Relationship between Salt Tolerance and Resistance to Chloramphenicol in *Staphylococcus pyogenes.** (23992)

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It has been recognized for some time that Gram positive cocci are capable of growth in comparatively high concentrations of salt. Hill and White(5) recommended media containing from 2 to 20% NaCl for selective isolation of staphylococci, and Koch(6) suggested that agar containing 7.5% NaCl or broth containing 15% NaCl be used as a selective medium for isolation of all staphylococci. Proteose-lactose agar containing 7.5% NaCl was found by Chapman(2) to be the medium of choice for isolation of micrococci from fecal material. McVeigh and Hobdy(7) found that 2 penicillin-resistant strains of *Micrococcus pyogenes* had lost the ability to grow in proteose-lactose medium containing 7.5% NaCl; strains resistant to streptomycin, aureomycin, chloramphenicol and subtilin retained their ability to grow in this medium. This paper reports a comparative study of salt tolerance in chloramphenicol sensitive and resistant strains of *Staphylococcus pyogenes*. Also, the combined effect of salt and chloramphenicol upon growth has been examined.

Methods. The organisms used were a strain of *S. pyogenes* which was sensitive to chloramphenicol (Strain S) and a resistant mutant (Strain R) derived therefrom(10). The resistance of Strain R was approximately 100 fold that of Strain S(10). The basal medium contained Difco Casamino acids, salts, vitamins and glucose(10). Glucose was autoclaved separately and added aseptically to sterile cooled medium to eliminate any error due to caramelization and preclude spurious results from uncontrollable stimulatory products of heat degradation of glucose(9). Riboflavin was omitted from the medium because of its toxicity for the test organisms(11). Inoculum cells were harvested by centrifugation from 20-24 hour cultures in AC broth (Difco), washed twice in basal salt solution, resuspended and diluted to the desired concentration. All tubes of test series were inoculated with approximately 250 cells. After incubation at 37°C, growth was measured turbidimetrically with Bausch and Lomb Spectronic 20 photoelectric colorimeter with setting of 525 mμ. Every experiment was performed in duplicate and repeated at least once.

Results. When the 2 strains were inoculated into the basal medium containing increasing concentrations of NaCl, KCl, and glucose, it was found that NaCl and KCl were more inhibitory toward Strain R than toward Strain S (Fig. 1). In some instances, this difference was quite marked. For example, 0.8 M KCl was completely inhibitory to Strain R, whereas 1.8 M was required to inhibit Strain S. Other experiments indicated that NH_4Cl was slightly less inhibitory on a molar basis than KCl or NaCl, whereas $MgCl_2$ was more inhibitory. However, precipitation of the latter upon autoclaving made growth measurements by nephelometry impossible. By subculture it was determined that 0.6 N $MgCl_2$ demonstrated primary toxicity toward Strain R, while 1.0 N was required to render Strain S non-viable.

The difference in tolerance to glucose is insignificant; however, it should be noted that the osmotic pressure of glucose at highest concentration tested is only about one-half that of the salts.

Effect of increased salt tolerance upon resistance to chloramphenicol. Since Strain R exhibited a decreased tolerance to salts, it was of interest to determine whether an inverse relationship existed between resistance to salt and to chloramphenicol. The salt tolerance of Strain R was increased by serial transfer in the basal medium containing increasing con-

* Supported by grant E-605 from U.S.P.H.S.

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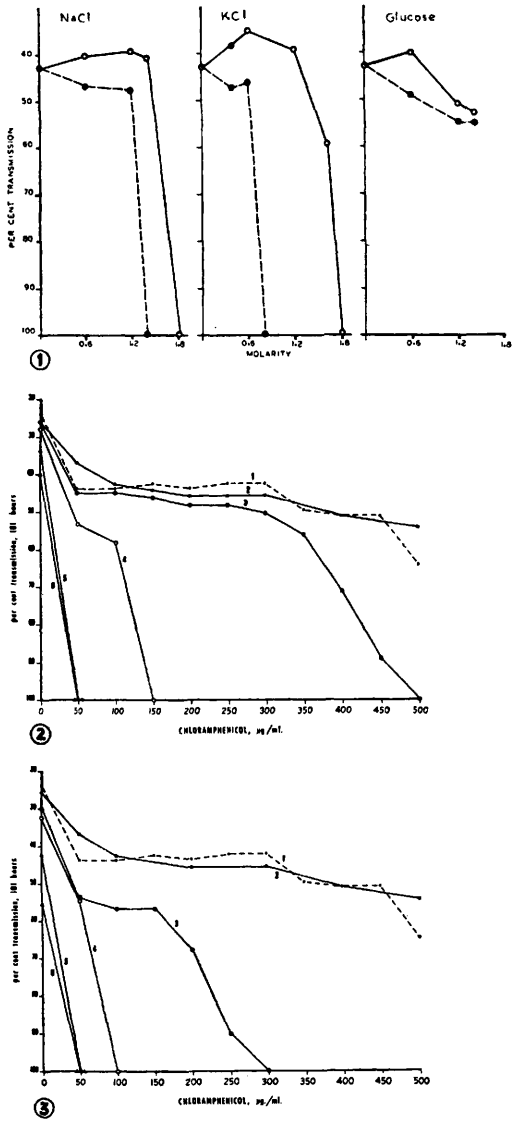

FIG. 1. Tolerance to NaCl, KCl, and glucose of *Staphylococcus pyogenes*, Strain S (solid lines) and Strain R (broken lines).

FIG. 2. Response of Strain R to chloramphenicol before and after enhancement of tolerance to NaCl. 1. Strain R after 9 transfers in the basal medium (control). 2. Strain R (stock strain). 3. Strain R after 9 transfers in increasing concentrations of NaCl. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M, and 2.0 M added NaCl, respectively.

FIG. 3. Response of Strain R to chloramphenicol before and after enhancement of tolerance to KCl. 1. Strain R after 9 transfers in basal medium (control). 2. Strain R (stock strain). 3. Strain R after 9 transfers in increasing concentrations of KCl. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M and 2.0 M added KCl, respectively.

centrations of either KCl or NaCl. The strains resulting after 9 transfers could grow in the basal medium containing 2.2 M KCl or 2.0 M NaCl respectively. This represented the maximum adaptation which could be obtained. Increased tolerance to one of these salts conferred increased tolerance to the other.

Fig. 2 and 3 show that increased salt tolerance resulted in a marked decrease in resistance to chloramphenicol. Strain R was only slightly inhibited by 450 µg of chloramphenicol/ml, but after passage through NaCl was markedly inhibited at this concentration. Similarly, the KCl-tolerant strain was completely inhibited by 300 µg/ml. Control cultures, transferred 9 times in the absence of salts, demonstrated no significant alteration in chloramphenicol resistance. Since Strain R has been shown to retain resistance to chloramphenicol after 25 transfers in drug-free medium (10), it would appear that transfer in presence of salt results in selection of a strain with increased salt tolerance and a parallel decreased resistance to chloramphenicol.

Fig. 2 and 3 also indicate a striking combined effect of chloramphenicol and salts upon salt-tolerant strains (lines 4, 5 and 6). The NaCl-tolerant variant of Strain R was completely inhibited by 0.8 M NaCl in combination with 150 µg chloramphenicol/ml (Fig. 2, line 4). These concentrations were without effect when used alone. A similar effect was noted with the KCl-tolerant variant (Fig. 3, line 4) and comparable results have also been obtained with the original Strains S and R. In most concentrations the effect of both KCl and NaCl appeared to be additive, and often synergistic, with chloramphenicol; KCl was the more effective of the two salts in this respect.

Discussion. The inverse relationship between salt tolerance and chloramphenicol resistance could be explained by at least 2 possible mechanisms. First, it might be anticipated that chloramphenicol interferes with some enzymatic sequence which is relatively resistant to salt. Alteration of this enzyme sequence as a result of chloramphenicol resis-

tance could be accompanied by a decreased resistance to salt. Alternatively, the inverse resistance patterns could be due to altered permeability. If salt and chloramphenicol gain access to the cell by different means, an increased activity of one mechanism might well be accompanied by a decreased activity of the other.

The mechanism of toxicity of salts for bacteria is poorly understood. While osmotic pressure may play a role, additional toxic characteristics must be ascribed to salts. Spiegelberg(13) noted that *Clostridium pasteurianum* was inhibited by a NaCl solution of only 18 to 38 atmospheres, whereas a sugar solution of 60 to 70 atmospheres was required for inhibition.

The additive, and often synergistic effect, noted with combinations of salts (KCl and NaCl) and chloramphenicol would indicate that their modes of action are not the same. No reports of synergism between salts and antibiotics have been found, although there are several reports of the converse. For example, Weinberg(14) noted that inhibition of *Pseudomonas aeruginosa* by oxytetracycline was strongly reversed by several multivalent cations. Berti(1) found that Ca^{++} and Mg^{++} antagonized the effect of streptomycin on *Escherichia coli* and Soncin(12) reported a similar antagonism between Mg^{++} and aureomycin, terramycin and chloramphenicol. Grossowicz, *et al.*(3) observed that Na^{+} and K^{+} antagonized the inhibition of *S. aureus* by spermine and spermidine. The action of tetracycline on *S. aureus* and *S. albus* was reversed by Mn^{++}(4).

It should be noted that even at salt concentrations which are slightly stimulatory, the addition of chloramphenicol results in more inhibition than the drug could produce alone. According to Winslow and Haywood(15), stimulatory concentrations of salts are associated with increased permeability of cells. This could explain the enhancement of inhibition by chloramphenicol at low salt concentrations. However, the decreased permeability at high concentrations of salts, postulated by Winslow and Haywood, would not explain the greater enhancement of chloramphenicol activity.

Summary. 1) A chloramphenicol-resistant strain of *Staphylococcus pyogenes* (Strain R) has been found less tolerant to NaCl and KCl than its chloramphenicol-sensitive counterpart (Strain S). The restoration of salt-tolerance in Strain R by serial sub-cultivation in increasing concentrations of salts resulted in a decrease in resistance to chloramphenicol. 2) The combined effects of chloramphenicol and salts on both Strains S and R were additive in most concentrations and frequently synergistic.

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Received March 27, 1958. P.S.E.B.M., 1958, v98.