THE CORRELATION BETWEEN THE INHIBITION OF DRUG RESISTANCE AND SYNERGISM IN STREPTOMYCIN AND PENICILLIN

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The problem of drug resistance has become an important limiting factor in the therapeutic efficiency of streptomycin (Buggs et al., 1946; Finland et al., 1946; Bondi et al., 1946). We have previously shown in the case of streptomycin that of 13 strains tested all had the ability to throw off, spontaneously, variants resistant to streptomycin (Klein and Kimmelman, 1946; Klein, 1947). The destruction by streptomycin of the mass of susceptible bacteria and the multiplication of the few highly resistant variants was indicated to be a mechanism for the development of streptomycin resistance. Alexander and Leidy (1947), working with *Staphylococcus aureus*, have recently obtained similar results. Clinically, the inhibition of the rapid development of streptomycin resistance may then require the destruction of a relatively small number of resistant bacteria, which might be effected by the addition of a low concentration of another drug. In the present work we have therefore studied the combined action of streptomycin, penicillin, and sulfadiazine in vitro and determined the relationship between the synergistic action of the compounds and the inhibition of the development of streptomycin resistance.

MATERIALS AND METHODS

*Staphylococcus aureus*, susceptible to streptomycin, penicillin, and sulfadiazine, was used as the test organism. A casein hydrolyzate medium (Straus, Dingle, and Finland, 1941) containing 0.5 per cent glucose provided a clear medium which was convenient in the determination of growth rates turbidimetrically in the Klett-Summmerson photoelectric colorimeter. The presence of the glucose resulted in a drop in pH after 24 hours that did reduce the streptomycin activity (Geiger, Green, and Waksman, 1946). However, this did not interfere with the interpretation of the results on the combined drug action.

The tests for drug activity were performed as follows: Six ml of the casein hydrolyzate medium, containing the various drugs singly or in combination, were added to the Klett-Summmerson tubes, and a standard inoculum of 0.1 ml of a 20- to 24-hour culture, diluted to give a reading of 50 on the Klett-Summmerson colorimeter (approximately 15,000,000 bacteria), was seeded into each of the tubes. This large inoculum provided a rapid initial growth, which permitted the taking of turbidity readings at 6 hours, in addition to the 12-, 24-, and 48-hour readings. In preliminary assays it was found that the 24-hour growth

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curves obtained on the basis of turbidity readings followed essentially the same pattern as the 24-hour growth curves obtained on the basis of viable counts. The 48-hour readings were included to show any delayed growth obtained with the inhibitors.

The increase in drug resistance was estimated after 48 hours' incubation at 37°C. The bacteria were subcultured from the initial drug assays after 48 hours and grown for 20 to 24 hours in the casein hydrolyzate medium. A standard 0.1-ml sample was inoculated into the same drug concentration initially used, and growth was again determined turbidimetrically. The increase in the growth rate was then a measure of the increase in resistance to the drug. Turbidity readings of the medium, plus the standard inoculum, were taken at the beginning of all experiments, and the increase in turbidity over the initial reading was recorded. Only increases in turbidity greater than a reading of 10 were recorded in the graphs, and the turbidity readings were plotted as ordinates on a log scale. All growth curves are representative experiments from at least four separate assays.

RESULTS

In figure 1 are shown the growth rates of the initially susceptible bacteria grown in partially inhibitory concentrations of penicillin, streptomycin, and sulfadiazine. The increase in resistance to each of the drugs is indicated for the 48-hour subcultures reassayed against the same concentration of the respective drugs. The bacteria subcultured after 48 hours from the initial assays of each of the drugs, and retested against the same concentration of each drug, showed a sharp increase in streptomycin resistance, a moderate increase in penicillin resistance, and no increase (frequently a slight decrease) in the rate of growth in the presence of sulfadiazine. The increase in resistance after 48 hours to one drug did not result in an increase in resistance to any of the other drugs.

We have found that the rate of increase in resistance to penicillin and streptomycin is a function not only of the specific drug but of the concentration of the drug used. It was found that the greater the partially inhibitory action of penicillin or streptomycin, the greater the increase in resistance. When bacteria were grown in 2 units of streptomycin per ml, subcultured after 48 hours, and retested against 8 units of streptomycin, they showed only a relatively small increase in resistance. Bacteria grown in 4 and 8 units of streptomycin per ml showed significantly greater increases in resistance. Likewise, bacteria subcultured after 48 hours' growth in 0.02 of a unit of penicillin per ml and retested against 0.06 units of penicillin per ml showed only a slight increase in resistance when compared with the increase in penicillin resistance of bacteria grown in 0.04 and 0.06 units of penicillin per ml. This role of drug concentration in the development of penicillin and streptomycin resistance can be explained on the basis of the selection and multiplication of resistant variants. In high concentrations of penicillin or streptomycin which did not completely inhibit growth, all but a few of the most resistant bacteria in the initial inoculum would be eliminated. These few bacteria could multiply and resistant variants would be
thrown off in the direction of greater drug resistance. However, at lower drug concentrations one would not obtain so effective a selection of the few resistant variants; more of the less resistant bacteria would survive and on subculture they would tend to overgrow the few most resistant variants. Upon reassaying, such a culture would show only a moderate or slight increase in resistance. We also observed that when there was no significant inhibition by

![Figure 1: Development of Resistance to Streptomycin, Penicillin, and Sulfadiazine (Staphylococcus aureus)](image)

**Fig. 1. Development of Resistance to Streptomycin, Penicillin, and Sulfadiazine (Staphylococcus aureus)**

Medium: casein hydrolyzate, pH 7.4. Inoculum: 0.1 ml of 20- to 24-hour culture (approx. 15,000,000 organisms). 

- **P** = penicillin, 0.06 u/ml seeded with susceptible bacteria.
- **S** = streptomycin, 8.0 u/ml seeded with susceptible bacteria.
- **SD** = sulfadiazine, 1:5,000 seeded with susceptible bacteria.
- **P(R)** = penicillin, 0.06 u/ml seeded with organisms grown for 48 hours in 0.06 u/ml penicillin.
- **S(R)** = streptomycin, 8.0 u/ml seeded with organisms grown for 48 hours in 8.0 u/ml streptomycin.
- **SD(R)** = sulfadiazine, 1:5,000 seeded with organisms grown for 48 hours in 1:5,000 sulfadiazine.

the drug, i.e., when no selection of the more resistant forms would occur, there was no demonstrable increase in resistance.

**Combined action of two drugs and the inhibition of streptomycin resistance.** We determined the relative effectiveness of sulfadiazine and penicillin when added to streptomycin both with respect to their ability to increase the inhibitory action of streptomycin and their effectiveness in decreasing the streptomycin resistance of bacteria surviving the action of the drug. The results are shown in figures 2 and 3. When 1:5,000 sulfadiazine or 0.06 of a unit of penicillin were added to 8 units of streptomycin, the combined action of the two drugs was
greater than either drug alone and the effect was not a simple additive one. Sulfadiazine in a 1:5,000 concentration, which was less inhibitory than 0.06 units of penicillin, was far more effective when combined with streptomycin than was penicillin, and this greater effectiveness of sulfadiazine as a synergist was related to its ability to reduce more effectively the resistance of bacteria surviving the action of streptomycin. In figure 3 is shown the increase in resistance of bacteria surviving the action of (1) 8 units of streptomycin, (2) 8 units of streptomycin plus 0.06 units of penicillin, and (3) 8 units of streptomycin plus 1:5,000 sulfadiazine. The bacteria grown in streptomycin alone showed a very marked increase in resistance, and the bacteria grown in the combination of streptomycin and penicillin showed almost the same increase in streptomycin resistance. However, the bacteria grown in streptomycin and sulfadiazine showed only a moderate increase in streptomycin resistance, indicating that the greater activity of sulfadiazine as a synergist was associated with a greater activity in inhibiting the development of streptomycin resistance. There was, however, in all cases an increase in streptomycin resistance over the initial susceptibility of the bacteria.

When two drugs are combined, each inhibits the development of drug res-
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Resistance to the other. Streptomycin was found to inhibit effectively the development of penicillin resistance, as did sulfadiazine. No increase in sulfadiazine resistance was ever observed after 48 hours when subcultures were tested from sulfadiazine alone or in combination with other drugs.

Combined action of three drugs. The combined action of 1:10,000 sulfadiazine, 0.04 units of penicillin, and 4 units of streptomycin was determined against S. aureus, and the results are shown in figure 4. The combination of streptomycin and sulfadiazine or the combination of streptomycin and penicillin effected

only a partial inhibition of growth, whereas the combination of all three drugs completely inhibited growth. Though inhibition was complete, there were always a few bacteria surviving the combined drug action. These bacteria when subcultured and reassayed against streptomycin, penicillin, and sulfadiazine, respectively, never showed any increase in resistance and regularly showed a slight decrease in growth rate in the presence of streptomycin and occasionally a slight decrease in resistance to penicillin and sulfadiazine. The absence of any increase in resistance can be interpreted as being due to the
prompt inhibition of all multiplication by the three drugs with the subsequent inability of resistant variants to arise. The few surviving bacteria can be considered as nondividing cells in a physiological state temporarily unaffected by the action of the drugs.

When the three drugs were combined in lower concentrations which permitted some multiplication, e.g., penicillin 0.02 units per ml, streptomycin 4 units per ml, and 1:15,000 sulfadiazine, the bacteria when reassayed after 48 hours showed neither an increase nor a decrease in resistance. It should be pointed out again that lowering the test concentration of penicillin or streptomycin is in itself a factor in effecting a decrease in the rate of development of drug resistance. However, this reduction in drug concentration is not in itself sufficient to eliminate completely the development of drug resistance in the case of the individual compounds.

It should be pointed out that both drugs must be present in concentrations which are in themselves inhibitory. We have found that if one exposes a streptomycin-resistant culture to streptomycin and sulfadiazine, or streptomycin and penicillin, one obtains the inhibitory action of the sulfadiazine or penicillin alone.
DISCUSSION

The relationship between synergism and drug resistance has been previously indicated by us in a report on the combined action of penicillin and the sulfonamides (Klein and Kalter, 1945). It was found that an important factor in the observed synergism was the ability of a small amount of an added drug, in this case the sulfonamide, to prevent the multiplication of the few bacteria resistant to the test concentration of penicillin.

Several factors may be considered in the present inhibition of drug resistance resulting from the simultaneous use of several drugs. We have already indicated that the use of lower concentrations of each drug is an important factor in the decreased resistance to the drug. Carpenter, Bahn, Ackerman, and Stokinger (1945) found that when bacteria were grown in sulfathiazole, rivanol lactate, promin, and penicillin, drug resistance did not develop against any of the compounds. In the combination of four drugs Carpenter and his coworkers used one-fourth the drug concentration initially used in the development of resistance to each agent. It would be of interest to know to what extent this reduction in the concentration of the individual drugs was related to the elimination of resistance when all four drugs were combined.

As a synergist with streptomycin, the greater activity of sulfadiazine as compared with penicillin may be related to the very high degree of sensitivity of sulfadiazine to the total number of bacteria present. We have found, for example, that a 1,000-fold decrease in the size of our S. aureus inoculum increased the sulfadiazine titer over 30-fold, but under similar conditions the penicillin titer was increased only 3-fold. Hence when only a small number of streptomycin-resistant cells are present, low concentrations of sulfadiazine would be particularly effective.

If one assumed that a drug had an all or none effect, i.e., it either inhibited a bacterium from dividing or left the cell essentially unaltered, then the combined effect of the drugs could be explained exclusively in terms of this independent action. A given concentration of streptomycin would therefore destroy all but a small number of bacteria completely resistant to it and the small concentrations of the added drug or drugs would independently inhibit the small number of surviving bacteria. If, however, a drug can significantly modify cellular metabolism though not inhibit cell division, then it is possible that two drugs acting on a single cell may together effect complete inhibition or killing when each alone could not (Mudd, 1945). One would then have in addition to the independent action, which must occur, this combined action on a single cell.

Apart from any consideration as to the precise mode of action and with due regard to possible toxic effects, one can state that drugs having some limited degree of action against streptomycin-resistant bacteria are potential tools for reducing or eliminating the development of streptomycin resistance. It may be mentioned that antibodies and phagocytes should play a role in inhibiting the development of resistance by suppressing the multiplication of resistant cells. It is of particular interest to note that Schnitzer, Lafferty, and Buck (1946) found that drug resistance of the trypanosomes developed most rapidly in those treated experimental animals in which there was little antibody activity.
SUMMARY

After 48 hour's growth in a casein hydrolyzate medium containing streptomycin, penicillin, or sulfadiazine, Staphylococcus aureus showed a marked increase in resistance to streptomycin and penicillin and no increase in resistance to sulfadiazine.

The greater the partially inhibitory concentration of streptomycin or penicillin the greater the increase in the rate of development of resistance.

Sulfadiazine, when added to streptomycin broth, was far more effective as a synergist and inhibitor of streptomycin resistance than was penicillin.

Low concentrations of streptomycin, penicillin, and sulfadiazine when combined were highly effective in inhibiting multiplication and prevented the development of drug resistance.

The results are interpreted on the basis of the selection and inhibition of resistant variants.

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