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.

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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.

TABLE II.
Bacterial Spectrum of Impure Streptomycin Residue as Compared to Impure and Purified Streptomycin Sulfate.

Streptomycin	Potency in μg/mg	Strain: Sensitivity in μg per mg			
		A. aerog.	*E. coli*	*Staph. aur.*	*E. typh.*
Pure sulfate (standard)	802	2.25	7.5	19	32
Impure sulfate	277	5	7	20	20
Impure residue					
Prep. No. 1	187	1.5	7	20	8
" 2	40	2	6	>20	<6
" 3	124	2	5	20	8
" 4	43	2	6	>20	<6
" 5	68	3	6	>20	<6
" 6	35	3	8	>20	<4

have been tested and found to show the same effect (Table II).

Four freshly isolated strains and one additional stock strain of *E. typhosa* were similarly tested for sensitivity to preparations of impure streptomycin sulfate, highly purified streptomycin sulfate prepared from crystalline material, and to the crude streptomycin residue. All 5 strains were at least 2 to 5 times more sensitive to the streptomycin residue than to highly purified streptomycin sulfate. The sensitivity to impure streptomycin sulfate was less than the sensitivity to the streptomycin residue although greater than the sensitivity to the highly purified sulfate preparations. The relative order of sensitivity to the 3 forms was consistent with the fact that they represent successive steps of purification (Table III).

A single preparation of streptomycin residue was partially purified and the resultant active fraction again tested against *Aer. aerogenes, E. coli, Staphylococcus aureus,* and *E. typhosa.* All of the strains tested were more sensitive to this partially purified residue than they were to the crystalline salt. The difference was most marked, however, with *E. typhosa* (Table IV).

TABLE III.
Action of Various Forms of Streptomycin on *E. typhosa.*

Strain	Sensitivity in μg per cc		
	Crude sulfate 453 μg/mg	Pure sulfate 802 μg/mg	Residue 165 μg/mg
E. typhosa (stock)	40	>40	8
(Ga)	36	>40	8
(Me)	24	>40	16
(Mc)	24	>40	8
(Ur)	40	>40	20

TABLE IV.
Comparison of Bacterial Spectrum of Streptomycin Residue and Crystalline $CaCl_2$ Double Salt of Streptomycin.

Strain	Sensitivity in μg per cc		
	Original residue 165 μg/mg	Partially purified residue 22000 μg/cc	Crystalline double salt 780 μg/mg
A. aerogenes	2.5	1.5	2.5
E. coli	>10	7	>10
Staph. aur. (H)	20	16	>20
E. typhosa	8	8	>40
M. tuberculosis ($H_{37}Rv$)	5	5*	

* No apparent difference in sensitivity as indicated by the No. of μg per cc necessary for complete inhibition of growth. In the presence of 5 μg/cc of the partially purified residue, however, growth appeared much more slowly and to a less extent than in the presence of 5 μg per cc of purified streptomycin sulfate, crude streptomycin sulfate or the original residue. All sensitivity determinations on *M. tuberculosis* were carried out in the Dubos medium.[4]

Antibacterial Action on Organisms Grown on Solid Media. Recognition of the various penicillins as distinct entities was facilitated by the early observation of Coghill[5] that the degree of antibacterial activity of these substances differed when tested against *Bc. subtilis* and against *Staphylococcus aureus* by the Oxford cup plate method. In view of the fact that the streptomycin residues de-

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.,* 1946, **83**, 409.

[5] Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.,* 1945, **49**, 411.

scribed above possessed such marked activity against *E. typhosa,* as compared to impure streptomycin sulfate or highly purified streptomycin sulfate prepared from crystalline material, it seemed likely that a similar difference could be detected by the Oxford method.

Using as standard a preparation of highly purified streptomycin sulfate, having a potency of 802 μg per mg and prepared from a crystalline salt, the potency of a number of preparations of the streptomycin residue was determined against *E. typhosa.* All of these residues showed a potency per mg against *E. typhosa* considerably higher than the official *Bc. subtilis-E. coli* value.‡ The *E. typhosa-Bc. subtilis (E. coli)* differential ratios of these preparations of streptomycin residue ranged from 1.94 to 3.14. All of these preparations were highly impure (Table V).

Effect of Various Forms of Streptomycin on Growth of Organisms in Vitro. The difference in the activity of the residual form of streptomycin as compared to the impure or purified streptomycin sulfate was further demonstrated in growth curves on *E. coli, E. typhosa,* and *Streptococcus hemolyticus.* Eighteen-hour broth cultures diluted in streptomycin broth immediately prior to use to give a concentration of 1:2000 organisms per cc were used. The concentrations of streptomycin used represented amounts slightly below and slightly above the sensitivity of the individual strain to the specific form of streptomycin. Incubation was carried out at 37°C. The number of organisms per cc was determined by plate counts at 0, 24, 48, and 72 hours.

TABLE V.
Activity of Streptomycin Residue Oxford Assay Method.

Sample No.	Potency (units per mg)		
	*B. subt.-E. coli**	*E. typhosa*	*E. typh.-B. subt.** ratio
1	165	363	2.20
2	210	407	1.94
3	40	95	2.38
4	124	283	2.28
5	43	135	3.14
Standard: Streptomycin sulfate	802	802	1.00

* Assay values with *B. subtilis* and *E. coli* are similar. Average of these potencies used throughout.

‡ The potency of streptomycin in terms of micrograms of streptomycin per mg of material is determined routinely according to the procedure recommended by the Food and Drug Administration. The Oxford cup plate method, using *Bacillus subtilis* as the test organism, is the official test. Similar results may be obtained by the turbidimetric method, using *E. coli* as the test organism, and the average on the 2 tests has been used in the present study.

No definite difference could be detected in the effect of the 3 forms of streptomycin on the growth of *E. coli* or *Streptococcus hemolyticus.*

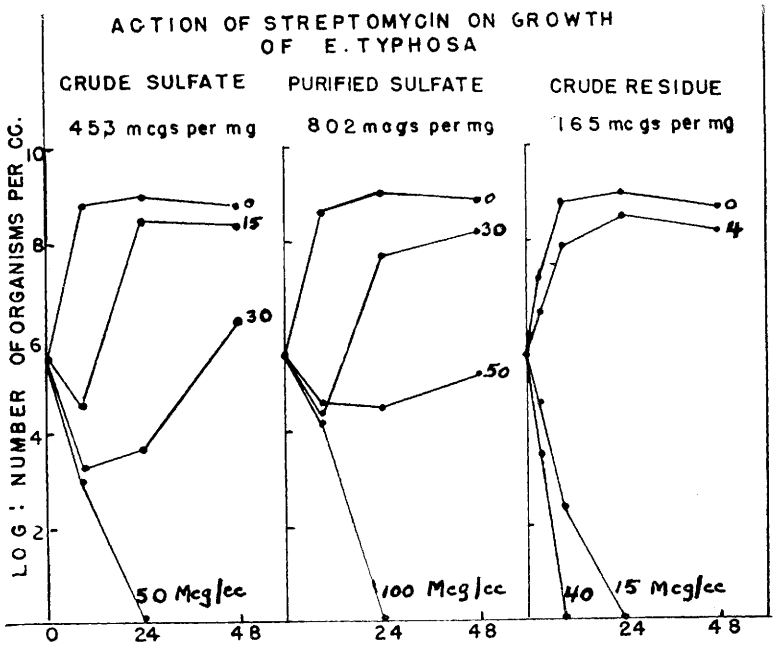
In the case of *E. typhosa,* the difference was marked. Fifteen μg of the crude residue per cc showed greater antibacterial activity against this organism than 30 μg of impure streptomycin sulfate or 50 μg of pure streptomycin sulfate per cc.

These results were consistent with the fact that the sensitivity of this organism to the crude residue was only 8 μg per cc whereas its sensitivity to the impure streptomycin sulfate was 40 μg per cc and to the purified streptomycin sulfate, >40 μg per cc (Graph I).

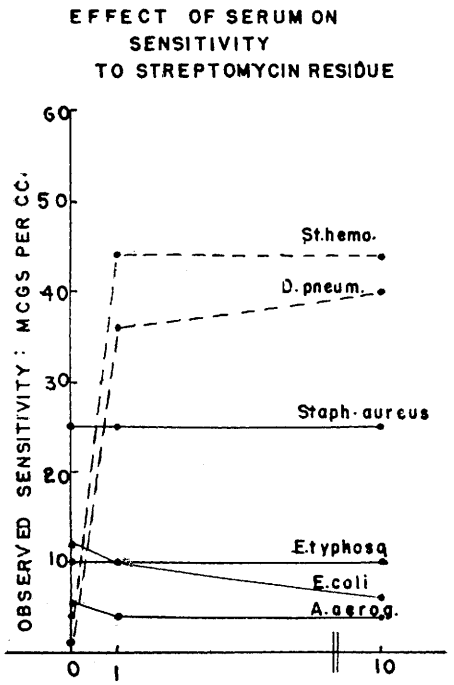
Effect of Serum on the Sensitivity of Organisms to the Residual Streptomycin. In a previous communication,[3] the effect of serum on the sensitivity of certain organisms to impure and highly purified streptomycin sulfate was described. It was shown that concentrations of 1 to 5% horse or human serum decreased the sensitivity of certain of the Gram-positive organisms to these forms of streptomycin.

Similar experiments were carried out to determine the effect of serum on the sensitivity of 6 different organisms to the streptomycin residue. Concentrations of 1, 10, 20 and 50% pooled normal human serum were used. The high buffering capacity of the broth medium served to maintain a constant pH of 7.8 throughout.

The resistance of the streptococcus and



Graph I.



Graph II.

pneumococcus strains tested was greatly increased whereas the single strain of staphylococcus tested reacted similarly to the Gram-negative strains whose sensitivity was unaltered (Graph II).

The effect of serum on the sensitivity of various organisms to the streptomycin residue was thus identical with its effect on their sensitivity to impure or highly purified streptomycin sulfate as previously reported.[3]

Effect of Residual Streptomycin in E. Typhosa Infections in Mice. A stock strain of *E. typhosa* was used as the infecting organism throughout these experiments. One cc of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of a 16-hour plain broth culture, diluted in 5% mucin according to the method of Miller,[6] was injected intraperitoneally into each of a series of 20 g white mice. A minimum of 8 to 10 mice was used for each dilution of each series. Treatment was started 2 hours after the infecting dose. The streptomycin was administered to part of the animals subcutaneously in peanut oil[7] in divided dosage. To the remainder of the animals it was administered in aqueous solution by mouth.

[6] Miller, C. P., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1136.

[7] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

TABLE VI.
Chemotherapeutic Action of Various Forms of Streptomycin on *E. typhosa* Infections in Mice.

Form of streptomycin		Impure sulfate	Crude residue
Potency: μg per mg		802	165
Ratio:	$\frac{E.\ typhosa}{Bc.\ subt.\text{-}E.\ coli}$	1.00	2.20
Route of administration*	Total dosage† μg	Therapeutic effect (% survival)	
Subcutaneous	60	21.7	26.1
	110	47.9	40.0
	175	45.8	35.0
	240	64.6	80.6
	500	80.8	78.2
Oral	100	10.7	10.7
	250	19.2	7.3
	500	37.9	16.6
Controls	0	15.3	

* All mice were infected by the intraperitoneal route; treatment was carried out by the subcutaneous or oral routes.

† 10^{-2}, 10^{-3}, and 10^{-4} dilutions of culture and a minimum of 8-10 mice per dilution were used for each dosage level tested.

Forty per cent of the total dose was administered 2 hours, 40% 7 hours, and 20% 24 hours after infection. Total dosages of 60, 110, 175, 240 and 500 μg were used in the subcutaneously-treated animals; 100, 250, and 500 μg in the animals treated by mouth. A control series of untreated animals was included in each experiment. The untreated infection uniformly produced death in 85% of animals. The results may be seen in Table VI. The streptomycin residue was active *in vivo* when administered by the intraperitoneal route. Little or no difference in the protective power of this material and of the purified streptomycin sulfate was observed. The conditions of the experiment, however, and the nature of the infection in mice may not have been such as would show a quantitative difference in the activity of these fractions. By the oral route no protection resulted from the administration of 100, 250, or 500 μg of either form of streptomycin.

Absorption of Residual Streptomycin. A comparison of the absorption of a single preparation of streptomycin residue, and of impure and highly purified streptomycin was carried out in a small series of rabbits following the intramuscular injection of 5000 μg per kg of body weight. The concentration of streptomycin per cc of serum was determined at 0, 1, 2, 3, 4, and 6 hours by the Oxford cup plate method using *B. subtilis* as the test organism. It is recognized that the rabbit is an unsatisfactory animal for absorption and excretion studies. Serum levels varied widely from one animal to another. The average levels on 5 to 6 animals per sample, however, showed no significant difference in the rate at which impure streptomycin sulfate, highly purified streptomycin sulfate, or crude streptomycin residue is eliminated from the blood stream.

Discussion. A residual form of streptomycin, which is a more efficient antibacterial agent against *E. typhosa in vitro,* has been described. The sensitivity of the typhoid bacillus to this residual fraction of streptomycin obtained during the purification process is at least 2 to 5 times greater than to the crystalline $CaCl_2$ double salt of streptomycin. By the Oxford cup plate method, the *E. typhosa-Bc. subtilis (E. coli)* differential ratio of impure preparations of this material is approximately 2.0-3.0.

The significance of the active material present in the streptomycin residue remains to be determined. Whether or not this residual substance is actually a different streptomycin than that with which we are familiar at present must await further chemical separation and chemical analysis. Fried and Titus[8] have recently described a form of streptomycin (B) differing in *in vitro* activity from purified streptomycin. Furthermore, present evidence suggests that crystalline streptomycin, as prepared today, may not be entirely uniform.

In the case of penicillin, 5 naturally occurring forms have been described. These forms differ quantitatively in their antibacterial efficiency both *in vitro* and *in vivo*.[1] Furthermore penicillin esters have been de-

[8] Fried, J., and Titus, E., *J. Biol. Chem.,* 1947, **168**, 391.

scribed which, although inactive *in vitro*, are highly effective *in vivo* in certain species of animals.[9] The residual form of streptomycin and crystalline streptomycin also differ quantitatively *in vitro*. The residual form is a more efficient antibacterial agent against the typhoid bacillus *in vitro*, and possesses definite *in vivo* activity as well. Its value as a chemotherapeutic agent, nevertheless, must await further pharmacological and therapeutic tests.

Summary. The sensitivity of *E. typhosa* to a residual fraction of streptomycin obtained during the purification process is at least 2 to 5 times greater than to the crystalline $CaCl_2$ double salt of streptomycin. By the Oxford cup plate method, the *E. typhosa-Bc. subtilis* (or *E. coli*) differential ratio of this material is approximately 2.0-3.0. This residual streptomycin is active *in vivo* as well as *in vitro*. Its activity *in vivo* is not as great, however, as might be anticipated from the *in vitro* results.

[9] Meyer, K., Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 100.

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Complement Fixation Studies with Pus Antigen in Granuloma Inguinale.

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Three types of antigens for use in complement fixation tests for granuloma inguinale have been described. Anderson and her associates[1] utilized "capsular substances" obtained by chemical treatment of embryonic yolk cultures of *"Donovania granulomatis."* Dunham and Rake[2] grew a strain obtained from Anderson on yolk-beef heart infusion agar and then on Levinthal's medium, from which they prepared culture antigens. Jennison *et al.*[3] used saline suspensions of Donovan bodies grown in the yolks of fertile eggs.

This report presents data from use of an antigen prepared from pus aspirated from an abscess of Donovan body origin. This abscess was one of 3 which followed an initial lesion of the cervix uteri. The diagnosis of granuloma inguinale of the cervix was established by smear and biopsy.[4]

Serological Studies. The pus antigen, used in complement fixation tests for granuloma inguinale, was prepared from pus aspirated from a large fluctuating abscess of the hand. Smears revealed typical Donovan bodies in abundance. No other organism could be demonstrated. The pus was diluted 1:6 with physiological saline solution, shaken thoroughly, and heated at 60°C for 1 hour on 2 successive days. Merthiolate (1:10,000) was added. All tests were negative for organisms which might be expected to grow on the media employed.

The pus antigen was used in complement fixation tests with sera from 25 patients with proven granuloma inguinale, from 14 hospitalized individuals with various infections, other than venereal, from 12 syphilitic patients with no evidence of granuloma inguinale, and from 5 healthy individuals who served as controls.

As shown in Table I, 21 (84%) of the

[1] Anderson, Katherine, Goodpasture, E. W., and De Monbreun, W. A., *J. Exp. Med.*, 1945, **81**, 41.

[2] Dunham, Wolcott, and Rake, Geoffrey, *J. Bact.*, 1946, **51**, 67.

[3] Jennison, David B., Helwig, Elson B., and Milstone, J. H., *Arch. Dermat. and Syph.*, 1947, **55**, 342.

[4] Packer, Henry, Turner, Henry B., and Dulaney, Anna Dean, *J. A. M. A.*, in press.