5 animals were autopsied 6 months after the institution of hormone treatment. Administration of hormone was suspended 53 days before autopsy in 2 NH females which received similar treatment.

Two ovariectomized NH females received the same treatment with the estrogen and in addition were injected weekly with 150 μg of progesterone in oil. Two additional ovariectomized females received the estrogen plus progesterone, but treatment was suspended 47 days before autopsy.

Two NH females ovariectomized at 21 days of age were given 17.5 μg of testosterone propionate in oil weekly by subcutaneous injection beginning 2 weeks after operation. This treatment was continued until the animals were autopsied 5½ months after ovariectomy. Three mice were similarly treated except that they received no injections of hormone for the 46 days preceding autopsy.

Histologic serial sections of the adrenal glands were studied following complete autopsy. Representative sections of the reproductive tracts, submaxillary glands and kidneys were observed to assay the hormonal status of the experimental animals.

Cortical adenomas appeared in gonadectomized mice of the NH strain receiving amounts of estrogenic hormone just sufficient to maintain constant estrus. Ovariectomized females receiving masculinizing doses of testosterone propionate (as judged by submaxillary gland histology) also developed cortical adenomas. Similar adenomas were present in the ovariectomized females which received estrogen and progesterone simultaneously, and in ovariectomized females receiving no hormone injections. A group of 4 ovariectomized NH females which received 0.1 μg of estradiol dipropionate weekly for 4 months following operation at 35 days of age had definite adenomas at autopsy.

Seventeen of 20 gonadectomized mice on hormone treatment developed adenomas which were in all respects similar to those observed in controls which had been merely gonadectomized. Weekly injections of 0.05 μg of estradiol dipropionate failed to prevent the development of spontaneous adrenal cortical adenomas in NH females.[3]

Since adrenal cortical adenomas developed in gonadectomized mice receiving maintenance doses of sex hormone, it appears that absence of functional gonadal tissue, rather than withdrawal of sex hormone, is the prime factor in the development of adrenal cortical adenomas in gonadectomized mice. The gonad, as compared to the adrenal, may preferentially utilize trophic hormone of the pituitary gland. When functional gonadal tissue is absent in a strain of mice with an extragonadal sensitive target organ, such as the NH adrenal cortex, adenomas develop.

[3] Frantz, M. F., unpublished data.

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Search for Antibiotics Active Against *Mycobacterium tuberculosis*. 1. A Streptothricin-like Antibiotic from a *Streptomyces sp.** (17408)

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In a search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* several hundred antagonists have been encountered and one called *Streptomyces sp.* EI_5 in our records was chosen for intensive study because of its marked activity against the mycobacteria and the low animal toxicity of its crude filtrate.[1] The present report is

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the account of a study of this organism and the antibiotic it produces.

Methods and Results. A. Morphological and Cultural Characteristics of Streptomyces sp. EI_5. Since preliminary tests of solubility and antibacterial activity indicated that the antibiotic produced by *S. sp* EI_5 is similar to streptomycin and streptothricin, a comparison of the organisms that produce these antibiotics was made.

Streptomyces sp. EI_5 was morphologically similar to *Streptomyces griseus*, and both organisms produced a soluble yellow to yellow-brown pigment when grown at room temperature on nutrient agar, glucose agar, starch agar, and gelatin. Only *S. griseus* produced pigment on potato agar. The color of the aerial mycelium varied from white to tea-green with *S. griseus* and from white to gray with *S. sp.* EI_5. The vegetative mycelium of *S. griseus* was white to cream colored and that of *S. sp.* EI_5 yellow to tannish orange. *Streptomyces sp.* EI_5 was not susceptible to the *S. griseus* actinophage of Reilly, Harris and Waksman.[2] These observations indicate that *S. griseus* and *S. sp.* EI_5, are similar but significantly different organisms.

Streptomyces lavendulae produced a darker brown pigment on glucose agar, nutrient agar and glucose broth than *S. sp.* EI_5, and in addition produced pigment on the potato plug. Further, slide cultures on Czapek's medium at 30°C showed the aerial mycelium of *S. lavendulae* to be pink to lavender, and the hyphae often coiled terminally, in contrast to the white to gray color and the straight hyphae of *S. sp.* EI_5. The conidia of *S. sp.* EI_5 were observed to be oval to globose and considerably smaller than those of *S. lavendulae*. These differences indicate that *S. lavendulae* and *S. sp.* EI_5 are not identical.

It is also probable that *S. sp.* EI_5 is distinct from the organism described by Trussell, Fulton and Grant[3] since, unlike our organism, their organism produces streptomycin and displays tightly coiled terminal hyphae. In the latter respect, our organism differs also from the two *Actinomyces sp.* described by Kelner and Morton[4] (A-10 and A-105) which produce the antibiotics, lavendulin and actinorubin.

B. *Production of Antibiotic* EI_5.‡ The 1% glucose medium of Waksman and Woodruff[5] proved to be as satisfactory as any medium tried including modifications of the soybean medium described by Rake and Donovick.[6]

Attempts to enhance antibiotic production by the procedure employed for *S. griseus* by Waksman, Reilly, and Johnstone[7] of growing the organism in high concentrations of its own antibiotic failed. The standard inoculum adopted was a heavy spore suspension of a high antibiotic-producing substrain. The organisms were grown in shake cultures for 5-7 days and the culture fluid harvested when the pH rose to 7.0 to 7.4. It was filtered through cotton and stored at –45°C prior to extraction.

C. *Concentration and Purification of* EI_5. Since the antibiotic was found to belong to the 3rd solubility group of Waksman,[8] the general scheme of the procedure used by Le-Page and Campbell[9] for the concentration of streptomycin was adopted. It consisted of adsorption on carbon, elution with acid methanol and precipitation with acetone. The total yield in this concentrate was approximately 30% of the amount present in the original filtrate.

Further purification was accomplished by the use of the synthetic zeolite Decalso. Spot plate analyses by the method of Loo and co-

1 Gardner, G. M., Master's Thesis, University of Washington, 1946.

2 Reilly, H. C., Harris, D. A., and Waksman, S. A., *J. Bact.*, 1947, **54**, 451.

3 Trussell, P. C., Fulton, C. O., and Grant, G. A., *J. Bact.*, 1947, **53**, 769.

4 Kelner, A., and Morton, H. E., *J. Bact.*, 1947, **53**, 695.

‡ Antibiotic EI_5 is subsequently designated as EI_5.

5 Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207.

6 Rake, G., and Donovick, R., *J. Bact.*, 1946, **52**, 223.

7 Waksman, S. A., Reilly, H. C., and Johnstone, D. C., *J. Bact.*, 1946, **52**, 393.

8 Waksman, S. A., Microbial Antagonisms and Antibiotic Substances, p. 171, The Commonwealth Fund, New York, N. Y., 1945.

9 Le Page, G. A., and Campbell, E., *J. Biol. Chem.*, 1946, **162**, 163.

workers[10] showed that most of the antibiotic was adsorbed on the Decalso column and all dark pigment eliminated.

The eluate was evaporated *in vacuo* to a small volume and the antibiotic brought down as a fine white precipitate by addition of acetone. Still further purification was carried out by the chromatographic method as employed by Carter and co-workers[11] for the purification of streptomycin.

The material was finally concentrated *in vacuo*, reprecipitated with acetone, dissolved in double glass-distilled water and sterilized by passage through a sintered glass filter.

D. *Methods of Assaying* EI_5. The potency of EI_5 was determined by a serial dilution method employing the broth base of the medium recommended by Loo and co-workers[10] for streptomycin assay. The unitage is expressed as dilution units using *Escherichia coli-communior* as the test organism. On the basis of dry weight, the final product was bacteriostatic for *E. coli-communior* in a dilution of approximately 1:200,000. The activity of various batches of antibiotic was observed to vary considerably and no consistent results were obtained which would make possible an accurate comparison of the effectiveness of the several purification methods used.

E. *Antibacterial Spectrum of* EI_5. Antibacterial tests readily established that EI_5 and streptomycin are different antibiotics. For example, the minimum concentrations inhibitive for *Bacillus subtilis* were EI_5, 1.6 units and streptomycin, 4.0 units. With *Serratia marcescens* the values were EI_5 30.0 units and streptomycin, 0.75 unit.

The differences between the antibacterial spectra of EI_5 and streptothricin were less marked.

In a further attempt to identify EI_5 resort was made to the development of strains of test organisms resistant to streptothricin and to EI_5. The parent strains of organisms used for this work were *B. subtilis*, *E. coli* M3G and *Micrococcus pyogenes var, aureus* 209-P. They were cultured at 37°C in nutrient broth pH 7.1, containing varying amounts of antibiotic covering the ranges of tolerance and subcultures made at 24-hour intervals with inocula from the highest concentration of antibiotic showing growth in the previous series.

Sixty or more serial transfers were made over a period of 3 months before cultures of appreciable resistance were obtained (cf. Kelner and Morton,[4] and Trussell, Fulton, and Grant[3]). It was noted that some of the organisms tended to lose their acquired resistance upon repeated subculture on nutrient agar.

The antibacterial spectra of streptothricin and purified EI_5, determined with the parent and resistant strains shown in Table I, clearly indicate that streptothricin and EI_5 are different substances. It is noteworthy that all of the resistant strains developed against EI_5 also showed marked resistance to streptothricin, whereas, resistant strains developed against streptothricin do not show as uniform and marked increase in resistance to EI_5. Of additional interest is the case of *B. subtilis* cultivated in EI_5 in which a marked resistance to streptothricin resulted without an increase in resistance to EI_5.

One explanation which may account for these findings is that the development of resistance to one antibiotic may result in the development of resistance to another closely related antibiotic (cf. Kelner and Morton[4]). Another possible explanation is that the preparation of EI_5 may contain a small amount of streptothricin as an impurity.

F. *Comparison of* EI_5 *with other Streptothricin-like Antibiotics.* Since lavendulin and actinorubin (Kelner and Morton[4]) were not available, resort was made to cross-streak antibacterial spectral analysis of *S. sp.* EI_5, *S. sp.* A-10, and *S. sp.* A-105 in an attempt to obtain information on the identity of the respective antibiotics. The results are presented in Table II.

The marked variation in the zones of inhibition shown by *S. sp.* A-10 and *S. sp.* EI_5 indicates strongly that the antibiotic produced

[10] Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, 1945, **50**, 701.

[11] Carter, H. E., Clark, R. K., Jr., Dickmans, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, 1945, **160**, 337.

TABLE I.
Antibacterial Spectra of Streptothricin and EI_5 Determined with Resistant and Parent Strains of Bacteria.
(Readings taken after 24 hr at 37°C in nutrient broth at pH 7.0).

Test organism	Min. inhibiting conc. of streptothricin in dilution units	Min. inhibiting conc. of EI_5 in dilution units
Bacillus subtilis (parent)	12	4
" " (streptothricin resistant)	120	8
" " (EI_5 resistant)	90	4
Escherichia coli (parent)	0.6	0.8
" " (streptothricin resistant)	8	8
" " (EI_5 resistant)	20	20
Micrococcus aureus (parent)	0.3	0.5
" " (streptothricin resistant)	22	4
" " (EI_5 resistant)	36	40

TABLE II.
Cross-streak of Antibacterial Spectra of *Streptomyces sp.* A-10, *Streptomyces sp.* EI_5, and *Streptomyces sp.* A-105.

Test organism	Zone of inhibition in mm		
	S. sp. A-10	*S. sp.* EI_5	*S. sp.* A-105
Achromobacter sp.	18	16	12
Aerobacter aerogenes	20	30	25
Bacillus cereus	10	3	5
" *megatherium*	17	15	10
" *mycoides*	25	30	30
" *sp. 290*	22	27	20
" *subtilis*	8	1	3
Corynebacterium xerose	12	2	7
Escherichia coli-communior	20	20	15
Micrococcus aureus 209-P	18	15	15
Sarcina lutea	20	15	10
Serratia marcescens	12	2	2
EI_5 resistant			
Bacillus subtilis	8	5	5
Micrococcus aureus	10	3	5
Streptothricin resistant			
Bacillus subtilis	5	1	1
Escherichia coli-communior	15	7	7
Micrococcus aureus	25	7	12

by *S. sp.* A-10 (lavendulin) is different from EI_5. In contrast, the zones of inhibition shown by *S. sp.* A-105 and *S. sp.* EI_5 are similar indicating that a close relationship between actinorubin and EI_5. Further both substances are soluble in water and acid-methanol, insoluble in ether and acetone, resist boiling, and, as shown later, have similar toxicities for mice.

G. *The Antibacterial Activity of Antibiotic EI_5 for the Mycobacteria.* By use of the method described by Youmans[12] EI_5 was shown to be bacteriostatic for *M. tuberculosis* H37Rv in a dilution of 1:100,000 and for *Mycobacterium phlei* in a dilution of 1:500,000.

H. *Toxicity of EI_5 for Mice.* Large doses of EI_5 ranging from 150,000 to 270,000 units per kg in the form of both the initial concentrate and purified antibiotic were injected intraperitoneally into 10 white mice weighing 20 to 35 g. One animal died on the 5th day and the others were sacrificed from the 5th

[12] Youmans G. P., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 119.

to the 19th day. The results demonstrated that EI_5 is apparently less toxic than streptothricin as judged by the findings of Molitor,[13] and more nearly equal to the toxicity of actinorubin as determined by Morton.[14] Both actinorubin and EI_5 in large dosage cause focal necrosis of the liver and renal epithelial damage.

Summary. Search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* has resulted in the isolation of a streptothricin-like antibiotic, termed EI_5, from an unidentified *Streptomyces sp.* It is bacteriostatic for *Mycobacterium tuberculosis* H37Rv in dilution of 1:100,000 and closely resembles actinorubin in its antibacterial spectrum, general properties, and toxicity.

The organisms that produce the two antibiotics, *Streptomyces sp.* A-105 and *Streptomyces sp.* EI_5 are morphologically distinct.

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13 Molitor, H., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 101.

14 Morton, H. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 327.

Multiplicity of Antigens in Ragweed Pollen Extracts as Demonstrated by the Technic of Oudin. (17409)

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The heterogeneity of ragweed pollen extracts has been demonstrated by chemical fractionation[1] and by electrophoretic analysis.[2] Precipitins to crude extracts of ragweed pollen have been produced by numerous workers,[3,4] but because of the heterogeneity of the extracts used, it would be expected that the precipitins so obtained were directed to a number of different antigenic substances. It is the purpose of this paper to show, by a new technic, the presence of a multiplicity of antibodies in the antisera produced against crude extracts of ragweed pollen, and consequently, the presence of a multiplicity of antigens in the extracts used for immunization.

The new technic used to demonstrate this antigenic complexity was that recently described by Oudin[5-9] which consists in overlaying a solid immune serum-agar mixture contained in a thin-bore tube with the antigen solution. An antigen-antibody precipitate is formed as a sharp band which moves down the tube as more and more antigen diffuses into the agar. Oudin showed that only one band is formed when a single antigen-antibody system is present, while multiple systems give

1 Stull, A., Sherman, William, and Wilson, W., *J. Allergy*, 1942, **13**, 537.

2 Abramson, H. A., *Ann. Allergy*, 1947, **5**, 19.

3 Kukla, A., and Hirsch, D., *J. Immunol.*, 1946, **53**, 391.

4 Eagle, H., Arbesman, C. E., and Winkenwerder, W. L., *J. Immunol.*, 1939, **36**, 425.

5 Oudin, J., *Compt. Rend. Acad. Sci.*, 1946, **222**, 115.

6 Oudin, J., *Bull. Soc. Chim. Biol.*, 1947, **29**, 140.

7 Oudin, J., *Annal. de l'Inst. Pasteur*, 1948, **75**, 30.

8 Oudin, J., *Annal. de l'Inst. Pasteur*, 1948, **75**, 109.

9 Oudin, J., *Compt. Rend. Acad. Sci.*, 1949, **228**, 1890.