, with growth in all tubes, at pH 6.0.

To determine the influence of pH on the activity of Savlon against other organisms, test organisms *Proteus vulgaris* no. NCTC4635 and *Ps. aeruginosa* no. NCTC6749 were used (Table III). Quadruplicate capacity tests were carried out with dilutions of Savlon H.C. that gave marginal results against the test organisms in order that changes in activity should be readily detectable.

P. vulgaris was used with Savlon H.C. at 1 in 40 dilution, in the presence of yeast. A significantly greater number of tubes showed growth after the tests at pH 6.0 than after those at pH 7.2 (P less than 0.005).

Ps. aeruginosa was used with Savlon H.C. 1 in 400 without yeast. There was no indication that the activity of the disinfectant against this organism was affected by the change from pH 7.2 to 6.0.

The long-term behaviour of adapted *Ps. cepacia* was examined in 1 in 30 dilutions of Savlon H.C. prepared in 0.1 M phosphate buffers at pH values 5.8, 6.2, 6.6, 7.0, and 7.4. A suspension of the adapted organism was divided between 10 test tubes and the organisms were deposited by centrifugation. The supernatants were discarded and replaced by the buffered solutions, giving duplicate tests at each pH. Subcultures were made after the tubes had stood for 14 days at room temperature. No growth was obtained from the tubes at pH 7.4, but survivors were obtained from the tubes at pH 7.0 and 6.6 and heavy growth was obtained from the tubes at pH 6.0 and 5.8.

To show that the ability to adapt to 1 in 30

Suspension			Savlon H.C.		Ratio of No. of Tubes Showing Growth to No. of Tubes Inoculated	
State of Organism	pH	Yeast	Dilution	Diluent		
Unadapted	7.2	2%	1 in 30	Hard tap water	7.2	0/60
Unadapted	7.2	2%	1 in 30	Distilled water	7.2	2/60
Unadapted	6.0	2%	1 in 30	Hard tap water	6.0	13/60
Unadapted	6.0	2%	1 in 30	Distilled water	6.0	11/60
Adapted	7.2	Nil	1 in 30	Distilled water	7.2	0/60
Adapted	6.0	Nil	1 in 30	Distilled water	6.0	60/60
Unadapted	7.2	Nil	1 in 250	Distilled water	7.2	6/30
Unadapted	6.0	Nil	1 in 250	Distilled water	6.0	30/30

Table II Effect of pH on capacity tests with Savlon H.C. and inocula of *Ps. cepacia*

Suspension			Savlon H.C.		Ratio of No. of Tubes Showing Growth to No. of Tubes Inoculated
Organism	pH	Yeast	Dilution	pH	
<i>P. vulgaris</i>	7.2	2%	1 in 40	7.2	4/60
<i>P. vulgaris</i>	6.0	2%	1 in 40	6.0	17/60
<i>Ps. aeruginosa</i>	7.2	Nil	1 in 400	7.2	11/60
<i>Ps. aeruginosa</i>	6.0	Nil	1 in 400	6.0	11/60

Table III Effect of pH on capacity tests with Savlon H.C. and inocula of *P. vulgaris* and *Ps. aeruginosa*

Savlon was not peculiar to one strain of *Ps. cepacia*, the adaptation of two other strains (NCTC10661 and A103/69) was carried out in the manner described above. Viable counts of 2.8×10^6 and 2.2×10^6 organisms per ml were obtained in 1 in 30 Savlon H.C.

Discussion

Savlon is a mixture of chlorhexidine with a quaternary ammonium compound, cetrimide, and it is therefore to be expected that it would be affected by changes in pH. The loss of activity by quaternary ammonium compounds in acid solutions was described by Dunn (1937) and by Gershenfeld and Milanick (1941), and the greatly reduced bactericidal effect of chlorhexidine against *Staphylococcus aureus* under acid conditions was noted in the original description of that disinfectant (Davies, Francis, Martin, Rose, and Swain, 1954).

The results reported here show that in a 1 in 30 dilution of Savlon H.C. in hard tap water a pH on the alkaline side of neutrality is obtained, and under these conditions both constituents of Savlon are present in concentrations effective against *Ps. cepacia*. However, when the dilution is made in distilled water and a more acid solution is produced, only the chlorhexidine is present in inhibitory concentration, and adaptation of the organism and multiplication in the disinfectant may occur. It has been shown that the adapted organism grown in 1 in 30 Savlon at pH 6.0 is still sensitive to the same concentration of Savlon at the higher pH.

Ps. cepacia is evidently more resistant to Savlon than is the strain of *Ps. aeruginosa* that was tested, and it seems reasonable to infer that a 1 in 30 dilution of Savlon H.C. at pH 7.2 would be proof against the multiplication of either organism, provided that no inactivating substance were present. One cannot assert that no organism exists that could multiply in such a solution, but the solution would be more resistant to contamination and more effective in use than one at a lower pH. If solutions of this strength must be held in stock, it is probably still desirable to add a protective agent, such as iso-propyl alcohol (4%).

Slight inactivation of Savlon occurs in hard water

because of the divalent cations present, and the solutions produced are slightly turbid. It is presumably for these reasons that distilled or de-ionized water is often used, but these, or indeed a mains water supply of low pH, may produce Savlon dilutions of reduced activity.

Dilutions of Savlon H.C. have virtually no buffering power, and contact in use with matter that has a low pH might well take the disinfectant beyond the narrow pH range considered in this study and produce greater loss of activity. The range of uses for which Savlon is suitable should be decided with this in mind.

It is clear that, although a disinfectant dilution may pass the capacity test, this does not preclude the possibility that the test organism may adapt to and multiply in that dilution. The test does not purport to give information on this point. However, the important effect of pH on disinfectants may need to be considered in the capacity test, particularly the pH of the bacterial suspension which has considerable buffering power, while the disinfectant solution may, as in this case, have little or none. It would seem desirable that the pH of the test should always be stated when a capacity test result is given, and that tests should cover the pH range that recommended uses might involve.

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