(one from Group I and the other from Group IV) showed symptoms of encephalitis on the seventh day. These animals were sacrificed and suspensions of each brain were injected intracerebrally into normal mice. In both cases virus was present in high titer and was identified as western equine encephalomyelitis virus by serum neutralization tests.

As shown in Table I, 20% of the control animals (Group I) survived the observation period of 10 days while 52% of the serum-treated animals (Group II) survived. Sixty-one and five-tenths percent of ether-treated animals survived, as compared with 90.7% of animals receiving combined ether anesthesia and hyperimmune serum (Group IV). Statistical analysis indicated that the difference in effectiveness of hyperimmune serum therapy alone as compared with that of ether anesthesia alone was not significant. The effect of combined therapy, however, is markedly greater than the effect of either method alone. Whether the serum therapy and anesthesia act independently, or whether there is a synergistic effect between the two cannot be stated from the data obtained. The possibility that anesthesia may facilitate union of virus and antibody is under investigation.

Summary. Either intraperitoneal injection of hyperimmune rabbit serum or deep ether anesthesia administered beginning 18 hours following intracerebral inoculation of virus effects a significant change in survival rate among Swiss mice infected with western equine encephalomyelitis. A combination of the two methods is more effective than either method alone.

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Production of Extracellular Penicillin-Inactivating Substances Associated with Penicillin Resistance in *Staphylococcus aureus.*

JOSEPH S. GOTS. (Introduced by A. B. Gutman.)

When Abraham and Chain[1] discovered the penicillin-destroying enzyme, penicillinase, the question was raised whether a bacterium's susceptibility to penicillin varied inversely with its ability to produce the enzyme. The present data do not favor complete agreement.[1-5] Too many inconsistencies exist to permit the acceptance of enzyme formation as the sole factor in explaining variation in penicillin susceptibility. In some bacteria, however, penicillin resistance may be definitely ascribed to their ability to produce penicillinase. When Kirby[6] and others[7,8] found that an active intracellular penicillin inactivator was present in resistant staphylococci and not in sensitive strains, it became apparent that perhaps in cases of varied susceptibility among various strains of the same species, the variation might be due to difference in enzyme formation. This investigation was formulated with the hope that the use of technics which are dependent on the extracellular nature of the inactivating enzyme, might throw further light on the latter concept.

Methods and Materials. The staphylococci studied were isolated primarily from cases of

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

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

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

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

5 Woodruff, H. B., and Foster, J. W., *J. Bact.,* 1945, **49**, 7.

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

7 Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

8 Manwaring, W. H., *Calif. and West. Med.,* 1944, **61**, 184.

eye infections and pyodermas. Detailed account of the latter group has been reported.[9] All of the organisms were hemolytic *Staphylococcus aureus*, positive for coagulase production and mannite fermentation. The majority of the resistant strains are known to be naturally resistant. However, in a few in which primary isolation was accomplished after penicillin therapy was started, the possibility that these had acquired resistance because of treatment, cannot be ignored. Penicillin sensitivity was determined by a tube dilution turbidimetric method.[9]

The first method employed for determining the existence of the penicillin inactivator, presumably penicillinase,* has been previously described.[10] In brief, it consists of the ability of the enzyme to diffuse from actively growing organisms into a penicillin agar medium which had been previously seeded with a very sensitive *Staphylococcus aureus*. The organisms under study are streaked on the surface of the plate, the enzyme elaborated during growth diffuses into the medium, inactivates the regional penicillin and the sensitive indicator organism is permitted to grow out as a satellitic zone of colonies around the line of streak. By measuring the width of this satellitic zone and by using a series of plates containing varying concentrations of penicillin, a fairly accurate quantitative estimation is possible. That this satellitic manifestation is actually ascribed to the inactivation of the penicillin has been shown by assaying agar plugs cut from the plate. The plugs taken from the area of satellitic growth contain no penicillin, whereas those taken from the other sections of the plate maintain original concentration.

The alternate method of penicillinase detec-

TABLE I.

Inactivation of Penicillin by *Staphylococcus aureus* on Penicillin Agar Seeded with Sensitive *Staphylococcus aureus*. Incubation: 24 hours at 37°C

Conc. of penicillin required for inhibition of organism Units per ml	Conc. of penicillin in test medium Units per ml	Total strains tested No.	Production of penicillin inactivator* (No. of strains)				
			—	+	++	+++	++++
More than 5	5.0	53	13	40			
	1.0			46	7		
	0.5			24	28	1	
	0.1				15	38	
	0.05					20	33
5.0-1.0	1.0	4	4				
	0.5		4				
	0.1		3	1			
	0.05		2	1	1		
1.0-0.5	0.5	4	4				
	0.1		4				
	0.05		4				
0.5-0.1	0.1	2	2				
	0.05		2				
0.1-0.05	0.05	7	7				

* Penicillin inactivation recorded as follows:

— = No growth of indicator organism, therefore no penicillin inactivation.

\+ = No zone of inactivation but indicator organism grew out under line of streak: slight inactivation of penicillin.

++ = Zone of inactivation 0-2 mm (measured from edge of streak).

+++ = Zone of inactivation 2-5 mm.

++++ = Zone of inactivation more than 5 mm.

[9] Waisman, M., and Gots, J. S., submitted for publication.

* No evidence is at present available that this substance is the same as the penicillinase described by Abraham and Chain.[1]

[10] Gots, J. S., *Science*, to be published.

TABLE II.
Inactivation of Penicillin by *Staphylococcus aureus* on Penicillin Agar (0.01 units per ml) Seeded with a Hemolytic *Streptococcus* (Group A). Incubation: 24 hrs at 37°C.

Conc. of penicillin required for inhibition of organism Units per ml	Total strains tested No.	Production of penicillin inactivator* (No. of strains)				
		—	+	++	+++	++++
> 5	10					7
5.0-1.0	4		1	2	1	
1.0-0.1	2		1	1		
0.1-0.05	11	11				
< 0.05	29	29				

* See Table I for explanation.

tion depends upon the ability of the enzyme to diffuse into a fluid medium. Supernatants obtained by centrifuging 24-hour broth cultures of the organisms were added in equal amounts to a penicillin dilution. The mixture was incubated for 2 hours at 37°C and residual penicillin was determined by the agar cup method using *Staphylococcus aureus*, Strain H, as the index of inhibition. By making serial dilutions of the supernatants, a quantitative estimation was possible.

Results. The results obtained by the first method are depicted in Table I. All of the highly resistant organisms, those which required more than 5 units of penicillin per ml for complete inhibition, showed a marked ability to produce a potent penicillin-inactivating substance. The amount of inactivator produced did not vary greatly. Slight production occurred in those few strains which were inhibited by penicillin concentrations of 5 to 1 units per ml, but in those which required less than 1 unit per ml for complete inhibition no production was found. Correlation with the average sensitive strains could not be accomplished because the lowest penicillin concentration possible to use in the test medium was sufficient to inhibit their growth. This difficulty was overcome by substituting for the sensitive *Staphylococcus* indicator organism a Group A hemolytic *Streptococcus*. All of the sensitive organisms could now be included in the analysis. The results with this modification are depicted in Table II. No difference could be found in the ability to produce the inactivator by the resistant organisms. Two organisms with a sensitivity range of 1 to 0.1 units per ml were added to the list of penicillinase producers. No evidence of production could be found in organisms whose sensitivity was less than 0.1 units per ml.

The results obtained with the broth culture supernatants (Table III) is in agreement with

TABLE III.
Inhibition of Penicillin by Supernatants of 24-hour Broth Cultures of *Staphylococcus aureus*. A typical experiment.

Conc.of penicillin required for inhibition of organism Units per ml	Supernatant* dilution	Zone of inhibition produced† mm
> 5.0	1/2	0
	1/4	0
	1/8	0
5.0-1.0	1/2	0
	1/4	V
	1/8	V
< 1.0	1/2	27
Broth control	—	27

* Final concentration of culture supernatant contained in supernatant-penicillin mixture

† Inhibition of *Staphylococcus aureus*, Strain H, by agar cup method.

V = variable results with different strains—all were less than control.

Preliminary incubation of mixture: 2 hr at 37°C.

the plate method. The resistant strains produced an inactivator whereas the sensitive strains did not. It is of interest that Bondi and Dietz[3] by a similar method were unable to demonstrate inactivation of penicillin by 5 *Staphylococcus aureus* strains studied. Inasmuch as no mention was made of the degree of susceptibility of their strains, their failure may well have been due to the use of sensitive strains.

Discussion. In seeking an answer for the mechanism of extreme variation of penicillin

susceptibility which occurs among various strains of the same species, such as *Staphylococcus aureus,* the first logical approach is to tag the resistant members with a quality lacking in the sensitive strains. The results presented here show a marked correlation of penicillin resistance with the ability to produce a penicillin-inactivating substance, presumably penicillinase. This substance is soluble and diffuses readily into surrounding liquid and solid environment. Kirby's[6] failure to demonstrate the inactivator in Seitz filtrates of culture fluid may be due to the observation[5] that part or all of the enzyme's activity may be lost by Seitz filtration, presumably by adsorption.

In view of the available data, it cannot be denied that an organism's ability to synthesize penicillinase is not the sole factor in explaining its sensitivity or insensitivity to the action of penicillin. Sensitive organisms capable of producing penicillinase and insensitive organisms incapable of producing it have been described. Our own findings with the Shigella-Typhoid-Salmonella group of organisms confirm the findings of Bondi and Dietz.[3] This group is relatively insensitive to the action of penicillin, yet the Shigella organisms produce an inactivator and the Typhoid and Salmonella do not. Suffice it to say that the complete explanation for resistance must be awaited.

In certain bacteria at least, the resistance to penicillin can be definitely ascribed to their ability to produce penicillinase. Rather convincing are Woodruff and Foster's studies[5] on a bacillus capable of producing extracellular penicillinase at pH of 6.0 or above. At this pH the organism was uninhibited in growth by several hundred units of penicillin per ml of culture medium. When they suppressed the production of penicillinase by lowering the pH to 5.5, the organism became easily inhibited by 10 units per ml.

Those who have produced penicillin-resistant strains *in vitro* by culturing in increasing concentrations of penicillin have found that neither the resistant nor the sensitive progenitor was able to produce penicillinase. Inasmuch as we have yet to encounter a resistant strain, isolated directly from an infected process, which is incapable of producing an inactivator, it appears that there may exist a difference in the mechanism between *in vitro* acquired resistance and natural and *in vivo* acquired resistance.

Summary. A potent penicillin-inactivating substance is produced by penicillin-resistant *Staphylococcus aureus* but not by penicillin-sensitive strains. The substance is soluble and diffuses readily into both liquid and solid culture medium. The variation of susceptibility to penicillin among various strains of *Staphylococcus aureus* appears to be associated with a difference in this enzyme formation.

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Adaptability of Gonococcus to Four Bacteriostatic Agents, Sodium Sulfathiazole, Rivanol Lactate, Promin, and Penicillin.*†

CHARLES M. CARPENTER, JEANNE M. BAHN, HELEN ACKERMAN AND HERBERT E. STOKINGER.

From the Department of Bacteriology, the University of Rochester, School of Medicine and Dentistry, Rochester, N.Y.

Chemotherapy of gonococcal infection has advanced markedly during the last decade. Sulfonamides appear to be less effective now, however, than when first introduced for the treatment of the disease. Two factors may be responsible for this evident decreased effi-

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