as the one giving a final electrolyte concentration which was most nearly optimal. Additional experiments confirmed this finding. They indicate further that the prozone effect was reduced or eliminated when the 5.0% diluent was employed, and that a more dilute antigen could be used (Table II).

The application of these observations has made possible the use of a screen test consisting of one or 2 low-serum dilutions, for the rapid detection of rickettsial antibodies in large numbers of sera at a considerable saving of antigen.

Summary. The use of a 5.0% solution of sodium chloride as a serum diluent in agglutination tests with *Rickettsia burneti* reduced or eliminated the prozone effect and permitted the employment of a more dilute antigen.

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Induced *in vitro* Resistance of Staphylococci to Streptomycin and Penicillin.

OTTO E. GRAESSLE AND BETTINA M. FROST. (Introduced by H. Molitor.)
From the Merck Institute for Therapeutic Research, Rahway, N.J.

It has been recognized that certain strains of staphylococci acquire resistance to penicillin when grown in a medium containing this antibiotic.[1-6] Streptomycin[7] resistant strains can also be produced *in vitro* by exposure to this agent.[8-10]

The following study was undertaken to determine (1) the rate and degree to which various staphylococcal strains acquire resistance to streptomycin and to crystalline penicillin (G); (2) whether strains made resistant to penicillin simultaneously acquire resistance to streptomycin; (3) whether the resistant strains revert to their original sensitivity after repeated transfer in nontreated broth or when stored on dry ice for long periods of time; (4) if differences occur in the metabolic activity of the resistant and sensitive strains with respect to carbohydrate fermentation, and (5) to observe if changes in morphological characteristics occur after the organisms develop resistance to streptomycin.

Materials and Methods. The potency of the penicillin used was constant since crystalline penicillin (G) was used, however the streptomycin varied in potency from 250 to 600 units per mg. Bacto Brain Heart Infusion Broth was used as the liquid broth and standard F.D.A. agar served as the solid medium. Possible changes in the carbohydrate reactions of the cultures rendered resistant to streptomycin were investigated in Bacto Peptone Colloid Medium to which was added 0.002% phenol red and 1% of the carbohydrate. The media were inoculated from a 24-hour culture, incubated at 37°C and read after 24, 30, 48 and 120 hours.

Six strains of staphylococci obtained from different sources were used and designated as

1 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

2 Rommelkamp, C. H., and Maxton, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

3 McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

4 Demerec, M., *Proc. Nat. Acad. Sci.*, 1945. **31**, 16.

5 Todd, E. W., Turner, G. S., and Drew, L. G., *Brit. Med. J.*, No. 4386, 1945.

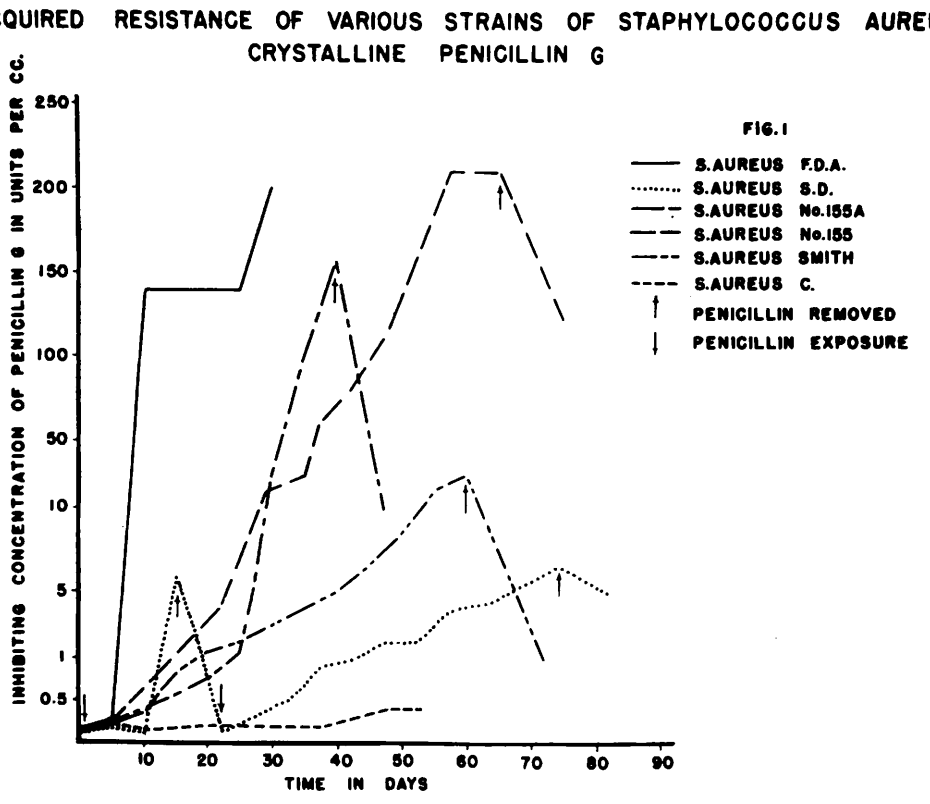
6 Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

7 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

8 Waksman, S. A., Reilly, H. G., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

9 Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clinic*, 1946, **21**, 126.

10 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.



S. aureus Smith, S. aureus F.D.A., S. aureus No. 155, S. aureus No. 155A, S. aureus S.D., and *S. aureus C.*

Regression of induced resistance to the antibiotics was studied by freezing the fast strains and storing them on dry ice for from 9 to 12 months. Upon removal they were grown through several transfers before testing for resistance.

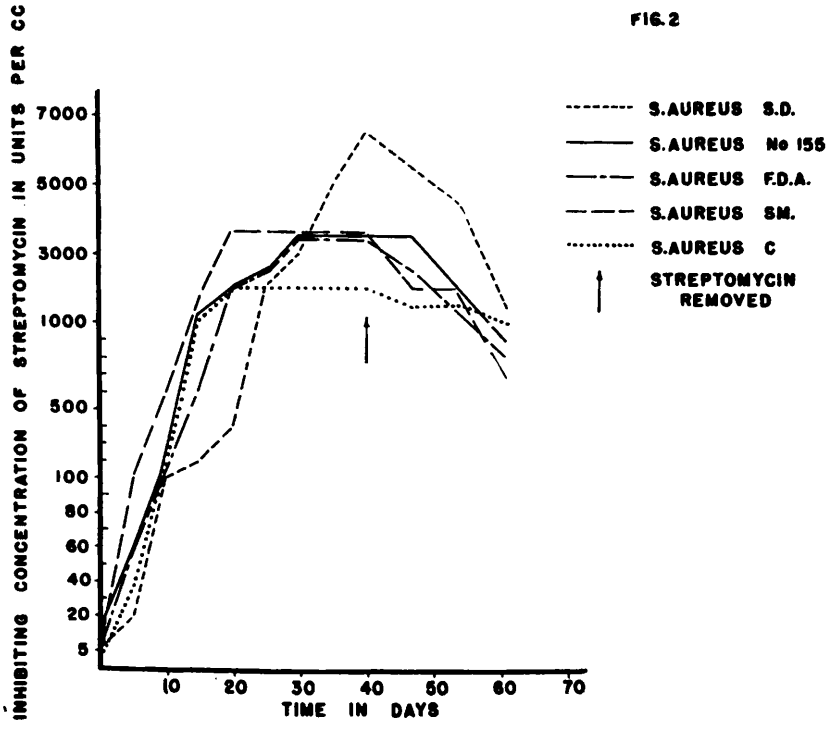
The sensitivity of each organism to the antibiotics was determined by the following method: A series of dilutions of the test agent in sterile distilled water was prepared in such a manner that they were 10 times more concentrated than the final dilution desired. To 1 cc of each dilution was added 9 cc of seeded culture medium containing a 10^{-5} dilution of a 24-hour culture of the test organism. The tubes were incubated at 37°C for 24 hours and examined for turbidity. The minimal concentration of the antibiotic in units per cc which resulted in complete inhibition of growth was taken as the inhibiting concentration.

Resistance was produced by transferring each culture into broth containing one-half of its inhibiting concentration of the antibiotic. After 5 such daily transfers, the bacteriostatic concentration for the culture was redetermined, and the drug concentration increased so as to equal one-half of the new inhibiting level. This procedure or a slight modification, was continued until no further significant change in resistance was evident. Control cultures were maintained by daily transfer in untreated broth.

Results. Induced Resistance. All of the cultures exposed to penicillin and streptomycin as described above decreased in sensitivity to the antibiotics. The degree to which resistance was acquired depended upon the strain and the duration of exposure. Thus, 3 strains of *Staphylococcus aureus* acquired a resistance to penicillin of at least 1500-fold within 10 to 52 days; whereas one culture became only 4 times more resistant when exposed in the same manner (Fig. 1). Furthermore, the rate at which resistance was ac-

ACQUIRED RESISTANCE OF VARIOUS STRAINS OF STAPHYLOCOCCUS AUREUS TO STREPTOMYCIN

FIG. 2



quired also varied as evidenced by the fact that one strain *(S. aureus F.D.A.)* developed a resistance of approximately 1000-fold in 10 days, whereas other strains required from 35 to 45 days to withstand the same increase in penicillin concentration.

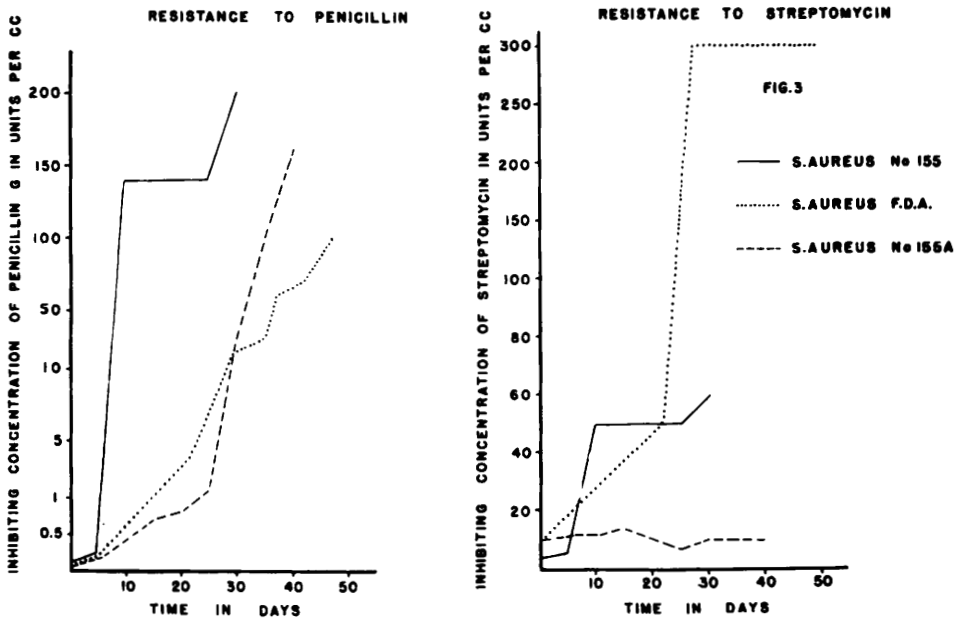
A similar resistance developed when the original cultures were exposed to increasing concentrations of streptomycin (Fig. 2), but showed less variation than when exposed to penicillin. It seemed to occur more rapidly, but to a lesser degree than to penicillin. In all cases resistance increased rapidly until the organisms tolerated approximately 300 to 700 times as much streptomycin as originally. It was of interest to note that none of the resistant strains destroyed streptomycin indicating that increased resistance is not due to the formation of a streptomycin-destroying enzyme.

Regression of Induced Resistance. In order to determine whether a resistant strain would revert to its original sensitivity when cultured in broth free of penicillin, strain *S. aureus S.D.* which had acquired a resistance of 60-fold in 15 days was transferred daily in nutrient broth over a 7-day period. The results show that with this strain resistance was lost within 7 days (Fig. 1). Upon re-exposure to penicillin the culture became resistant to the same degree, requiring however, a longer period of time. Similar tests were performed with *S. aureus No. 155A, S. aureus Smith* and *S. aureus No. 155* after these cultures acquired an increased resistance of 1600-, 300- and 2100-fold respectively.

All of the penicillin-resistant strains tested showed some decrease in resistance upon transfer in nutrient broth.

When the streptomycin-resistant strains were transferred in a similar manner they also became more sensitive to the antibiotic. Over the 21-day period during which these strains were followed in broth they showed less variation in the rate at which resistance regressed than was observed with the penicil-

ACQUIRED RESISTANCE TO PENICILLIN AND STREPTOMYCIN OF STAPHYLOCOCCAL STRAINS EXPOSED TO CRYSTALLINE PENICILLIN G



lin-resistant strains.

When stored on dry ice for from 9 to 12 months 3 of the 5 strains which had acquired resistance to penicillin were found to be more sensitive. The resistance of strains S.M., S.D. and No. 155A was reduced from 300-, 65- and 1600- to 40-, 3.5- and 70-fold respectively when stored for this period of time. On the other hand, strains F.D.A. and No. 155 remained practically unchanged. It is notable that the strains which retained their resistance were those which showed the greatest degree of increased resistance during exposure to penicillin. In contrast to the foregoing findings all streptomycin-fast strains retained their resistance to this antibiotic when stored at a low temperature for long periods of time.

Cross Resistance. During the period in which the staphylococci were being made resistant to penicillin they were also tested for sensitivity to streptomycin. A change in the inhibitory effect of penicillin on the streptomycin-fast strains was also investigated. Under these conditions it was observed that *S. aureus F.D.A.* and *S. aureus No. 155* while acquiring resistance to penicillin had simultaneously become 15 and 30 times more resistant to streptomycin. These strains had also shown the greatest increase in resistance to penicillin. Three additional strains did not show this increase in resistance. For simplicity the results observed with 3 representative strains are presented (Fig. 3). In contrast, all 5 streptomycin-resistant strains remained sensitive to penicillin.

Morphology. Cultures acquiring resistance to commercial penicillins have been reported to show morphological changes,[6,11,12] marked pleomorphism,[6,11] loss of pigmentation,[6,11] and slowing of metabolic reactions.[1,6,11,12] Similar morphological changes and pleomorphism as have been described by others were observed in the staphylococcal strains made resistant to crystalline penicillin (G).

Studies on the growth characteristics of the streptomycin-resistant strains showed no

11 Gardner, A. D., *Nature*, 1940, **146**, 837.

12 Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 210.

TABLE I.
Carbohydrate Reactions of Streptomycin Resistant Staphylococcal Strains.

	S. aureus S. M.								*S. aureus F. D. A.*							
	Control Time in hr				Streptomycin Resistant Time in hr				Control Time in hr				Streptomycin Resistant Time in hr			
Carbohydrate	24	30	48	120	24	30	48	120	24	30	48	120	24	30	48	120
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
Lactose	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	—	—	—	+	+	+	+	+	±	+	+	+
Sucrose	+	+	+	+	—	—	±	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	—	±	+	+	+	+	+	+	+	+	+	+
	S. aureus Cameron								*S. aureus S. D.*							
Glucose	+	+	+	+	±	+	+	+	+	+	+	+	±	±	±	+
Lactose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Maltose	+	+	+	+	±	±	+	+	+	+	+	+	±	±	±	+
Sucrose	+	+	+	+	±	+	+	+	+	+	+	+	±	+	+	+
Mannitol	—	—	—	—	—	—	—	—	+	+	+	+	±	+	+	+
	S. aureus No. 155															
Glucose	±	+	+	+	±	+	+	+								
Lactose	±	±	+	+	±	±	±	+								
Maltose	+	+	+	+	+	+	+	+								
Sucrose	+	+	+	+	+	+	+	+								
Mannitol	+	+	+	+	+	+	+	+								

+ Acid. ± Some acid. — No acid.

marked differences in the appearance of the broth cultures or colony formation on agar, except for a loss of pigmentation by the resistant *S. aureus F.D.A.* and *S. aureus Smith.*

Carbohydrate Fermentation. At peak resistance the streptomycin-resistant staphylococcal cultures showed no change in the sugars fermented (Table I), but an appreciable increase in the time required for the fermentation was observed. Two weeks after the cultures had been returned to broth without streptomycin they were retested, and with the exception of *S. aureus No. 155* which continued slow in fermenting lactose, produced acid at the same rate as the control cultures.

Summary. 1. Under certain conditions strains of *S. aureus* develop resistance *in vitro* to streptomycin or to penicillin (G). 2. Greater variation occurs in the degree and rate at which resistance is acquired to penicillin than to streptomycin. 3. All of the streptomycin and penicillin-resistant strains studied showed some decrease in resistance upon transfer in broth free of the antibiotics. 4. Two of the penicillin-fast strains increased in resistance to streptomycin whereas the streptomycin-fast strains remained sensitive to penicillin. 5. Some streptomycin-fast staphylococcal strains show a change in pigment production and a reduction in the rate of carbohydrate fermentation.