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# Mercury resistance and tetracycline resistance in *Staphylococcus aureus*

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## INTRODUCTION

Since resistance to mercury salts was first described as a useful marker for identification of epidemic strains of *Staphylococcus aureus* (Moore, 1960), attempts have been made to correlate mercury resistance with other physiological markers. Relationships have been described between mercury resistance, tetracycline resistance and multiple antibiotic resistance (Turner & Willis, 1962; Willis & Turner, 1963; Willis, Jacobs, & Goodburn, 1964), and between mercury resistance, high penicillinase production, tetracycline resistance and multiple antibiotic resistance (Richmond, Parker, Jevons & John, 1964).

Most workers have noted that mercury resistant strains are found predominantly among staphylococcal strains of phage groups I and III, and particularly among those of the 'pure 80/81 strains' (Parker & Jevons, 1963), and among strains of type 83A (Green, 1962; Jessen *et al.* 1963). Parker & Jevons (1963) also noted the close relationship between mercury and tetracycline resistance, and the multiple antibiotic-resistant nature of mercury resistant strains has been confirmed by Vogelsang (1965).

This paper is concerned with the relationship between mercury and tetracycline resistance. Mercury resistant staphylococci are usually, though not invariably, resistant to tetracycline, whereas tetracycline resistance occurs only sporadically in mercury sensitive strains. Evidence is produced which shows a qualitative difference between the tetracycline resistance of the two groups. Tetracycline resistance in mercury resistant strains is regarded as genetic in origin—the result of mutation, followed by selection. Resistance to tetracycline in the mercury sensitive strains is possibly the result of 'training' or adaptation to the drug.

## MATERIALS AND METHODS

### *Staphylococcal strains*

Tetracycline resistant strains were obtained from a number of different sources. All strains were independently isolated and differed in phage pattern, antibiogram or place of isolation. The 119 strains in survey I consisted of 101 staphylococci isolated at St Thomas's Hospital over a period of about $2\frac{1}{2}$ years in the course of other investigations, and 18 strains (supplied by the Central Public Health Laboratory, Colindale) which had caused outbreaks of sepsis in hospitals before 1963.

The 137 strains in survey II were also supplied by the Central Public Health

Laboratory and were selected from the annual surveys of 1965 and 1966. These surveys are carried out on staphylococci isolated consecutively over a given period in a number of hospitals in the London area.

All strains but one were resistant to both penicillin and tetracycline. Penicillin sensitive strains were excluded because of the close genetic linkage between mercury resistance and penicillinase production (Richmond & John, 1964) as it was not possible to decide whether these strains had originally been resistant to mercury or not. The one penicillin sensitive exception had the phage pattern 3C/55/71: phage group II strains are generally mercury sensitive (Green, 1962) and, as only three other tetracycline resistant examples from this group were found, this strain was retained.

*Mercury sensitivity and antibiograms*

Mercury sensitivity was tested by the method of Green (1962). Antibiotic sensitivity was tested by applying 'Multodisks' (Oxoid) to surface lawns of growth on nutrient agar ('Oxoid' Blood agar base, no. 2). The composition of the multodisks was: tetracycline, 50 $\mu$g.; chloramphenicol, 50 $\mu$g.; erythromycin, 50 $\mu$g.; neomycin, 30 $\mu$g.; novobiocin, 30 $\mu$g.; cloxacillin, 5 $\mu$g.; streptomycin, 25 $\mu$g.; penicillin, 5 units.

*Phage typing*

Phage typing was carried out by the method of Blair & Williams (1961).

*Resistance of strains to tetracycline*

Minimum inhibitory concentration (M.I.C.) of tetracycline for each strain was estimated by absence or presence of growth on nutrient agar containing the antibiotic. Doubling dilutions of pure tetracycline hydrochloride (Lederle) were incorporated in 20 ml. amounts of nutrient agar to give a range of concentrations of 3·125 $\mu$g./ml. to 200 $\mu$g./ml. All plates were freshly prepared. After drying, these were inoculated by application of overnight broth cultures with a multiple loop device, and incubated at 37° C.

*Growth curves*

Growth patterns of selected strains in a therapeutic concentration of tetracycline (3 $\mu$g./ml.) (Barber & Garrod, 1963) were investigated turbidimetrically in an EEL nephelometer (Evans Electroselenium Ltd.). Strains were selected to provide two or more representatives of each of a series of M.I.C.'s tetracycline in the mercury resistant and mercury sensitive groups (Table 1).

Metal-capped 6 in. × $\frac{1}{2}$ in. test tubes containing 10 ml. peptone water (Evans peptone, 1 %; sodium chloride, 0·5 %; w/v) and tetracycline hydrochloride, 3 $\mu$g./ml., were each inoculated with 0·02 ml. of an overnight peptone water culture (approximately 50,000 viable organisms). Control tubes containing peptone water only were inoculated at the same time. Peptone water was selected as a culture medium so that growth would not occur too rapidly.

Tubes were incubated at 37° C. and turbidity readings taken hourly from 4 to 12 hr., and again at 24 hr. Because of the relative insensitivity of the nephelo-

meter during the early period of growth, readings were not consistently recordable until 4 hr. incubation had elapsed.

Table 1. *Strains selected to represent different M.I.C.'s of tetracycline in growth curve measurements*

| M.I.C. tetracycline ($\mu$g./ml.) | Mercury resistant strains. Phage pattern | Mercury resistant strains. Phage pattern |
|---|---|---|
| 100 | 52/52A/80/81 | 52/52A/80/81 |
| 100 | 83A | 77 |
| 100 | 47/53/75/77 | — |
| 50 | 80* | 29/52/52A/79/80 |
| 50 | B5 | 71 |
| 50 | 80/47* | 77* |
| 50 | — | 3C |
| 25 | 80* | 29/80/47/53/75 |
| 25 | 53* | 42D/81 |
| 12·5 | — | N.T.† |
| 12·5 | — | 3C/55/71‡ |
| Totals | 8 strains | 10 strains |

* Reactions with phages at 1000 × R.T.D. (1000 × Routine Test Dilution of phage).
† This strain died during training to growth at high levels of tetracycline.
‡ Penicillin sensitive strain.

Readings were recorded as logarithms to base 2 according to the tables of Finney, Hazlewood & Smith (1955), and the turbidities were checked against viable counts (Miles & Misra, 1938) as recommended by Monod (1949).

Two sets of growth measurements were taken on the selected strains. The first set was obtained from staphylococci that had not been pretreated in any way. The second set was made from the same strains after they had been adapted to grow on agar containing high levels of tetracycline (100 $\mu$g./ml.).

*Adaptation of strains*

Adaptation was initiated by plating the staphylococci on agar containing tetracycline, 3 $\mu$g./ml., and thereafter on increasing concentrations until all the strains grew on agar containing tetracycline, 100 $\mu$g./ml. On completion of this process, the strains were grown in plain peptone water overnight, and the growth pattern investigated as described on the following day.

One nontypable strain, M.I.C. tetracycline 12·5 $\mu$g., died during adaptation to growth at higher concentrations of the antibiotic.

## RESULTS

The staphylococci in both surveys were divided into two groups—mercury resistant and mercury sensitive.

*Distribution of minimum inhibitory concentrations of tetracycline*

Percentage distribution of different levels of M.I.C. tetracycline in each group in both surveys are shown in Fig. 1. The mean percentage distributions of M.I.C.'s for all strains studied are shown in Fig. 2.

Resistance to tetracycline in both surveys is generally higher in the mercury resistant than in the mercury sensitive strains. The two sets of mercury resistant strains show a close similarity in distribution of resistance to tetracycline, and the lower distribution of M.I.C.'s in the mercury sensitive strains shows close corres-

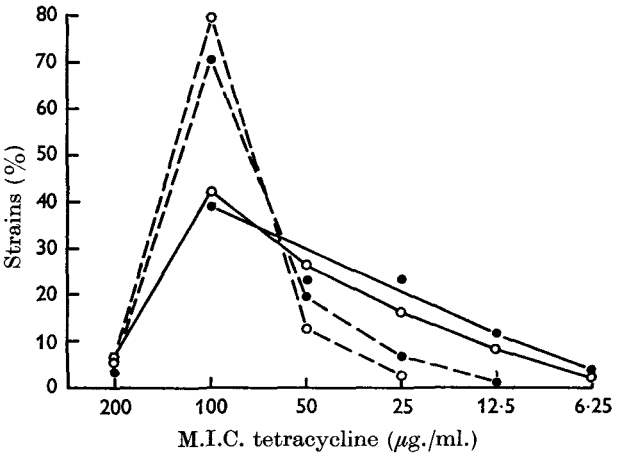


Fig. 1. Distribution of M.I.C.'s tetracycline in strains of survey 1 and survey 2. Survey 1: ●- - -●, mercury resistant strains (93); ●——●, mercury-sensitive strains (26). Survey 2: ○ - - - ○, mercury resistant strains (87); ○——○, mercury sensitive strains (50).

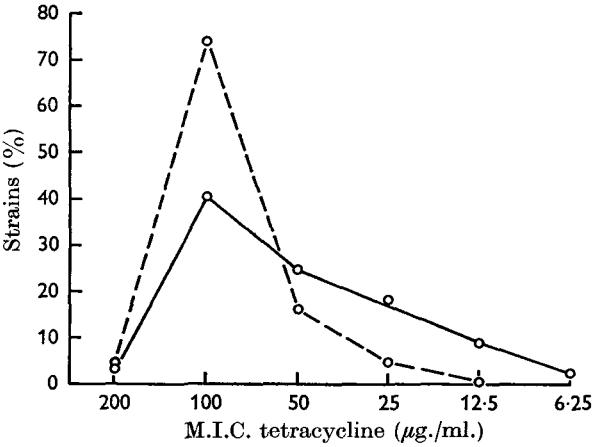


Fig. 2. Mean distribution of M.I.C.'s tetracycline for all strains (surveys 1 and 2). ○- - -○, mercury resistant strains (180); ○——○, mercury sensitive strains (76).

pondence in the two surveys. The number of mercury sensitive strains in survey 1 is considerably smaller than in survey 2. Nonetheless, there is no significant difference between these groups in both surveys.

Differences in distribution of M.I.C.'s tetracycline between mercury resistant and mercury sensitive strains are clearly seen if an arbitrary level (tetracycline, 50 μg./ml.) is taken to divide all the strains studied into those of high resistance and those of lower resistance (Table 2). The mercury resistant group has a pre-

ponderance of strains (70–85 %) resistant to tetracycline, 100 or 200 μg./ml. The majority of mercury sensitive strains (50–62 %) have an M.I.C. tetracycline of 50 μg./ml. or less.

Table 2. *Percentage distributions of M.I.C.'s tetracycline in mercury resistant and mercury sensitive strains*

| | Mercury resistant | | | Mercury sensitive | | |
|---|---|---|---|---|---|---|
| M.I.C. tetracycline | Survey 1 | Survey 2 | Mean | Survey 1 | Survey 2 | Mean |
| 100 and 200 μg./ml. | 73·2 | 84·9 | 78·8 | 38·5 | 48 | 44·7 |
| 50 and < 50 μg./ml. | 26·7 | 15 | 21·1 | 61·5 | 52 | 55·2 |
| Totals | 99·9 | 99·9 | 99·9 | 100 | 100 | 99·9 |

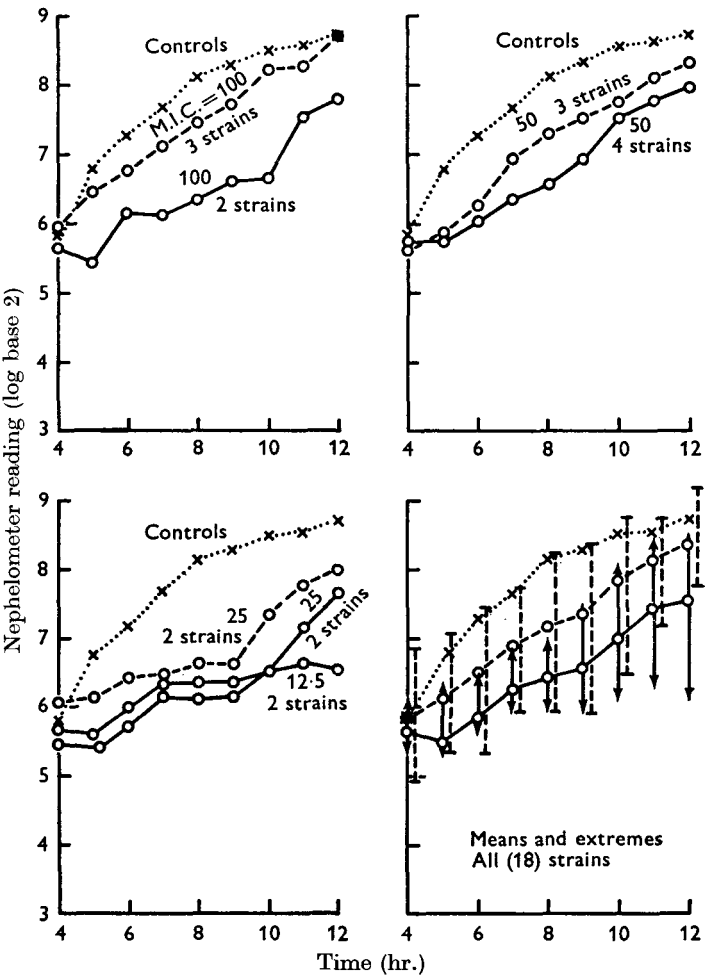


Fig. 3. Growth of representative strains (see Table 1) in peptone water containing tetracycline, 3 μg./ml. These strains have not been 'trained'. × .... ×, Controls; O——O, mercury sensitive strains; O - - - - O, mercury resistant strains.

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*Growth curves in therapeutic concentrations of tetracycline*

Results of the growth curves of untrained strains in a therapeutic concentration of tetracycline (3 $\mu$g./ml.) are shown in Fig. 3. The curves cover the period of growth from 4 to 12 hr. Mean growth rates for the strains representing the various M.I.C.'s tetracycline—see Table 1—are plotted so that the mercury sensitive and mercury resistant groups can be compared. Mean growth rates for all the strains, together with extremes of readings are also shown.

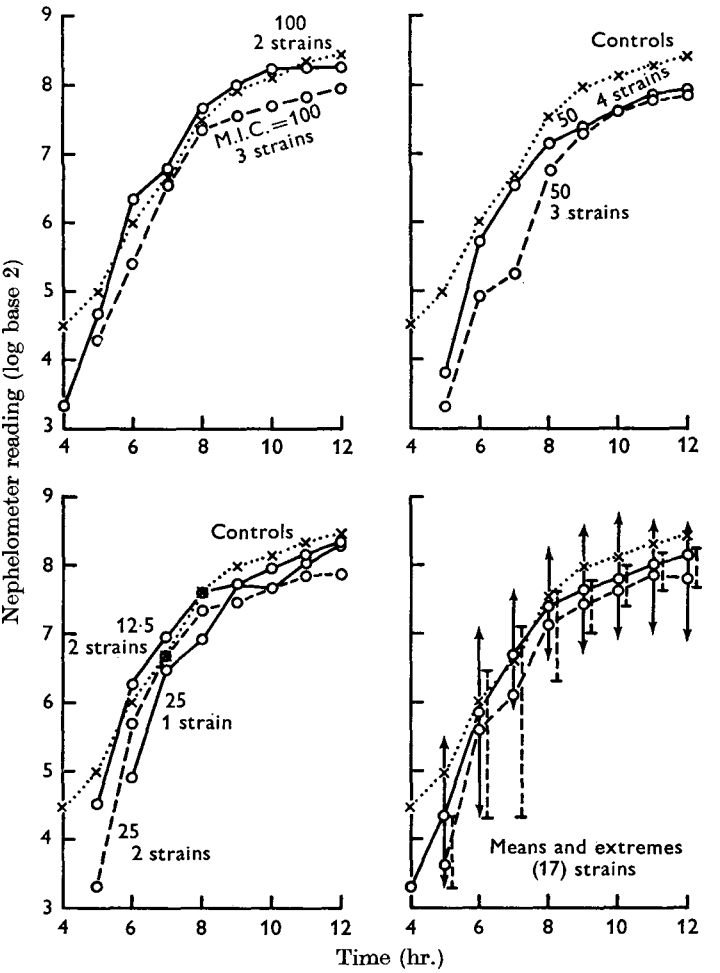


Fig. 4. Growth of representative strains in peptone water containing tetracycline, 3 $\mu$g./ml., after they had been 'trained' to grow at high concentrations of tetracycline. × ..... ×, Controls; ○——○, mercury sensitive strains; ○— —○, mercury resistant strains.

Controls are plotted as means of all readings taken during growth of the strains in plain peptone water: in terms of viable counts, control readings reached a maximum of about $2{\cdot}5 \times 10^6$ organisms/ml. in the 12 hr. period of incubation.

Mercury resistant strains show a gradual decline in growth rate with decrease in

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resistance to tetracycline, but, even so, each group of mercury resistant strains grows better than the corresponding mercury sensitive groups representing each M.I.C. tetracycline.

Apart from the difference in growth rate, mercury sensitive strains show an appreciable delay in reaching their maximum growth rate in comparison with the mercury resistant strains. Such a delay is often found during 'training' or adaptation of bacteria to drug resistance (Dean, personal communication). The possibility of adaptation as a mechanism of tetracycline resistance in these mercury sensitive strains is supported by the incubation period, which is not sufficiently long to permit any appreciable selection of tetracycline resistant mutants that may have arisen spontaneously (Dean & Hinshelwood, 1966).

The readings for growth of untrained strains up to 12 hr. were analysed by the '$T$' test (Industrial Experimentation, War Office, H.M.S.O., 1949 reprinted 1960, page 35). The $T$ value is 2·33 (S.D. = 5·4). This $T$ value is for 16 degrees of freedom significant at the 5% level of confidence ($P < 0{\cdot}05$).

Readings taken at 24 hr. show a wide scatter in both groups, and there is no consistent difference between mercury resistant and mercury sensitive strains.

The growth curves for the same strains after adaptation to growth on agar containing tetracycline, 100 $\mu$g./ml. (with the one exception that died during 'training'—see Table 1) are shown in Fig. 4. After 'training' the growth rate of the mercury sensitive strains is noticeably increased in the therapeutic concentration of tetracycline, and they now grow somewhat better than the mercury resistant strains.

Furthermore, there is no longer any obvious difference between the growth patterns of the controls—which are the same 'trained' strains grown in plain peptone water—and the two groups under investigation. The marked alteration in the growth pattern of the mercury sensitive strains suggests a more complete adaptation to growth in the presence of tetracycline.

Mercury resistant strains also show some difference in growth pattern after 'training', though these are not so pronounced as in the mercury sensitive group. The slope of the curve is now steeper, suggesting that growth has commenced earlier than in the untrained strains, and the extremes of readings during the growth of the various strains are now closer together. However, allowing for experimental variation, there is no great alteration in the pattern of growth of mercury resistant strains following 'training'.

## DISCUSSION

A number of associations of mercury resistance in *Staphylococcus aureus* have been described by different workers. These include tetracycline resistance, high penicillinase activity, multiple resistance to antibiotics and susceptibility to lysis by phages of groups I and III. A further relationship, albeit incomplete, has been suggested between mercury resistance and ability to withstand drying (Rountree, 1963).

These factors are of undoubted importance in ensuring survival of strains within

the hospital environment, and possession of all or most of these markers ensures the success of 'hospital' or epidemic staphylococci. Two markers that are virtually constant in these strains are mercury resistance and tetracycline resistance.

The clinical behaviour of mercury resistant strains (Moore, 1960) has been further studied by Jessen *et al.* (1963), who reported that a higher mortality rate (50 %) results when the strains causing bacteraemia are mercury resistant than when the infecting strains are mercury sensitive (33 %). Richmond *et al.* (1964) showed that 31 out of 38 strains responsible for endemic sepsis were mercury resistant and, of these, 29 were tetracycline resistant.

The importance of tetracycline resistance as a marker of hospital strains has long been recognized. Many reports (Rountree & Thomson, 1952; Clarke, Dalgleish & Gillespie, 1952; Lowbury, Topley & Hood, 1952; Shooter *et al.* 1958; Williams, 1959; and Barber *et al.* 1960) have emphazised the dangerous nature of tetracycline resistant strains, their emergence and predominance in cases of hospital sepsis, and their ability to spread by cross-infection.

Tetracycline resistance alone, however, is not a reliable marker for recognition of hospital strains, as many non-epidemic strains now possess this characteristic, though there may be a qualitative difference between tetracycline resistance in mercury resistant and mercury sensitive strains, as the results given in this paper suggest.

Tetracycline resistance in mercury sensitive strains is shown to be generally lower than in the mercury resistant group. This finding suggests that strains sensitive to mercury have a less efficient mechanism for developing resistance to tetracycline under natural conditions, and the growth patterns suggest that the appearance of resistance is the result of 'training' or adaptation to the drug.

This argument is supported by the different behaviour of the mercury sensitive strains after they have been 'trained' to grow at high concentrations of tetracycline. Exponential growth occurs much sooner than in the same strains before 'training', and the growth rate increases.

The mechanism of 'training' of bacteria to drug resistance has been studied by Dean & Giordan (1964) who trained *Bact. lactis aerogenes* at various concentrations of terramycin. They demonstrated the continuous gradation of the resistance of 'trained' strains to the training concentration of the drug, and pointed out that the lag in growth in the presence of the antibiotic was shortened with each stage of training. They considered that 'training' was complete when no further appreciable shortening of the lag occurred in a given concentration of antibiotic.

In the present work, the concentration of tetracycline used in 'training' is far in excess of any that the bacteria would meet under actual hospital conditions, and it is apparent that, once the ability to become resistant has been developed—whatever the mechanism of that resistance—the degree of resistance can be greatly increased.

The changes in behaviour of the mercury resistant strains as a result of 'training', however, are not so marked as in the mercury sensitive staphylococci that have undergone the same treatment. The training process has less effect on the growth rate of the mercury resistant strains, and this suggests that their resistance is less

dependent on the presence of antibiotic in the environment, and is probably genetically determined.

The effect of 'training' on the mercury sensitive strains does not, however, preclude the possibility of mutation being the starting point in development of resistance to tetracycline. Drabble & Hinshelwood (1961), in investigations of streptomycin resistance in *Bact. lactis aerogenes*, described preliminary selection of pre-existing mutants at low concentrations of the drug. These mutants could then be 'trained' to become more resistant, and the degree of resistance finally achieved was dependent on the concentration of streptomycin used in 'training'.

A similar process could account for tetracycline resistance in mercury sensitive strains of staphylococci isolated from clinical sources. The resistance in these strains before 'training' is stable, as shown by repeated estimation of M.I.C. tetracycline, but this is not surprising, as the relative stability of adaptive changes in bacteria has long been recognised (Penfold, 1910). The degree of resistance to tetracycline of the mercury sensitive strains may well be a reflection of the amount of the drug used in the environment from which they were isolated.

Because of the clinical importance of epidemic staphylococci, genetic studies have been largely confined to strains having their characteristics, and little attention has been directed to the mechanism of resistance in the other groups.

Studies of genetic markers in resistant staphylococci have shown that the genes responsible for mercury resistance and penicillinase production are closely associated (Richmond & John, 1964) and can be co-transduced to sensitive strains; and that the genes responsible for tetracycline resistance and penicillin resistance are probably carried cytoplasmically and are found on different plasmids (May, Houghton & Perret, 1964) and particularly among strains of the '52, 52A, 80, 81' complex (Asheshov, 1966). However, these reports refer to strains that are undoubtedly 'hospital' staphylococci.

Published reports of transduction of tetracycline resistance suggest that successful transduction has been achieved from epidemic (or mercury resistant) donor strains. Only one author (McDonald, 1966) states that the donor strains were mercury resistant. However, descriptions of donor organisms in other studies (Mitsuhashi, Nakano, Fukutome & Kakinuma, 1961; Pattee & Baldwin, 1961; Collins & McDonald, 1962; and Mitsuhashi, Oshima, Kawaharada & Hashimoto, 1965) though incomplete in many respects, suggest that tetracycline resistance was transduced from mercury resistant donor strains or, at least, from strains that had the characteristics of epidemic staphylococci. No reports have been found in which transduction is described as being carried out from strains known to be mercury sensitive.

Further study on the nature of tetracycline resistance in mercury sensitive strains is required. A comparison between the incidence of tetracycline resistance in these strains and the amount of tetracycline used in the environment would be of interest.

The mercury test (Green, 1962) is easily performed and yields valuable information. Its use as a routine test does not demand additional media or glassware as it can be performed on the same plate as a 'Multodisk' test involving eight anti-

biotics. The information so gained is essential in genetic and epidemiological studies, and descriptions of staphylococcal strains in published reports should include mercury resistance or sensitivity.


## SUMMARY

Minimum inhibitory concentrations of tetracycline to 256 tetracycline-resistant strains of *Staphylococcus aureus* were determined. M.I.C.'s tetracycline were appreciably higher among mercury resistant than among mercury sensitive strains.

Mercury resistant strains representing various M.I.C.'s tetracycline grew significantly better in peptone water containing a therapeutic concentration of tetracycline than mercury sensitive strains representing the same range of resistance. The experiment was repeated after both groups had been adapted—or 'trained'—to grow on agar containing tetracycline, 100 $\mu$g./ml. The mercury sensitive strains now grew better than the mercury resistant group.

The significance of these findings is discussed. It is concluded that tetracycline resistance is more stable and efficient in mercury resistant strains, and that it is probably genetic in origin—the result of mutation and selection. Tetracycline resistance in mercury sensitive strains is possibly the result of 'training'.

The associations and significance of both mercury resistance and tetracycline resistance in *Staphylococcus aureus* are discussed.


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