Effect of Monosodium \( n \)-Alkylsalicylates on Spores of \textit{Bacillus subtilis}

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The effects of several monosodium \( n \)-alkylsalicylates on washed spores of \textit{Bacillus subtilis} have been examined. None of the compounds was sporidical, but in general they prevented dormancy induced by incubating the spores in water. This effect was related to the position in the ring and size of the alkyl groups substituted on the salicylic acid nucleus.

A series of alkylsalicylic acids have been synthesized (1) because of their similarity to anacardic acid, a naturally occurring mixture of alkyl salicylic acids having the average constitution of 6-pentadecadienyl-salicylic acid (1, 8, 9). Monosodium anacardate possesses high in vitro activity against vegetative bacteria, but it has no significant sporidical action (4). Recently Buckley and Thomas (3) reported that monosodium \( n \)-alkylsalicylates also possess significant antimicrobial activities and that these activities are influenced by the size and position of the alkyl side chains in salicylic acid. The effects of monosodium \( n \)-alkylsalicylates against the spores of \textit{Bacillus subtilis} have now been examined to determine whether any compounds of the series have significant sporidical activity.

MATERIALS AND METHODS

\textbf{Preparation of spores.} Vegetative cells of \textit{B. subtilis} (supplied by Microbiology Department, University of Sydney; catalog no. 21.6) were grown from freeze-dried spores through two subcultures in Oxoid nutrient broth no. 2 (20 ml, pH 7.3; 37 C for 18 hr with shaking at 80 oscillations/min). Spores were produced by flooding the second vegetative culture onto the surface of sporulation medium A of the \textit{British Pharmacopoeia, 1963} ed., layered in 180-ml flat, screw-capped bottles. After 5 days at 37 C, the culture was virtually all spores which were harvested with sterile distilled water, centrifuged from water three times, suspended in sterile distilled water, and maintained at 80 C for 10 min. The heated suspension was diluted to about 4 \( \times \) 10\(^8\) spores/ml (viable count) and subsequently stored at 4 C.

\textbf{Drug-spore mixtures.} Drug solution prepared as previously described (3) was added aseptically to 1 ml of aqueous spore suspension, previously equilibrated to 37 C in a 5-ml McCartney bottle. If necessary, sterile distilled water was added to achieve a final volume of 2 ml, a maximum concentration of 10% (v/v) ethanol, and 10\(^4\) to 2 \( \times \) 10\(^4\) spores/ml (viable count). Spore concentrations in these suspensions were determined before (zero) and after 2 and 24 hr of incubation at 37 C from samples (0.1 ml) which were diluted in separate 50-ml volumes of sterile distilled water at room temperature. From each dilution, five samples (0.1 ml) were separately plated in nutrient agar (20 ml, 60 C). The plating medium (Oxoid nutrient broth no. 2, solidified with Oxoid agar no. 3, 1.2%; pH 7.3) was sterilized (121 C for 15 min) in 150-ml amounts in screw-capped bottles. Plates were dried (37 C for 2 hr) and incubated (32 C for 16 hr) before colony counts were made with an electric counter.

\textbf{RESULTS AND DISCUSSION}

The results are summarized in Table 1 from which it can be seen that none of the alkylsalicylates is sporidical but, in general, the compounds facilitate spore germination. This becomes evident when the reduced counts which followed incubation (2 to 24 hr) in water, dilute ethanol (10%, v/v), and phenol [0.5% (w/v); ethanol (10%, v/v)] are compared with the counts derived from the salicylate mixtures. These reduced counts are considered to be due to an induced dormancy rather than a sporidical effect since none of these agents would be sporidical in this test (14).

In the case of water, the observed effect may be related to that reported by Keynan et al. (5), who demonstrated a temperature-dependent reversal of heat activation in spores of \textit{B. cereus} incubated in water. In the present study, reduced germination levels were observed only after 2 hr of incubation at 37 C, although greater reductions were apparent after 24 hr. After 2 hr of incubation in the presence of salicylates, except in the case of monosodium 3-\( n \)-heptylsalicylate, the mean spore counts were similar to or higher than
## Table 1. Effect of monosodium n-alkylsalicylates on spores of B. subtilis

| System                  | Mean spore concn after incubation | Per cent change in mean spore concn after incubation | Standard error of mean spore concn | Limits of error of mean spore concn (t = 2.78, P = 0.05)* |
|-------------------------|-----------------------------------|------------------------------------------------------|------------------------------------|----------------------------------------------------------|
|                         | Zero 2 hr 24 hr 2 hr 24 hr       | Zero 2 hr 24 hr                                      | Zero 2 hr 24 hr                     | Zero 2 hr 24 hr                                           |
| Water                   | 1,944 1,503 1,060 –23 –45        | 13.73 38.26 127.34                                  | 1,906–1,982 1,397–1,609            | 706–1,414                                                |
| Ethanol (10%, v/v)      | 1,955 1,183 620 –29 –63          | 35.36 37.80 13.51                                  | 1,557–1,753 1,078–1,288            | 588–658                                                  |
| Phenol (0.5%, w/v)c     | 1,531 1,048 832 –32 –46          | 52.57 69.81 26.58                                  | 1,385–1,677 854–1,242              | 758–906                                                  |
| Sodium salicylate (5%, w/v)c | 852 686 547 –23 –36          | 57.11 32.69 21.13                                  | 693–1,011 596–777                  | 488–606                                                  |
| Phenol (0.5%, w/v)c     | 1,223 965 608 –21 –51           | 65.15 18.64 16.70                                  | 1,042–1,404 913–1,017              | 561–655                                                  |
| Sodium salicylate (5%, w/v)c | 1,684 1,241 856 –26 –49          | 34.76 23.04 25.96                                  | 1,587–1,781 1,177–1,305            | 784–928                                                  |
| 3-n-Octylc (0.5%, w/v)c | 1,749 1,399 1,727 –20 Nil       | 72.65 81.54 106.8                                  | 1,376–1,748 1,353–1,857            | 954–1,286                                                 |
| 3-n-Octylc (0.5%, w/v)c | 1,942 1,767 1,924 –9 Nil         | 56.34 69.47 55.41                                  | 1,547–1,951 1,172–1,626            | 1,387–2,067                                               |
| 5-n-Octylc (0.5%, w/v)c | 1,414 1,898 1,575 +25 Nil       | 26.29 66.27 79.23                                  | 1,785–2,099 1,574–1,960            | 1,770–2,078                                               |
| 3-n-Dodecylc (0.25%, w/v)c | 1,735 1,819 1,274 +5 –27        | 48.39 70.68 45.65                                  | 1,600–1,870 1,623–2,015            | 1,147–1,411                                               |
| 4-n-Dodecylc (0.25%, w/v)c | 1,946 1,930 2,125 Nil +9        | 53.11 93.53 38.11                                  | 1,798–2,094 1,670–2,190            | 2,019–2,231                                               |
| 5-n-Dodecylc (0.25%, w/v)c | 1,830 1,841 1,826 Nil          | 70.23 119.53 85.23                                  | 1,635–2,025 1,510–2,172            | 1,589–2,063                                               |
| 3-n-Tetradecylc (0.5%, w/v)c | 1,617 1,823 1,432 +13 –12       | 73.9 99.72 25.38                                  | 1,412–1,822 1,546–2,100            | 1,361–1,503                                               |
| 3-n-Tetradecylc (0.5%, w/v)c | 2,120 2,020 1,658 Nil –22        | 50.07 54.66 51.10                                  | 1,981–2,259 1,968–2,172            | 1,516–1,800                                               |

* Mean count derived from five plates.
† Values expressed × 10⁷/ml.
‡ Solution also contained ethanol (10%, v/v).
§ Nature of alkyl substituent in monosodium n-alkylsalicylate.
¶ Solution in water.
∥ Mean count derived from four plates.
* t = 3.18; P = 0.05, 3 degrees of freedom.
preincubation counts. After 24 hr of incubation, sodium salicylate, monosodium 5-n-octylsalicylate, and monosodium 3-n-tetradecylsalicylate were less effective in maintaining the spore count than after 2 hr of incubation. However, in none of these cases was the reduction in spore count as large as that which followed 24 hr of incubation in water, phenol, or dilute ethanol.

It is inferred from the 24-hr results that the effect of alkylsalicylates on spore germination may be influenced by the size and position of the alkyl substituents, since monosodium 5-n-octylsalicylate was less effective than its 3-n-isomer and, although the three dodecyl homologues were effective, there was a fall off in activity in the case of monosodium 3-n-tetradecylsalicylate. A similar pattern of activities has been observed previously in relation to the antibacterial activities of these compounds (3). Rode and Foster (12) have demonstrated that the length of the alkyl chain, in a group of primary alkylamines, has an influence on the germination stimulant activity of these compounds.

It is possible that the alkylsalicylates were acting as triggers of germination as defined by Sussmann and Halvorson (13). The alkylsalicylates have the chemical structure typical of anionic surface-active agents (6) and, as salicylates, are potential chelating agents (7). Since it is known that certain chelating agents and anionic surface-active agents promote spore germination (10, 12), it is suggested that the function of alkylsalicylates as germination triggers may be related to their surface activity and chelating ability.

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