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The Activity of Streptomycin in the Presence of Serum and Whole Blood.*

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(Introduced by P. H. Long.)

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Previous studies[1] have shown that streptomycin resistant variants of Staphylococcus, Pneumococcus and Streptococcus grow as well in a bactericidal test as their parent susceptible strains. It was presumed that, in the presence of streptomycin, the additive effect of fresh whole blood could be demonstrated on both the susceptible and resistant strains. The effect on the latter, it was felt, would be minimal, but such investigation was of importance because of the numerous examples of streptomycin resistance which had been observed to develop during the course of therapy. No studies have been reported in which the susceptibility of the resistant organism was tested by the bactericidal technique in the presence of added streptomycin—a condition which would naturally occur in a patient under therapy.

The following studies were made to investigate the inter-relationships which may be present when bactericidal tests are performed with a strain of *Staphylococcus aureus* in the presence of streptomycin. In addition, the various important known components of the bactericidal test such as phagocytosis, hemolysis of red blood cells, immune serum and labile constitutents were investigated. All

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories, Inc., Parke Davis and Company, and the Upjohn Company.

1 Chandler, C. A., and Schoenbach, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 208.

these factors were controlled, wherever possible, by simultaneous duplicate observations in which the sensitive parent strain of the staphylococcus was used.

Materials and Methods. The strain used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital. The strain of staphylococcus resistant to 1000 μg of streptomycin was derived from the parent strain as previously described.[1] Following initial experiments all cultures were transferred at intervals of 3 to 4 weeks on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth (PDW)† containing streptomycin in appropriate concentrations. Each tube, within the range, contained 10% less streptomycin as measured against the initial tube containing the most streptomycin in a regular progression, so that the differences from tube to tube were uniform. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of about 200,000 organisms per one cc of medium. The tubes were incubated at 37°C and readings were made at the end of 24 hours.

The bactericidal power of freshly defibrinated human blood on the staphylococcus was determined in the following manner. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours, after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions. This technique was varied as described in each group of experiments by the substitution of serum as a substrate and by the addition of immune serum, streptomycin, etc.

Phagocytic activity was measured in the following manner: 0.25 cc of whole, defibrinated blood was measured into sterile pyrex tubes. To the above tubes, 0.1 cc serum, either normal or immune was added. Streptomycin, in a final concentration of 500 μg was then introduced into some of the tubes. Fluid volumes in all tubes were made equal by the addition of appropriate amounts of broth. Finally each tube was inoculated with 0.05 cc of a 3-hour culture of either the susceptible or resistant strain. The tubes were then sealed in an oxygen flame, slowly rotated for 30 minutes at 37°C, broken open, and one drop of the contents of each was smeared on a slide as a blood film. The slides were then stained with Wright's stain and examined with the oil immersion lense. A count was made of the number of intracellular staphylococci contained in 25 polymorphonuclear leukocytes and the percentage of cells taking part in the phagocytosis was noted.

Growth curves were obtained by making bacterial counts at 2, 4, 6 and 24 hours. The number of organisms was estimated by making count plates of suitable dilutions of the culture. Estimation of turbidity in a photoelectric colorimeter with readings at hourly intervals was used in each instance to confirm these counts.

Immune rabbit serum was obtained in the following manner. A white albino rabbit was injected intravenously with a washed, heat-killed suspension of organisms. The suspension was prepared from an 18-hour broth culture of the susceptible parent strain of the staphylococcus. The rabbit received injections 4 times a week for 3 weeks. During the final week living organisms were used. The final agglutinative titre of the serum prepared as described above was 1-20 against both the susceptible strain and the resistant strain. Thread agglutination was observed when both these strains were grown in media containing this immune serum.

One lot (Winthrop-Pfizer Lot No. P4713) of streptomycin was employed throughout this study. It had a labeled potency of 1000

† Peptone-Dextrose-Water.

TABLE I.
Bactericidal Studies.

Strain	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum				
		Streptomycin, 0 μg	Streptomycin, 5 μg × million	Streptomycin, 10 μg × million	Normal rabbit serum	Immune rabbit serum
Parent susceptible	Whole blood	420	4.2	42	640	640
	Serum	520*	52.0*	52*	—	—

* Human serum.

TABLE II.
Proportion of Organisms in Resistant Staphylococcus Culture Growing at Indicated Streptomycin Concentrations.

Concentration of streptomycin in medium (μg per cc)							
0		500		750		1000	
colonies		colonies		colonies		colonies	
No.	%	No.	%	No.	%	No.	%
700	100	700	100	600	85.7	600	85.7

units per milligram of pure base.

Results. (1) The sensitivity to streptomycin of the susceptible strain was re-evaluated in the light of previous reports that serum or whole blood inhibited the action of streptomycin.[2,3,4] In broth, the bacteriostatic range of streptomycin for the susceptible strain was 5 to 12 micrograms on repeated determinations. In the presence of 60% fresh human or rabbit serum (final concentration), this same strain was inhibited by 5 μg of the antibiotic when the inocula varied from 520 organisms to 5.2 million per cc. Growth occurred in the presence of 5 and 10 μg of streptomycin when a large inoculum was used (52,000,000 organisms).

In fresh whole human blood, inhibition occurred in a concentration of 5 μg with inocula ranging from 420 to 4.2 million. With 10 μg of streptomycin present, an inoculum of 42 million organisms was necessary to produce growth. In both the serum and whole blood assays, these findings were confirmed by sub-culture after 48 hours. This was deemed necessary to rule out erroneous interpretations which may arise because of nonspecific turbidity or hemolysis.

In summary, as shown in Table I, the susceptibility of this strain of staphylococcus to streptomycin was not affected by the presence of serum or whole blood within the limits of measurement which were employed. In addition, there was no real indication that the bacteriostasis achieved with streptomycin was augmented by the bactericidal activity of whole blood. In the absence of streptomycin, there was no indication that this strain of staphylococcus was affected by normal whole blood or when immune serum was added to the system.

(2) The resistant strain of staphylococcus had been derived in this laboratory from the parent strain and had been found to grow readily in broth containing more than 1000 μg of streptomycin. Colony counts performed by seeding agar plates containing either no streptomycin or up to 1000 μg of streptomycin revealed no essential difference in the number or type of colonies. This is shown in Table II. In the bactericidal test, as shown in Table III, the resistant strain was not inhibited by normal human blood or when immune serum was added to the system. Thus in all respects, except for its resistance to streptomycin, it resembled the parent strain.

When the resistant strain was grown in the

[2] May, J. R., Voureka, A. E., and Fleming, A., *Brit. Med. J.*, 1947, **1**, 627.

[3] Henry, R. J., Berkman, S., and Housewright, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 42.

[4] Hobby, G. L., and Lenert, T. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 242.

TABLE III.
Bactericidal Studies.

Strain	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum						
		Streptomycin, 0 μg	Streptomycin, 125 μg	Streptomycin, 500-1000 μg	Normal rabbit serum	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500-1000 μg*
Resistant	Whole blood	320	320	320	340	340	340	340
	Serum	360†	360†	360†	280‡	280‡	280‡	280‡

* Streptomycin.
† Human serum.
‡ Rabbit serum.

TABLE IV.
Bactericidal Studies.

Strain	Duration of growth, hr	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum					
			Normal rabbit serum	Normal rabbit serum + 125 μg*	Normal rabbit serum + 500 μg*	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500 μg*
Resistant	24	Whole blood	3600	3,600,000	360,000	360	360,000	3,600,000
	48	" "	340	—	—	340	340	340
Resistant	24	Rabbit serum	280	2800	280,000	280	280	28,000
	48	" "	280	280	2800	280	280	280

* Streptomycin.

TABLE V.
Bactericidal Studies.

Strain	Time inoculated after mixing	Substrate	Minimum No. of organisms required to initiate growth per cc of serum					
			Normal rabbit serum	Normal rabbit serum + 125 μg*	Normal rabbit serum + 500 μg*	Immune rabbit serum	Immune rabbit serum + 125 μg*	Immune rabbit serum + 500 μg*
Resistant	0	Rabbit serum	280	280	2800	280	280	280
	24 hr	Rabbit serum	160	1600	1600	160	160	16,000

* Streptomycin.

TABLE VI.
Phagocytic Studies.

Strain	Normal rabbit serum		Immune rabbit serum		500 μg streptomycin		Normal rabbit serum + 500 μg streptomycin		Immune rabbit serum + 500 μg streptomycin	
	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci
Parent susceptible	204	68%	625	94%	126	40%	—	—	—	—
Resistant	960	92%	1100	92%	832	72%	997	84%	775	72%

presence of 60% rabbit serum containing streptomycin, definite inhibition of growth was observed. With as little as 125 μg of streptomycin per cc, this strain, resistant to 1000 μg per cc, showed retardation in growth after 24 hours when small inocula (200 to 300,000 organisms per cc) were used. At the end of 48 hours, however, growth equal to that in the controls was observed. A similar pattern of growth inhibition with streptomycin was noted in the media containing whole blood with or without added immune serum. Inhibition was somewhat more apparent in these latter experiments because hemolysis was delayed. These results are summarized in Table IV.

An investigation was then undertaken into the cause of this transient inhibition of growth. That streptomycin might be inactivated or bound by one of the serum constituents, was considered a distinct possibility. If so, this would account for the subsequent escape from the inhibitive effects of streptomycin. Accordingly, duplicate experiments were devised in which normal rabbit serum which had been inactivated by heat, with and without added immune rabbit serum, and to which streptomycin had been added in concentrations of 125 and 500 μg per cc was inoculated with organisms at 2 different times. One set was inoculated immediately and incubated for 24 hours while the duplicate set was incubated for 24 hours after which it was inoculated with organisms and again incubated for 24 hours. As shown in Table V, no difference was observed between the set inoculated immediately and that inoculated after 24 hours and, therefore, the escape from inhibitive effects of streptomycin after 24 hours could not be attributed to inactivation of streptomycin.

The lag observed when the resistant strain was grown in the presence of streptomycin cannot be attributed to the non-homogeneous distribution of streptomycin sensitive organisms as suggested by Berkman *et al.*[5] As shown in Table II, almost all organisms were able to grow at higher streptomycin levels

[5] Berkman, S., Henry, R. J., and Riley, D. H., *J. Bact.*, 1947, **53**, 567.

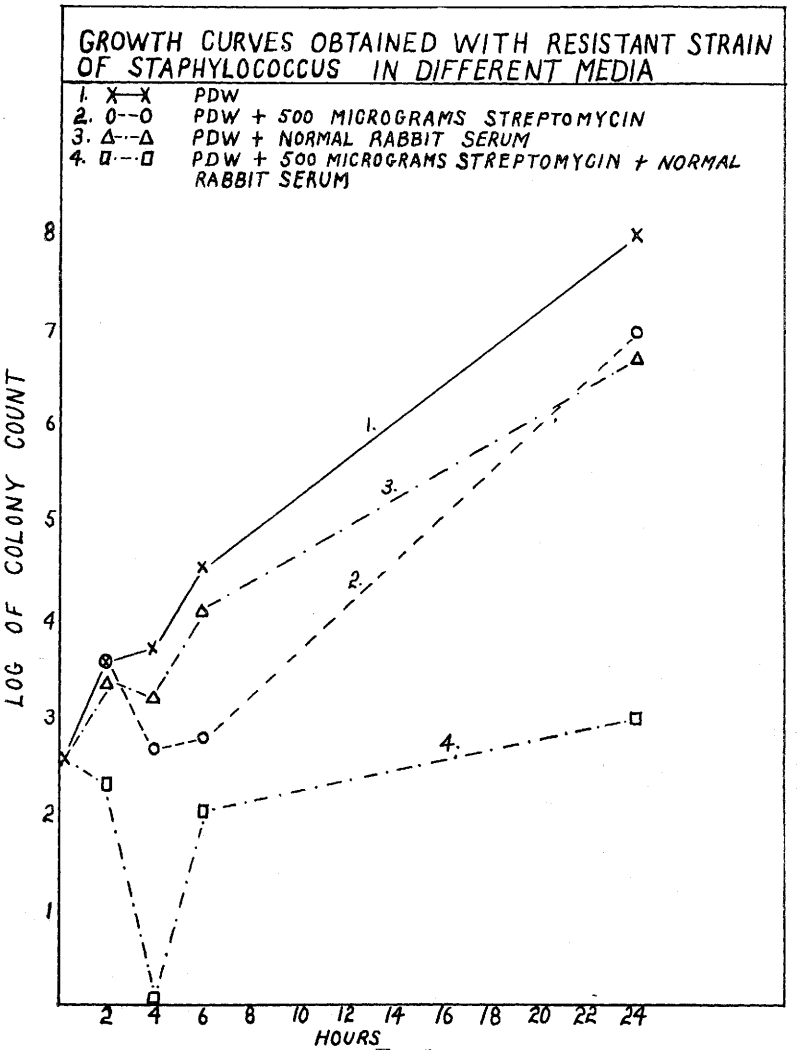

FIG. 1.

than were necessary to demonstrate this lag.

These observations are not necessarily in conflict with Price *et al.*[6] and Berkman and his co-workers[5] who found a marked difference in sensitivity to streptomycin when assayed in a somewhat deficient as compared with a highly nutritive medium. It would appear, however, from our observations that beyond a certain point progressive enrichment, although promoting growth of organisms, does not affect the final streptomycin titre.

[6] Price, C. W., Randall, W. A., Chandler, V. L., and Reedy, R. J., *J. Bact.*, 1947, **53**, 481.

Another possibility which was entertained was that streptomycin had stimulated phagocytosis and that, with the death of the leukocytes after 24 hours, growth had reappeared.

As shown in Table VI, it was found that in the presence of streptomycin, the phagocytic activity of the white blood cells was depressed in every instance and, therefore, the initial bacteriostasis could not be explained on the basis of enhanced phagocytosis at these streptomycin levels.

In the light of the above results, it seems probable that the only explanation of the in-

hibition of growth in the first 24-hour period in the presence of streptomycin, was a retardation in the rate of growth. Despite the observation that growth of the resistant strain in the absence of streptomycin did not differ materially from the susceptible strain in media containing broth or serum, this strain of staphylococcus, resistant to more than 1000 μg of streptomycin per cc, was markedly inhibited in its rate of growth by much lower concentrations of the antibiotic in the first 24-hour period. This inhibitory activity could be demonstrated with as little as 125 μg of streptomycin per cc. It was augmented when serum was present in the medium. Sample growth curves in streptomycin and non-streptomycin containing media are presented in Fig. 1. This type of growth curve resembles that noted with the sulfonamides which is apparent in the early phase of growth when small inocula are used.

Discussion. With the susceptible parent strain of staphylococcus, the addition of fresh whole blood to streptomycin did not augment streptomycin activity. On the other hand, the efficiency of the streptomycin was apparently not affected by the presence of blood and its constituents. It was, therefore, difficult to believe that the streptomycin was bound to some protein constituent in the serum unless the resultant combination retained the antibacterial properties of the antibiotic.

Inhibition of the rate of growth of a highly resistant strain in the presence of streptomycin was observed during the first 24 hours of incubation when the concentration of the latter was far below 1000 μg per cc. An explanation of this observation may be that the nutritional requirements of the organism had been modified with the acquisition of streptomycin resistance. The resistant strain appeared to grow as readily as the parent susceptible strain in the usual nutrient media, in the presence of serum and even in fresh whole human blood. When streptomycin was present in these media, however, the delay in the rate of growth of the resistant strain seemed to indicate that some nutritional factor or necessary enzymatic process had been materially affected. Eventually the resistant organism escaped from this streptomycin inhibition. The nature of this reaction may be of prime importance for the elucidation of the mode of action of streptomycin on microorganisms and possibly also the nature of the escape mechanism associated with resistance. These investigations are being continued at present.

These data may indicate that under normal conditions 2 alternative growth mechanisms are available to the normal organism. A general discussion of such mechanisms has been reviewed by Dubos.[7] In this study it would appear that one metabolic or synthetic pathway is blocked by streptomycin and another is insufficiently developed to permit normal growth. With the development of resistance, the second mechanism is augmented but apparently is still insufficient for optimal growth when streptomycin is blocking the first mechanism.

It is possible that the inhibitory activity of streptomycin is related to its combination with desoxyribonucleic acid in the bacterial cell.[8] Nucleoprotein metabolism is important in the growth and reproduction of bacteria. The combination of nucleic acid with streptomycin may interfere with the synthesis or utilization of nucleoproteins. Studies on the mode of action of penicillin have demonstrated that with *Staphylococcus aureus* dissimilation of ribonucleic acid was inhibited without any effect upon glucose oxidation.[9] The reaction inhibited by penicillin is probably the dissimilation of a pentose.[10] It has been claimed that streptomycin would also exhibit an inhibitory effect on the same reaction.[10]

When a strain resistant to streptomycin is isolated from a patient, it may be inferred that despite its ability to grow in high concentrations of the antibiotic *in vitro,* its rate of

[7] Dubos, R. J., *The Bacterial Cell,* 1945, Harvard University Press, Chapter VIII, Bacteriostatic and Bactericidal Agents, 275.

[8] Cohen, S. S., *J. Biol. Chem.,* 1947, **168**, 511.

[9] Krampitz, L. O., and Werkman, C. H., *Arch. Biochem.,* 1947, **12**, 57.

[10] Krampitz, L. O., Green, M. N., and Werkman, C. H., *J. Bact.,* 1947, **53**, 378.

growth in the body may be retarded by even small concentrations of the antibiotic. One should expect, therefore, that such resistant strains would have difficulty in maintaining themselves due to phagocytic mechanisms available *in vivo*. The impairment of phagocytosis by streptomycin should be studied in greater detail so that this important cellular factor in immunity may be more clearly defined.

Summary 1. The activity of streptomycin on a susceptible strain of *Staphylococcus aureus* was not changed in the presence of serum or whole blood. 2. The addition of fresh whole blood with or without immune serum did not augment streptomycin activity. 3. A resistant strain of *Staphylococcus aureus* was inhibited during the first 24 hours by streptomycin in low concentrations. This effect was augmented when serum was added to the medium. 4. Direct growth curves indicate that this inhibition by streptomycin of a resistant strain was attributable to interference with growth of the organism rather than inactivation of labile constituents. 5. Phagocytosis in the presence of high concentrations of streptomycin appears to be impaired. 6. The bacteriostatic activity of streptomycin was manifest on both susceptible and resistant strains. With the latter, this inhibition was transitory. The bacteriostatic mechanism and bactericidal mechanism are dissociated phenomena.

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Culture Procedures in Microbiologic Assays with *L. arabinosus* and *L. casei*.

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In the application of microbiological assay procedures, there have arisen questions relative to possible differences in the response of the test organisms as a result of variations in handling the stock cultures. Several investigators have mentioned the possible influence of stock culture media and conditions to which it is subjected on the subsequent response of an organism under assay environment, but few have made studies to determine the nature and extent of these influences. The most detailed work which has come to our attention has been the culture studies on *L. arabinosus* and *L. casei* by Nymon and Gortner.[1]

The present report is concerned with some notes made on frequency of transfer of stock cultures, preparation and dilution of inoculum and temperature and time of incubation of the assay tubes as these factors affect the assay.

Observations were made for a period of 6 months on the effect of frequency of transferring stock cultures of *L. arabinosus* on the subsequent growth response of this organism to graded amounts of nicotinic acid in the U.S.P.[2] assay medium. From a stab culture of *L. arabinosus* carried in agar medium (agar 1.5%, dextrose 1% and yeast 1%) 5 transfers were initially made into fresh agar medium of the same composition. After incubation at 30°C for 24 hours these cultures were stored in a refrigerator. One, designated "original", was maintained in the original stab without further transfer. The others were transferred at intervals of one, 2, 3, and 4 weeks respectively throughout the experiment. After incubation of the transplant for 24 hours it was returned to the refrigerator

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1 Nymon, M. C., and Gortner, W. A., *J. B. C.*, 1946, **163**, 277.

2 First U.S.P., XII Bound Supplement, 1943, 76.