are thermostable at 56°C, but are partially destroyed at 70°C.

The inhibitory or lethal action is observed *in vitro* in a medium prepared with the immune whole blood or immune serum on which the homologous organisms are grown. The reaction takes place in 3 stages, viz.,

(1) The organisms lose their motility and become agglutinated.

(2) They round up and become markedly swollen.

(3) Finally, the internal structure undergoes hyalinization which ends in the death of the protozoon.

Complement is not necessary for this reaction. This inhibitory or lethal factor appears to differ from ablastin.

15027

Penicillin Inhibitor from Staphylococci Which Have Developed Resistance to Penicillin in the Human Body.*†

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In previous reports[1,2] from this laboratory it was pointed out that strains of coagulase-positive staphylococci from human patients varied in their *in vitro* sensitivity to penicillin. None of these naturally resistant strains had been previously exposed to penicillin. Three strains of staphylococcus were made highly resistant to penicillin by exposing the organisms to increasing concentrations of penicillin. At that time, it appeared that acquired *in vitro* resistance to penicillin, like acquired sulfonamide resistance, was probably a permanent characteristic of these strains of staphylococcus. These resistant strains were subcultured on veal infusion agar slants every 3 to 4 weeks, and it was surprising to observe that within a year, the property of penicillin insensitivity had been completely lost. This was in contrast to 2 strains which had become resistant in patients as a result of penicillin therapy and still retained their resistance to penicillin after having been handled and subcultured in the same manner. The observations prompted further investigations to determine if staphylococci made resistant to penicillin by exposure *in vitro* differed from strains which had become resistant in the human body following the administration of penicillin.

Previous investigators[3] had shown that an enzyme-like substance called penicillinase, and extracted from *E. coli,* inhibited the action of penicillin. However, such an inhibitor could not be obtained from at least 6 strains of staphylococcus which were sensitive to penicillin, nor from 2 strains of staphylococcus which had been made resistant to penicillin *in vitro*.[4,5,6] Kirby,[7] employing a meth-

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1 Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 207.

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

3 Abraham, E. P., and Chain, E., *Nature,* 1940, **146**, 837.

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

5 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 132.

6 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 135.

7 Kirby, W. M. M., *Science,* 1944, **99**, 452.

od described by Harper[8] for paracolon bacilli, extracted with acetone and ether 7 strains of coagulase-positive staphylococci which were naturally resistant to penicillin, and reported that the dried bacterial residue contained a potent inactivator for penicillin. He could not obtain inactivator from 7 coagulase-positive sensitive strains. Kirby concluded that the inactivator from staphylococci destroyed penicillin.

No reports have appeared concerning the presence or absence of penicillin inhibitor in staphylococci which had become resistant to penicillin in the human body following administration of the drug. The following results show that extracted staphylococci, which became resistant to penicillin in the body, possess an inhibitor for penicillin. This is in contrast to the absence of demonstrable inhibitor in parent-sensitive strains which were isolated before therapy was instituted. Furthermore, staphylococci which had developed a high degree of resistance to penicillin following exposure *in vitro* to increasing concentrations of penicillin did not produce demonstrable inhibitor.

Methods of Study. Source of strains. Sixteen strains of coagulase-positive staphylococcus, including 4 strains which became resistant to penicillin *in vivo*, and their 4 parent strains which were sensitive, were studied. Strains 22J and 23B were supplied to us through the courtesy of Dr. Donald Anderson of Boston. The parent strains of these cultures were isolated from patients with chronic osteomyelitis. The penicillin-resistant organisms were recovered from the same patients after penicillin had been administered. Strains 22J and 23B have retained their resistance on culture medium for over 2 years. Strain Long was cultured from the infected operative wound of a patient following nephrectomy. Cultures after penicillin therapy revealed the presence of insensitive staphylococci. The strain Eden was isolated from a patient with chronic osteomyelitis. This patient had been treated with sulfathiazole, and then penicillin, and subsequent cultures showed the presence of cocci which were resistant to both sulfathiazole and penicillin. Strain Rutgers was obtained from a skin lesion, while strains Ked and Nelson were isolated from patients with chronic osteomyelitis. These 3 strains along with Eden II were sensitive to penicillin.

Development of in vitro resistance. Strains Rutgers, Ked, Nelson, and Eden II were made resistant to sodium penicillin by exposing the organisms in Gladstone's medium[9] to increasing concentrations of the same lot of commercial penicillin. Similar results were obtained when veal infusion broth was used. Briefly, the technic was as follows: each of several tubes contained 9 cc of the medium to which was added 1 cc of freshly prepared sodium penicillin dissolved in isotonic saline solution. A standard loopful of a 24-hour culture of organisms in liquid medium was seeded to medium containing the added penicillin in a concentration which permitted demonstrable growth at 37°C within 24 hours. Loopful transfers of the organisms were made every 24 to 48 hours in a given concentration of penicillin until maximum growth could be attained within 24 hours. Then the concentration of penicillin was increased and the serial transfers repeated. In this manner, 100 to 200 transfers were made, and the resistance attained was related to the number of transfers.

In vitro test for penicillin sensitivity. From 100,000 to 300,000 organisms of a 24-hour culture of each strain were seeded into a series of test tubes containing Gladstone's medium. Freshly prepared aqueous solutions of sodium penicillin were added in varying concentrations to the resulting bacterial suspensions. The final volume in each tube was 10 cc. Incubation was carried out for 48 hours at 37°C. Inhibition of bacterial growth was ascertained by the absence of turbidity, and the end point determined by selecting that tube which remained clear with the lowest concentration of penicillin.

Detection of penicillin inhibitor. Each strain of staphylococcus was extracted with acetone and ether according to Harper's method.[8] Sterile suspensions of the dried extracted bacteria in physiologic saline solution were added in varying amounts to solutions

[8] Harper, G. J., *Lancet*, 1943, **2**, 569.

[9] Spink, W. W., and Vivino, J. J., *J. Clin. Invest.*, 1944, **23**, 267.

TABLE I.
Relation Between Resistance to Penicillin and Production of an Inhibitor.
A. Strains Developing Resistance to Penicillin in the Human Body.

Strain	Parent strains (Control)		Resistant variant-strains	
	Oxford units penicillin per cc inhibiting growth	Presence or absence of penicillin inhibitor	Oxford units penicillin per cc inhibiting growth	Presence or absence of penicillin inhibitor
22J	0.05	Absent	1.0	Present
23B	0.05	"	2.0	"
Long	0.05	"	1.0	"
Eden	0.1	"	0.8	"
B. Strains Developing Resistance to Penicillin *in vitro*.				
Rutgers	0.2	Absent	40.	Absent
Ked	0.2	"	200.	"
Nelson	0.2	"	10.	"
Eden II	0.2	"	20.	"

of commercial penicillin in culture medium containing a standardized inoculum of penicillin-sensitive staphylococci. After incubation at 37°C for 48 hours, the presence or absence of viable bacteria was determined by culturing the contents of each tube on blood agar plates.

Results. In Table I is presented a summary of findings concerning the presence or absence of penicillin inhibitor in the extracted organisms of the 16 strains of staphylococcus studied. The significant feature is that an inhibitor of penicillin was obtained only from those 4 strains which had developed resistance to penicillin in the human body. No penicillin inhibitor was demonstrated in the dried, extracted bacteria of the remaining 12 strains even in a concentration of 1 mg of the dried bacteria per cc of penicillin solution. The extracted bacteria of strains which yielded inhibitor produced varying degrees of penicillin inactivation. The penicillin-resistant strain 23B produced the most potent inhibitor. A concentration of 0.1 mg of dried bacteria inhibited the action of 400 units of sodium penicillin per cc.

Summary. Strains of staphylococci which have developed resistance to penicillin in the human body yield an inhibitor for penicillin. Penicillin inhibitor was not present in 4 strains which had been made highly resistant by exposure *in vitro*. Resistance developed *in vivo* was found, among the strains tested, to be a more permanent characteristic of staphylococci than that acquired *in vitro*.

15028 P

Effect of Pituitary Implants and of Estradiol on Newly Metamorphosed Frogs, *Rana pipiens*.

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Primary and secondary sex organs respond markedly to treatment with hormones. It has been shown that the treatment of some immature salamanders with pituitary extracts results in marked testicular hypertrophy and increased spermatogenesis.[1] Also, other experiments have demonstrated that some estro-

[1] Burns, R. K., and Buyse, A., *J. Exp. Zool.*, 1934, 67.