Bursa of Fabricius is known to play a principal role in regulating formation of humoral antibodies, and the thymus is known to play a principal role in cellular (lymphocyte) immunity in birds(1,22). Our results further support the concept that cellular immunity plays an important part in the resistance of the host against tumor formation and that humoral antibodies are not importantly involved in this resistance.

Summary. Chickens were thymectomized during the first 72 hours of life. Controls were intact or sham-operated chickens of the same age. All chickens were simultaneously inoculated with Rous sarcoma virus at the age of 10-12 days. Thymectomy had a stimulating effect on induction of the Rous sarcomas and tumor growth rate.

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Effect of Antimicrobial Drug Combinations on Enzymic Lysis of *Staphylococcus aureus.* (32356)

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Although there have been numerous investigations of the antibacterial activities of antibiotic combinations and certain pairs of drugs have often been reported as synergistic or antagonistic, many of the reports are conflicting(1-6). Frequently the results of published studies reveal that the antagonistic or synergistic effect depends to a great extent upon the specific species or strain of bacteria employed and the relative proportion of the agents to which it has been exposed(1,5).

Previous studies from this laboratory(7-9) have demonstrated that the semisynthetic penicillin nafcillin has a profound effect on the integrity of the *Staphylococcus aureus* cell wall; in sublethal concentration, nafcillin renders the cell highly susceptible to lysis by lysozyme and trypsin.

An attempt has now been made to use this lytic system as a model for determining the activities of antibiotic combinations. In the present work, several other antibiotics were compared with nafcillin for the effect on the enzymic lysis of *S. aureus* CHP, and in addition their effect when combined with nafcillin was determined. The drugs presently investigated in this manner were tetracycline, chloramphenicol, novobiocin, erythromycin, actinomycin D, ampicillin, cephalothin and cloxacillin. Our observations that

the bacteriostatic drugs chloramphenicol and the tetracyclines inhibit nafcillin-induced lysis of *S. aureus* is consistent with the generally recognized antagonism between these two types of antibiotics.

Materials and methods. Bacterial strain. The experiments were performed with the CHP strain of *S. aureus,* a penicillinase producer highly resistant to penicillin G but susceptible to the semisynthetic penicillins nafcillin, oxacillin, cloxacillin and methicillin(10-12).

Medium and cultural conditions. The materials and methods were, for the most part, as described previously(9). The essential information can be summarized as follows: The organism was grown in Blake bottles containing 200 ml of brain heart infusion agar (Baltimore Biological Laboratories) adjusted to pH 7.6-7.8. The bottles were sterilized, seeded with 1.0 ml of a saline suspension of an 18-hour nutrient agar culture standardized to contain 1.4×10^9 bacteria per ml, and incubated for 4 hours at 37°C. Cells were harvested from the medium with distilled water and centrifuged; the deposits were washed twice with distilled water and suspended in 0.1 M phosphate buffer, pH 7.0.

Antibiotics. Nafcillin, chloramphenicol, tetracycline hydrochloride, actinomycin D, novobiocin, erythromycin hydrochloride, cephalothin, cloxacillin, and ampicillin were used in amounts that were inadequate to completely inhibit the growth of the test organism. Stock solutions (1 mg/ml) of the antibiotics were individually dissolved, diluted in sterile buffered 0.85% salt solution, and aseptically added to previously liquefied brain heart infusion agar. When not in use, the solutions of drugs were stored at −20°C.

To determine the influence of the antibiotic combinations on enzymic lysis, these mixtures were incubated for 30 minutes at 37°C before their inclusion in the liquefied media.

Enzyme preparations. The enzymes used were crystalline egg white lysozyme (Armour) and crystalline trypsin (Worthington Laboratories) dissolved in 0.1 M phosphate buffer, pH 7.0.

Determinations of enzymic lysis. The extent of lysis was determined by measuring the turbidities of the preparations on a Klett-Summerson photoelectric colorimeter fitted with a red filter (No. 66). Suspensions of bacteria were adjusted to a final Klett reading of 400. To each tube 1 ml of lysozyme (20 μg/ml) and/or trypsin (10 μg/ml) in 0.1 M phosphate buffer, pH 7.0, was added, and the contents were thoroughly mixed. The tubes were incubated in a water bath at 37°C, and changes in optical density were determined for several time intervals.

Antibiotic sensitivity test. Minimal inhibitory concentrations (MIC), *i.e.,* the highest dilution of the antibiotic which will completely inhibit the growth of the test organism, were determined by the conventional agar diffusion procedure in brain heart infusion agar in Blake bottles. The inoculum size and other experimental conditions were the same as those used for evaluating the influence on lytic response.

Results. Effect of antibiotics on growth of S. aureus. Table I gives the MIC of the antibiotics when tested either alone or in combination with 0.1 μg/ml of nafcillin against *S. aureus* CHP. Addition of a fixed, ineffective concentration of nafcillin (0.1 μg/ml) to the cultures doubled the MIC's of erythromycin, cephalothin and tetracycline but caused no change, either positive or negative, in the activity of the other antibiotics.

Effect of antibiotics on enzymic lysis. The action of lysozyme and trypsin on *S. aureus* grown in the presence of sublethal concen-

TABLE I. Amounts of Antibiotics Required for Inhibition of *S. aureus* CHP in the Presence of Nafcillin.

Antibiotic	MIC (μg/ml)* No nafcillin	Nafcillin (0.1 μg/ml)
Nafcillin	1.6	—
Novobiocin	2.5	2.5
Erythromycin	.2	.4
Cloxacillin	1.6	1.6
Actinomycin D	.8	.8
Ampicillin	400.0	400.0
Cephalothin	2.0	4.0
Chloramphenicol	8.0	8.0
Tetracycline	.25	.5

* Minimal inhibitory concentrations were determined as described in text. A large inoculum (1.4×10^9 cells) was used and readings were taken after a 4-hr incubation period.

TABLE II. Influence of Various Antibiotics Alone and in Combination with Nafcillin on Lysis of *S. aureus* CHP by Lysozyme and Trypsin.

Antibiotic	Conc (μg/ml)	Nafcillin (μg/ml)	Klett readings*
Control	—	—	370
Nafcillin	—	.1	50
Chloramphenicol	2.0	—	365
	.5	.1	76
	1.0	.1	182
	2.0	.1	280
Tetracycline	.1	—	360
	.03	.1	80
	.06	.1	120
	.12	.1	230
Erythromycin	.1	—	350
	.025	.1	80
	.05	.1	100
	.10	.1	180
Actinomycin D	.2	—	330
	.2	.1	60
	.3	.1	100
	.4	.1	205
Novobiocin	.15	—	300
	.15	.1	175
Ampicillin	200	—	210
	200	.1	35
Cephalothin	1.0	—	175
	1.0	.1	30
Cloxacillin	.2	—	185
	.2	.1	40

* Each system incubated for 2 hr at 37°C in the presence of lysozyme (20 μg/ml) and trypsin (10 μg/ml).

trations of a number of antibiotics is shown in Table II. The enzyme mixture brought about no significant change in control cells but a rapid decrease in turbidity of nafcillin-treated cells. Cells grown in the presence of sublethal concentrations of chloramphenicol, tetracycline, erythromycin or actinomycin D were not rendered susceptible to significant enzymic lysis. Novobiocin, cephalothin and ampicillin did activate enzymic lysis, but not to the extent of nafcillin.

Effect of antibiotics in combination with nafcillin. The enzymic lysis of *S. aureus* when grown in the presence of a fixed level of nafcillin (0.1 μg/ml) and a sublethal concentration of either chloramphenicol, tetracycline, erythromycin or actinomycin D is also shown in Table II. In various degrees these antibiotics antagonized the nafcillin effect. The effect on the lytic response was unrelated to the growth-inhibiting activity: thus chloramphenicol, which had the greatest inhibitory effect on lysis, exerted this effect at only one-fourth its MIC, while tetracycline, actinomycin D and erythromycin attained significant activity only at concentrations just short of their respective MIC's. Those antibiotics, therefore, which act primarily through inhibition of protein synthesis antagonized the nafcillin effect, and each did so at its own characteristic concentration. It is apparent that the lytic response is a sensitive indicator of this antagonistic effect.

To determine what effect antibiotics known to inhibit cell wall synthesis would have on lysis, we combined cloxacillin, cephalothin, novobiocin and ampicillin with nafcillin. In this experiment, *S. aureus* was seeded in a culture medium containing a sublethal concentration of nafcillin (0.1 μg/ml) and a concentration of the second antibiotic that induced a lytic response. Such a design does not allow precise quantitative comparisons. However, cloxacillin, ampicillin and cephalothin produced little apparent change in the nafcillin-promoted lytic response (Table II) and novobiocin caused a significant reduction. Novobiocin, therefore, unlike other inhibitors of cell wall synthesis, antagonizes the action of nafcillin on this strain of *S. aureus*.

Effect of chloramphenicol in combination with ampicillin. S. aureus was also lysed after having been grown in the presence of sublethal concentrations of ampicillin, and it was of interest to determine the influence of chloramphenicol on this response. Experiments of the same sort as described for nafcillin were performed. Although much higher concentrations of ampicillin were required to activate the lytic effect with lysozyme and trypsin, the results were otherwise similar to those obtained with nafcillin. The reversal of the ampicillin effect by chloramphenicol, while significant, was smaller than that observed for nafcillin.

Discussion. The *in vitro* antagonisms encountered in the present study demonstrated that despite their common ability to inhibit protein synthesis, antibiotics, possibly because of different sites of action, have a variable capacity for blocking the lytic response mediated by nafcillin. On the basis of the MIC's, chloramphenicol was the most active nafcillin

antagonist, followed in order by tetracycline, actinomycin D and erythromycin. The antagonism was not specific for nafcillin, as shown by the experiments with ampicillin, in which chloramphenicol also inhibited the ampicillin-promoted enzymic lysis, but to a lesser degree.

It has been reported that novobiocin acts primarily on cell wall synthesis(13) or on the cell membrane of *S. aureus*(14); hence the present demonstration of a significant interference of this antibiotic in the nafcillin-promoted enzymic lysis of *S. aureus* was unexpected and suggests a different primary site of action, one perhaps more closely associated with antibiotics that interfere with protein synthesis than those that inhibit cell wall synthesis. Another possibility is that several mechanisms are concurrently involved. The results are in substantial agreement with the more recent studies of Shockman and Lampen(15) and of Hancock and Fitz-James (16), who demonstrated that novobiocin acts in a manner that is different from penicillin even though both antibiotics cause an accumulation of mucopeptide precursors within the cell.

Summary. Sublethal concentrations of nafcillin, ampicillin, cloxacillin and cephalothin react with growing cells of *S. aureus* CHP and bring about a characteristic lytic response when these cells are subsequently incubated with lysozyme and trypsin. The same lytic effect is apparent to a much lesser degree with novobiocin but not with inhibitors of protein synthesis, *e.g.*, chloramphenicol, tetracycline, erythromycin or actinomycin D, which in fact antagonize this effect. In combination with nafcillin, novobiocin was shown to antagonize the nafcillin-promoted enzymic lysis, while ampicillin, cloxacillin and cephalothin exerted neither antagonism or significant synergism.

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