concentration of drug so greatly influences the effect of the streptomycin is probably a limiting factor in its usefulness. In like manner the effectiveness of the sulfonamides has been dependent at least in part on the concentration of organisms in relation to the amount of sulfonamide present. The number of units of penicillin necessary for inhibition of a culture, on the other hand, is altered only by large variations in the number of organisms present.

One can only speculate on the relationship this may have to the apparent development of bacterial resistance to streptomycin. It is now recognized however that in contrast to penicillin, the effectiveness of streptomycin *in vivo* is at times limited by the ease with which susceptible strains of microorganisms develop resistance to it.

Under the experimental conditions used, no differences have been demonstrated in the sensitivity of most organisms to impure streptomycin sulfate, to the crystalline $CaCl_2$ double salt of streptomycin, or to highly purified streptomycin sulfate prepared from a crystalline salt. Impure streptomycin and highly purified or crystalline streptomycin appear equally effective *in vitro* against all organisms except *E. typhosa*. The significance of the fact that certain preparations of impure streptomycin sulfate are more effective against strains of *E. typhosa* than highly purified streptomycin will be discussed in detail elsewhere.

Summary. 1. A standardized procedure for determination of sensitivity of microorganisms to streptomycin is described.

2. The sensitivity of an organism to streptomycin is influenced by age of culture, concentration of organisms, growth phase of culture, and constituents of medium used. Providing these factors are held constant, the sensitivity of a given strain will remain constant from day to day.

3. The action of streptomycin is bacteriostatic rather than bactericidal. Its action is inhibited by certain growth stimulating substances such as peptone, as well as by certain reducing substances.

4. The sensitivity of 84 strains belonging to 7 species is described. Marked variation in sensitivity exists between different strains within a single species and at times between different cells within a given strain.

5. The sensitivity of 9 strains belonging to 8 species is essentially the same when tested against crude streptomycin sulfate (453 μg/mg), against 3 preparations of the crystalline $CaCl_2$ double salt of streptomycin (685 to 708 μg/mg) and against a preparation of streptomycin sulfate (802 μg/mg) prepared from a crystalline salt.

6. The sensitivity of 4 strains of *E. typhosa* to certain preparations of impure streptomycin sulfate is greater than to highly purified streptomycin sulfate.

15923

Observations on the Action of Streptomycin *in vitro* (II).*

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It is recognized that a wide variety of factors may influence the observed sensitivity of an organism to streptomycin. Certain of these factors have been discussed in a recent communication from this laboratory.[1] The sensitivity of a number of strains belonging to 10 different species was described and it was shown that with the exception of *E. typhosa* the strains tested were equally sen-

* A part of this work was presented at the Conference on Antibiotic Research held at Washington, D.C. on January 31 and February 1, 1947 under the auspices of the Antibiotics Study Section of the National Institute of Health.

[1] Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 235.

sitive to impure streptomycin sulfate, to highly purified streptomycin sulfate and to the crystalline $CaCl_2$ double salt of streptomycin.

The differences in sensitivity of an organism caused by alteration in medium, or variation of culture from day to day, are no greater than the difference in sensitivity of a strain when tested against various preparations on the same day or the difference in sensitivity of individual cells within a given strain.[1] An actual increase in the resistance of strains of bacteria to streptomycin both *in vitro* and *in vivo* has been observed by many investigators, however.[2-11]

Experimental. It has been amply demonstrated that the presence of serum alters the sensitivity of an organism to penicillin.[12-15] Since the chemotherapeutic effect of any such agent is dependent upon its activity in the presence of body fluids and since the demonstration of blood or serum levels is dependent upon the determination of the sensitivity of a standard organism in the presence of serum, the present study was undertaken to determine the effect of serum upon the sensitivity of various organisms to streptomycin. In addition, the effect of certain related factors have been investigated.

Effect of Serum on Sensitivity of Organisms to Streptomycin. Pooled normal human serum and normal horse serum were used throughout. The sensitivity of a number of organisms belonging to several species was determined against a preparation of streptomycin sulfate† having a potency of 802 μg per mg. The procedure used was identical with that previously described[1] except that broth containing varying amounts of serum was used in place of plain broth.

A total of 31 strains belonging to 7 different species were tested in broth containing 0, 1, 5, 10, 20 and 50% normal horse serum. Thirteen strains, also belonging to 7 different species, were tested in broth containing the same amounts of human serum.

The sensitivity of the Gram negative organisms tested (i.e., *E. coli, E. typhosa, K. pneumoniae,* and *A. aerogenes*) was unaltered by addition of 1 to 5% of either human or horse serum to the medium. Larger amounts of serum increased the sensitivity of these strains slightly. Certain strains of *Staphylococcus aureus* were similarly affected by serum whereas others became considerably less sensitive to streptomycin when concentrations of 1 to 50% serum were present. All strains of *Streptococcus hemolyticus, Streptococcus viridans,* and *Diplococcus pneumoniae* showed a marked increase in resistance to streptomycin in the presence of 1 to 5% serum. (Tables I, II).

The average sensitivity for each species of organisms tested again illustrates the marked increase in resistance of the Gram positive organisms. (Graph I).

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† All streptomycin used throughout this study was prepared by Chas. Pfizer and Co.

TABLE I.
Effect of Horse Serum on Sensitivity of Organisms to Streptomycin.

Strain	Sensitivity in μg per cc % serum 0	1	5	10	20	50
E. coli						
(W)	5.4	4.4	4.5	4.4	3.6	1.5
(B)	3.0	3.0	4.0	3.0	2.0	1.0
(T)	7.0	3.0	6.0	4.0	3.0	2.0
(Y)	4.0	3.0	4.0	3.0	3.0	<1.0
(A)	4.0	3.0	4.0	3.0	3.0	<1.0
(H)	4.0	3.0	4.0	4.0	3.0	2.0
E. typhosa						
(S)	32.0	24.0	24.0	32.0	32.0	<8.0
(Ga)	36.0	36.0	(20.0)	28.0	16.0	8.0
(M)	32.0	32.0	28.0	24.0	20.0	16.0
(Mc)	>40.0	>40.0	40.0	36.0	24.0	8.0
(U)	28.0	32.0	36.0	36.0	32.0	8.0
(G)	>40.0	40.0	32.0	36.0	32.0	12.0
A. aerogenes	1.5	1.5	1.25	1.25	1.0	1.0
Staph. aur.						
(H)	<4.0	20.0		20.0	8.0	<4.0
(T)	2.0	6.0	5.0	4.0	3.0	3.0
(HT)	2.0	2.0	4.0	3.0	2.0	<1.0
(HS)	4.0	5.0	6.0	4.0	3.0	2.0
(M)	2.0	6.0	4.0	2.0	3.0	<1.0
(K)	<1.0	5.0	5.0	3.0	3.0	3.0
(HS_2)	24.0	24.0	24.0	40.0	32.0	16.0
D. pneumoniae						
(I/230)	4.0	36.0	28.0	32.0	36.0	
(D/39)	4.0	16.0	28.0	20.0	28.0	
(A66)	<1.0	20.0	20.0	24.0	28.0	
Strep. hem.						
(C203Mv)	<4.0	36.0	40.0	>40.0	40.0	36.0
(Ch)	<4.0	20.0	24.0	24.0	24.0	16.0
(090)	10.0	>50.0	>50.0	>50.0	>50.0	45.0
(H_{76})	<5.0	40.0	40.0	40.0	45.0	35.0
(C_1)	20.0	>50.0	>50.0	>50.0	>50.0	>50.0
Strep. vir.						
(R)	32.0	>80.0	>80.0	>80.0	>80.0	>80.0
(T)	15.0	>50.0	>50.0	50.0	45.0	35.0
(L)	30.0	>50.0	>50.0	>50.0	>50.0	>50.0

Sensitivity = least amount of streptomycin causing complete inhibition of growth after 72 hours at 37°C.

Strains C203Mv and "Ch" belong to the Lancefield Serological Group A; Strain 090 to Group B; Strain H_{76} to Group C; Strain C_1 to Group D.

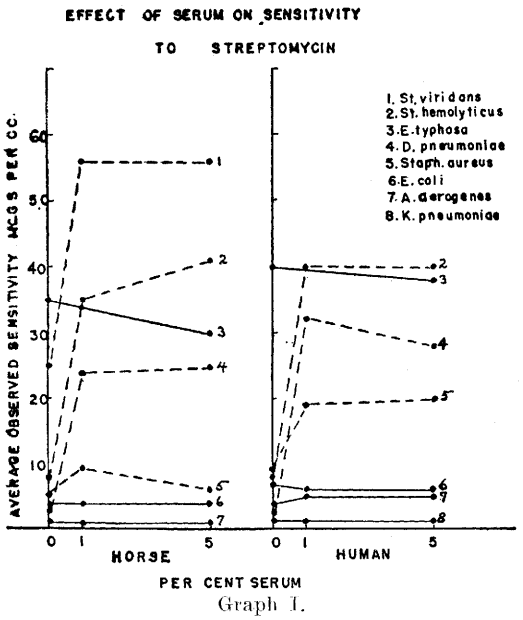
Effect of Serum on Growth of Organisms with and without Streptomycin. In view of the possibility that the decrease in the sensitivity of the Gram positive organisms to streptomycin in the presence of serum might be merely a reflection of their greater ability to multiply in the presence of serum, further experiments were carried out to determine the effect of serum on 3 representative strains: (1) a standard strain of *E. coli* (W), (2) the Oxford strain of *Staphylococcus aureus* (Strain H), and (3) *Streptococcus hemolyticus,* strain C203Mv.

Streptomycin sulfate having a potency of 802 μg per mg was used throughout this experiment. To each of 6 tubes was added broth containing 0, 1, 5, 10, 20 and 50% pooled human serum. To each tube, sufficient streptomycin was added to give a final concentration of 2 μg per cc. Each tube was

TABLE II.
Effect of Human Serum on Sensitivity of Organisms to Streptomycin.

Strain	Sensitivity in μg per cc % serum 0	1	5	10	20	50
E. coli						
(W)	8.5	8.0	8.5	6.0	4.5	3.0
(Y)	5.0	5.0	4.0	4.0	4.0	2.0
(H)	7.0	7.0	6.0	5.0	4.0	2.0
E. typhosa						
(S)	>40.0	>40.0	>40.0	>40.0	32.0	8.0
(Ga)	40.0	20.0	32.0	36.0	32.0	12.0
(U)	>40.0	32.0	>40.0	24.0	28.0	8.0
A. aerogenes	2.0	2.5	2.0	2.0	1.5	1.0
K. pneumoniae	1.0	1.0	1.0	<0.5	<0.5	<0.5
Staph. aur.						
(H)	16.0	>40.0	>40.0	40.0	40.0	20.0
(M)	5.0	7.0	10.0	5.0	3.0	3.0
(HS)	8.0	>10.0	10.0	>10.0	8.0	4.0
Strep. hem.						
(C203Mv)	8.0	>40.0	>40.0	>40.0	>40.0	>40.0
D. pneumoniae						
(I/230)	4.0	36.0	40.0	32.0	32.0	16.0
(D/39)	4.0	20.0	24.0	20.0	20.0	8.0
(A66)	<1.0	40.0	20.0	24.0	20.0	8.0

Sensitivity = least amount of streptomycin causing complete inhibition of growth after 72 hours at 37°C.

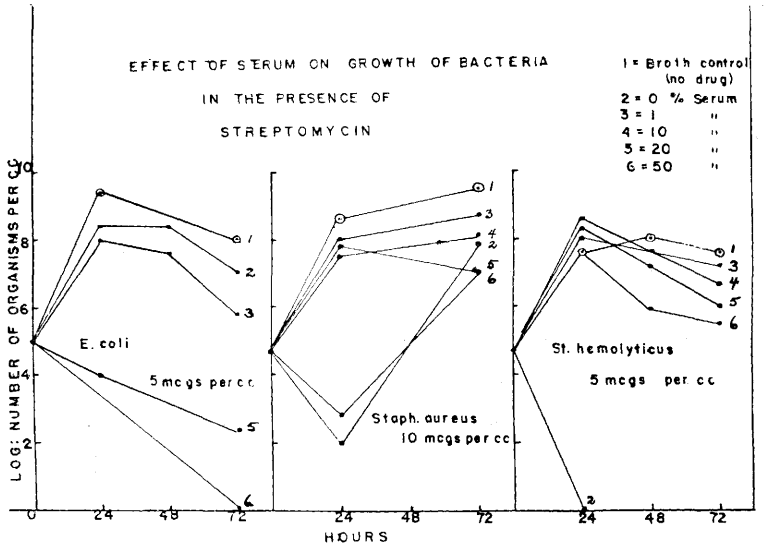


Graph I.

seeded with a sufficient amount of a 6 hour plain broth culture of *E. coli* (W) to give a final dilution of 1:2000. Prior to dilution, the culture had been adjusted to 78% transmission, Photovolt lumetron No. 400. The number of organisms per cc was determined by plate counts at 0, 24, 48, and 72 hours. Similarly, *E. coli* was tested using 5 μg of streptomycin per cc, *Staphylococcus aureus* (H) was tested using 5, 10, and 20 μg per cc and *Streptococcus hemolyticus* (C203Mv) using 2, 5, and 40 μg per cc. Similar tests were carried out on each organism using horse serum.

In the case of *E. coli,* there was no evidence that growth was altered by the presence of 1, 20 or 50% horse or human serum. A concentration of 2 μg of streptomycin caused no inhibition of growth either in plain broth or in serum broth. Five micrograms of streptomycin per cc, on the other hand, produced a slight bacteriostatic effect in plain broth. Its action was greatly enhanced in the presence of 20 to 50% human serum or 50% horse serum. (Graph II).

The growth of *Staphylococcus aureus* (strain H) was likewise unaffected by con-



Graph II.

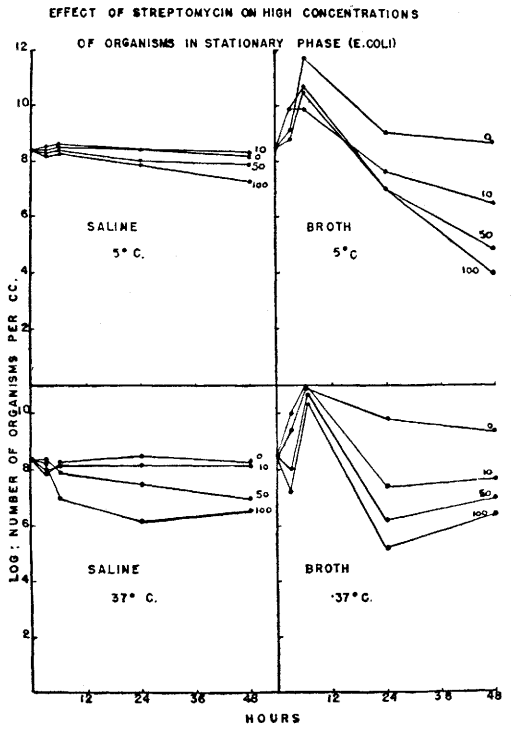
centrations of 1 to 50% horse or human serum. In the presence of 5 μg of streptomycin per cc, there was no bacteriostatic action either in the presence or absence of serum. In the presence of 10 or 20 μg of streptomycin per cc of plain broth, temporary bacteriostasis occurred. This bacteriostatic action was completely inhibited by 1, 10 or 20% human or horse serum, whereas the effect in 50% serum was comparable with that in plain broth. (Graph II).

The growth of *Streptococcus hemolyticus* was most markedly affected by the presence of serum. Concentrations of 1 to 50% serum caused a slight but definite increase in the rate of growth. In the presence of 2 μg of streptomycin per cc of plain broth, temporary bacteriostasis occurred whereas 5 μg per cc was adequate to produce sterilization of the culture within 24 hours. This effect was completely inhibited by 1, 10, 20 or 50% horse or human serum. The bactericidal action of 40 μg of streptomycin per cc was likewise completely inhibited by 1, 10, and 20% horse or human serum. (Graph II).

These results are comparable with those indicated by the alteration in sensitivity of organisms to streptomycin, in the presence of serum. The effect of serum on the sensitivity of an organism is greater than any effect of serum on the rate of growth of the organism, as shown by growth curves.

Effect of Streptomycin on Stationary or Slowly Dividing Cells. In view of the possible correlation between rate of growth and the effect of serum on the sensitivity of various organisms to streptomycin, experiments were carried out to determine the effect of streptomycin on stationary or slowly dividing cells and on rapidly dividing cells.

An 18 hour broth culture of *E. coli* was centrifuged and the organisms were resuspended (1) in saline and (2) in broth. Each suspension was divided into 4 parts and to 3 of each sufficient streptomycin was added to give final concentrations of 10, 50, and 100 μg per cc respectively. The fourth tube from each series was held as control. The saline suspensions were incubated at 5°C. Similar suspensions were prepared and incubated at 37°C. Colony counts were made at 3, 6, 24, and 48 hours. No multiplication took place in saline at either 5°C. or 37°C. Streptomycin in a concentration of 10 μg per cc exerted no action against the organisms present. In the presence of 50 and 100 μg of streptomycin per cc, a slight decrease in the number of organisms occurred at 37°C. after a lag of from 3 to 6 hours. The effect at 5°C. was not significant. In broth the number of organisms increased many fold. A slow bactericidal action was observed with 10 as well as with 50 and 100 μg per cc of streptomycin. The decrease in the number



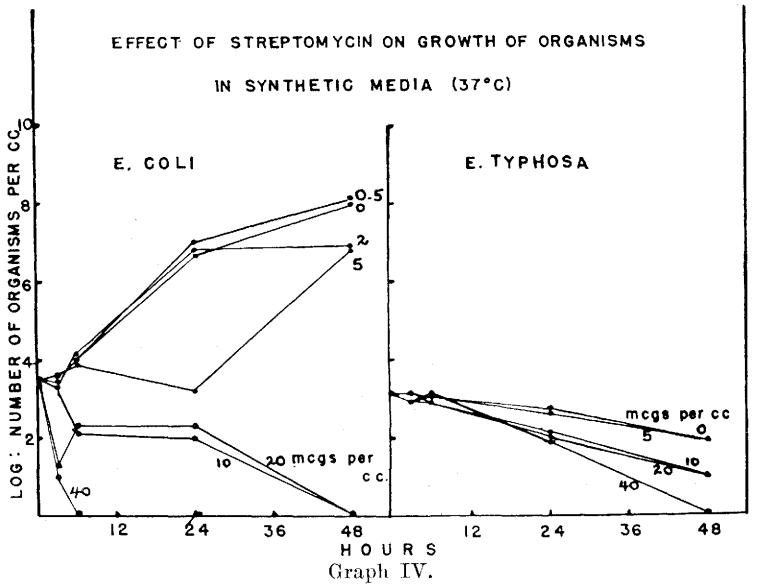
Graph III.

of organisms occurred only after a lag of at least 6 hours during which time definite multiplication took place. The decrease in broth was far greater than that in saline. It was apparent that under certain conditions streptomycin is capable of acting on both stationary and dividing cells provided a sufficiently high concentration of drug is used. The effect is greater on dividing cells however. (Graph III).

The difference in the action of streptomycin on rapidly dividing cells and on slowly dividing or resting cells was further demonstrated in the following typical experiment.

McLeod's synthetic medium containing asparagin and no glucose was prepared in such a manner as to contain 0, 0.5, 2, 5, 10, 20 and 40 μg of streptomycin per cc. Two tubes of each concentration were set up; one was inoculated with a sufficient amount of an 18 hour plain broth culture of *E. coli* to yield a final dilution of 10^{-5}, the other was similarly inoculated with *E. typhosa*. In each case, the cultures used were centrifuged and washed 3 times in the synthetic medium prior to dilution. Incubation was carried out at 37°C. and the number of organisms per cc determined by plate counts at 0, 3, 6, 24, and 48 hours.

E. coli multiplied rapidly in the synthetic medium. Likewise, in the presence of 0.5 and 2.0 μg of streptomycin per cc, rapid multiplication took place. A temporary bacteriostatic action occurred during the first 24 hours of incubation in the presence of 5 μg per cc. With larger amounts of streptomycin, there was a definite and permanent



Graph IV.

decrease in the number of organisms.

E. typhosa showed no growth in this synthetic medium. The number of organisms per cc remained constant. In the presence of 2.0 and 5.0 μg of streptomycin per cc, the number of organisms also remained constant. With 10, 20, or 40 μg per cc, however, there was a slight and gradual decrease in the number of organisms. (Graph IV).

It was again apparent that streptomycin, if in sufficient concentration, can act at 37° C. on slowly dividing or stationary cells. In view of the large initial number of organisms present in these experiments and the sensitivity of the strains used, the concentrations of streptomycin necessary to demonstrate bacteriostatic action were not exceptionally high, however.

Discussion. The relation of number of organisms and concentration of drug to the sensitivity of an organism to streptomycin has been discussed elsewhere. It is recognized that, in contrast to penicillin, the effectiveness *in vivo* is limited by the fact that susceptible strains of microorganisms frequently develop resistance to it. Undoubtedly, many factors influence this development of resistance. It has been suggested by Reimann and his coworkers that certain factors in the body may interfere with the bacteriostatic action of streptomycin.[16,17] Wolensky and Steenken[8] on the other hand were unable to demonstrate any destruction of streptomycin or any alteration in its bacteriostatic or bactericidal power by contact with broth, serous body fluids, pus or normal tissue juices.

In the present study, data have been presented which further suggest that factors within the body may enhance development of resistance. It has been demonstrated that serum markedly increases the resistance of certain of the Gram positive organisms to streptomycin. The effect of other body fluids or tissue substances on these or other bacterial species remains to be determined. It is obvious however that the sensitivity of an organism to streptomycin, as determined *in vitro* in the usual broth medium, is not necessarily an index of the *in vivo* sensitivity of the organism.

The importance of serum in the bioassay determination of penicillin in blood has been amply demonstrated by Tompsett, Schultz, and McDermott[15] and by Richardson and his associates.[18] Since, of necessity, serum is present in all such determinations, and since the effect is so marked in the case of streptomycin, it is apparent that the organism used in such assays must be one that is affected little if any by the concentration of serum present.

Whether the alteration in sensitivity to streptomycin, induced by the presence of serum, is due to an actual change in the organism or to the outgrowth of resistant forms already present cannot be determined from the data available here. That the effect is not due to an actual destruction of streptomycin by serum is suggested by growth curves and by the fact that the sensitivity of many organisms is unaltered in the presence of serum. It seems more likely that the apparent resistance to streptomycin is due to an alteration in the metabolic processes of certain bacterial species when grown in serum.

Summary. 1. The presence of 1 to 5% or more horse or human serum enhances the resistance of *Streptococcus hemolyticus, Streptococcus viridans, Diplococcus pneumoniae,* and certain strains of *Staphylococcus aureus.*

2. Serum in concentrations of 1 to 5% has no effect on the sensitivity of *E. coli, E. typhosa, K. pneumoniae,* or *Aer. aerogenes* to streptomycin. Higher concentrations of serum may at times enhance the action of streptomycin on certain of these organisms.

3. Streptomycin is effective under certain conditions against stationary or slowly dividing cells, as well as against rapidly dividing cells, provided a sufficient concentration of the drug is used.

4. No direct correlation between the rate

[16] Reimann, H. A., Price, A. H., and Elias, W. F., *Arch. Interm. Med.*, 1945, **76**, 269.

[17] Elias, W. F., and Durso, J., *Science*, 1945, **101**, 589.

[18] Richardson, A. P., Miller, I., Schumacher, C., Jambar, W., Pansy, F., and Lapedes, D., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 514.

of growth of an organism with or without serum and the effect of serum on the sensitivity of an organism to streptomycin can be made at this time.

15924

Biological Activity of a Residual Form of Streptomycin against *Eberthella typhosa.**

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The fact that a number of naturally occurring forms of penicillin have been recognized[1] suggests that other antibacterial agents of biologic origin may also occur in more than one form. The antibacterial action of impure and highly purified streptomycin sulfate and of the crystalline $CaCl_2$ double salt of streptomycin is discussed in detail elsewhere.[2,3] Under the experimental conditions used, a slight difference in their activity was apparent against *E. typhosa* but not against the other organisms tested.

Experimental. In the preparation of a crystalline salt of streptomycin from partially purified material (400 to 500 μg per mg), a residual fraction remains. This fraction possesses antibacterial activity. The present report covers preliminary observations which demonstrate that in certain biological aspects it differs from the impure or highly purified streptomycin sulfate,† as produced commercially, and from the crystalline $CaCl_2$ double salt of streptomycin.

Bacterial Spectrum. A comparison was made of the sensitivity of 7 different organisms to the residual form of streptomycin, to impure streptomycin sulfate, and to highly purified streptomycin sulfate prepared from a crystalline salt. The procedure used was identical with that described in a previous communication.[2] Sensitivity has been accepted throughout as the least amount of streptomycin in μg per cc causing inhibition of growth as evidenced by absence of gross turbidity at the end of 72 hours incubation at 37°C.

No significant differences in the sensitivity of *Aer. aerogenes, E. coli, Bc. subtilis* or *Staphylococcus aureus* to these streptomycins was observed. The difference in the sensitivity of *E. typhosa* however was marked. Whereas 40 or more μg of impure or highly purified streptomycin sulfate per cc were necessary for inhibition of this strain, only 8 μg of the crude residue were necessary (Table I).

Other preparations of streptomycin residue

TABLE I.
Bacterial Spectrum of 3 Forms of Streptomycin.

Strain	Sensitivity in μg per cc		
	Crude sulfate 453 μg/mg	Pure sulfate 802 μg/mg	Residue 165 μg/mg
A. aerogenes	2.	2.	2.
B. subtilis (W)	1.5	1.	1.
B. mycoides (W)	1.5	1.5	2.
E. coli (W)	6.	7.	7.
Staph. aur. (H)	20.	18.	18.
E. typhosa	40.	>40.	8.
M. tuberculosis ($H_{37}Rv$)	5.	5.	5.

* A part of this work was presented at the Conference on Antibiotic Research held at Washington, D.C. on January 31 and February 1, 1947 under the auspices of the Antibiotics Study Section of the National Institute of Health.

1 Report by the Committee on Medical Research, O.S.R.D. Washington, and the Medical Research Council, London, on Chemistry of Penicillin, *Science*, 1945, **102** (2660), 627.

2 Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 235.

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

† All streptomycin used throughout this study was prepared by Chas. Pfizer and Co.