TABLE I. Effect of Cortisone on Inhibitory Action of Antigonadotropic Sera.

Series	Total dose of pituitary antigen* (mg)	Daily dose of antigonadotropic sera (cc)	Daily dose of cortisone acetate† (mg)	Ovarian wt (mg)
A	0	0	0	14 ± 3
	10	0	0	38 ± 6
	10	1	0	12 ± 2
	10	1	.5	13 ± 3
B	0	0	0	17 ± 3
	10	0	0	54 ± 11
	10	1‡	0	18 ± 3
	10	1‡	1	26 ± 7
	10	1§	0	21 ± 2
	10	1§	1	30 ± 5
C	0	0	0	15 ± 3
	15	0	0	28 ± 4
	15	1‡	0	14 ± 4
	15	1‡	.75	13 ± 3
	15	.5‡	0	12 ± 2
	15	.5‡	.75	13 ± 2

* The antigen preparation used in Series C was less potent than that used in Series A or B.
† Cortisone given for 11 days beginning 8 days before antigen in Series A and for 5 days beginning 2 days before antigen in Series B and C. All animals were 23-29 days of age at autopsy. ‡ Sheep serum #5. § Sheep serum #13.
Series A includes 15 animals per group and Series B and C include 6 animals per group.

prepared by the prolonged administration of hog pituitary gonadotropin.

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Antibiotic Synergism Requires Simultaneous Presence of Both Members of a Synergistic Drug Pair.* (19201)

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Much information is available on the effect of synergistic pairs of antibiotics *in vitro* and *in vivo*, but the mechanism of synergistic action is not known. An understanding of this mechanism might permit more rational and efficient use of drug combinations. A number of theories, not necessarily mutually exclusive, may be proposed to explain synergism, among them the following: (a) The two drugs of a synergistic pair combine chemically to form a compound more effective than either drug alone; (b) one drug kills those organisms that are resistant to the other agent; (c) one drug acts principally on multiplying bacteria, the other on resting organisms; (d) one drug may potentiate the action of another, *e.g.*, by increasing the permeability of the cell wall; (e) potentiation may occur through the simultaneous blocking of alternate enzymatic pathways, *i.e.*, if a given anabolic process essential for growth may proceed by two different

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routes, there may be little or no inhibition of growth by the blocking of one pathway, but complete inhibition by the blocking of both. It seems likely that more than one of these possible mechanisms participate in synergistic effects. Certain ones appear less probable than others. Thus recent work indicates that the members of a synergistic pair of drugs do not interact to form a single compound(1). Synergism has been observed at times when one member of the pair alone had no apparent antimicrobial effect, whereas the combination killed all exposed bacteria(2). In such situations it is unlikely that theories (b) and (c) above play a significant role.

The theories involving some form of potentiation are in agreement with the finding that synergism can be demonstrated in certain pairs of drugs provided at least one of them manifests biological activity(2). If it is acknowledged that synergism may result not from an interaction of two drugs, but rather from the action of a "potentiating" agent on a microorganism which is likewise subjected to another drug, it becomes of interest to determine whether the "potentiating" agent and the active drug must be present simultaneously. It may be that blockage of alternate metabolic pathways would involve simultaneous presence of both members of a synergistic pair of drugs, whereas if a potentiating drug acted through an alteration of cell membrane permeability its effect might persist for some time after its removal. The experiments reported here attempt to differentiate between these possibilities by permitting one drug of a synergistic pair to act for some time, then removing it and substituting the other member of the pair and observing the effect on the viable bacterial population.

Materials and methods. Bacteria. Enterococcus (*Streptococcus faecalis*) strain 16 was tested with penicillin and streptomycin, and *Staphylococcus albus* strain H (Heatley) was tested with terramycin and bacitracin since these combinations were known to be synergistic against these organisms(2,3). Bacteria were grown in proteose peptone No. 3 (Difco) broth and serial dilutions were plated on proteose No. 3 agar. All cultures were incubated at 37°C. *Antibiotics.* Crystalline potassium penicillin G and streptomycin sulfate were obtained from commercial sources. Terramycin hydrochloride (Lot WBW507019) was supplied by Dr. H. H. Anderson, and bacitracin (Lot B-480420) by Dr. L. S. Smith. Stock solutions of all drugs were prepared in 0.85% sodium chloride solution and stored at 4°C. Further dilutions were prepared in distilled water as required. *Technic of test.* Broth containing antibiotic was distributed in 15 ml amounts in large, metal-capped test tubes containing a small amount of sterile sand to aid in resuspending the bacteria after centrifuging. Each tube was inoculated with 0.75 ml of an 18-hour broth culture of the organism under test, to give an initial concentration of 1 to 5 x 10^7 viable organisms per ml. All tubes, including suitable controls, were incubated at 37°C for 4 hours. The number of viable organisms was then determined by plate count and the tubes centrifuged for 20 minutes at 3000 rpm. The supernatant was removed and the sediment washed once in broth warmed to 37°C. The sediment was then resuspended in the original volume of warm broth containing antibiotic and incubation at 37°C was continued. These manipulations took approximately 50 minutes. Subsequently the number of viable organisms was estimated by plate counts at intervals indicated in the figures. In several experiments uncentrifuged control tubes were included. Plate counts of these controls did not differ significantly from the centrifuged tubes and indicated that centrifugation, resuspension, and exchange of menstruum did not result in a measurable loss of bacteria. A typical experiment included the following tubes:

Tube No.	Drug present in 1st 4 hr, μg	Drug present after centrifugation and washing, μg
1	0	0
2	Streptomycin, 100	Streptomycin, 100
3	" 100	0
4	" 100	Penicillin, 3
5	0	Streptomycin, 100
6	Penicillin, 3	Penicillin, 3
7	" 3	0
8	" 3	Streptomycin, 100
9	0	Penicillin, 3
10	Penicillin, 3, + streptomycin, 100	Penicillin, 3, + streptomycin, 100

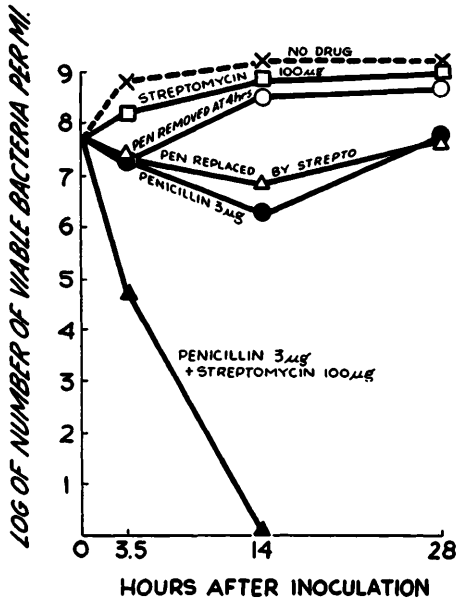

FIG. 1. Synergistic action of penicillin and streptomycin on enterococci. Rapid bactericidal effect when penicillin 3 μg/ml and streptomycin 100 μg/ml act simultaneously, not when penicillin is present alone 4 hr, then removed, and replaced by streptomycin. Likewise there was no synergism when this sequence was reversed (not shown).

Results. A representative experiment illustrating the action of penicillin and streptomycin on enterococci is shown in Fig. 1. In order to avoid crowding of the figure, the curves of 3, 4, and 5 of the above outline have been omitted. The results with these tubes are entirely parallel to those discussed below. When both drugs were present simultaneously throughout incubation the number of viable bacteria fell rapidly to zero. Synergism of the drug pair was evidenced by the great increase in bactericidal rate over that of either penicillin or streptomycin alone. The concentration of streptomycin employed (100 μg ml) had little apparent effect when acting alone. Penicillin (3 μg ml) caused only a moderate and temporary decrease in the viable bacterial count. Synergism was not evident when either penicillin or streptomycin acted on the bacteria for 4 hours and then was removed and replaced by the other member of the pair. Unless the two drugs were present simultaneously in significant concentrations synergistic action failed to occur.

Fig. 2 illustrates typical results obtained with terramycin and bacitracin acting on staphylococci. Alone, bacitracin (1.5 units/ml) had a barely noticeable effect, whereas terramycin (1.0 μg/ml) exhibited transient bacteriostasis only. When both drugs acted simultaneously the rate of bactericidal action was sharply increased and the entire population was killed. No such striking synergism could be observed when the two drugs were permitted to act in sequence.

From these results it was concluded that synergism as defined by a rapid rate of bactericidal action and complete killing of the exposed bacterial population could occur only when both members of a synergistic pair of drugs were present simultaneously, not when one preceded the other, and was subsquently removed from contact with the bacteria. In the latter case, there was sometimes no evidence of additive action whatever. In some instances, however, *e.g.*, when terramycin was replaced by bacitracin (Fig. 2), or when penicillin was followed by streptomycin, multiplication of bacteria was inhibited for a longer

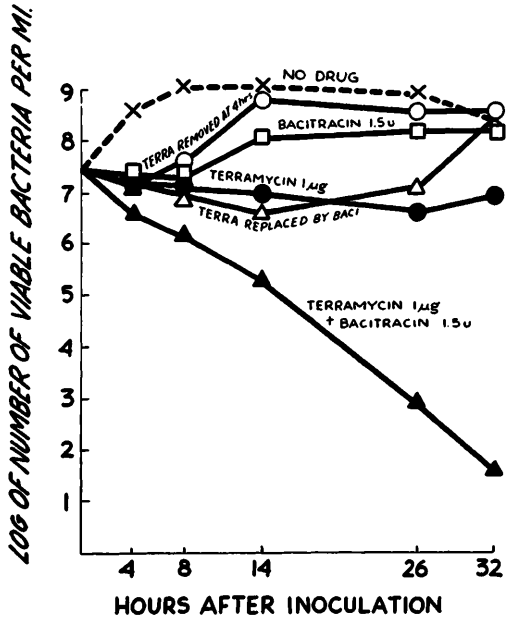

FIG. 2. Synergistic action of terramycin and bacitracin on staphylococci. Marked bactericidal effect when terramycin 1 μg/ml and bacitracin 1.5 units/ml act simultaneously, not when terramycin is present alone for 4 hr, then removed and replaced by bacitracin. Likewise there was no synergism when this sequence was reversed (not shown).

period of time than if the first drug was simply removed and the bacteria permitted to multiply in drug-free broth. That this effect might be attributed to summation (though not synergism) of drug action (perhaps attributable to incomplete removal of the first drug) was suggested by the following experiment. At the time of centrifugation an untreated broth culture was diluted so as to approximate the bacterial concentration of the tube in which the first drug had acted for 4 hours. When a drug was added to this smaller number of untreated bacteria, the rate of multiplication was somewhat more rapid than that of organisms exposed to a sequence of drugs.

This experiment indicates that the additive, though never synergistic effect of two drugs in sequence, can not be attributed to the smaller number of microorganisms acted upon by the second drug. It has not been ruled out that this minor effect could be the result of small, subinhibitory concentrations of the first drug remaining adsorbed on bacteria after washing.

It has been reported that a sudden increase in bactericidal rate followed the addition of streptomycin to an enterococcal population previously exposed to penicillin action for 24 to 72 hours(3). It was similarly observed that in another synergistic drug pair acting on staphylococci, the addition of bacitracin at intervals of 2 to 6 hours to a population exposed to terramycin (or vice versa) resulted in a prompt increase in the rate of bactericidal action. Here, too, even though both drugs were not added initially, simultaneous exposure of microorganisms to both members of a synergistic drug pair resulted in prompt synergism.

Summary and conclusions. (1) It has been demonstrated in two systems that antibiotic synergism is observed only when both members of a synergistic pair of drugs are acting simultaneously on the bacterial population, not when they act in sequence. It may, therefore, be suggested that antibiotic synergism is not usually the result of a modification in a bacterial population induced by one drug, which renders the microorganisms more susceptible to the other drug of a synergistic pair. (2) These findings would tend to favor a hypothesis of synergism involving simultaneous blocking of alternate enzymatic pathways.

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A Quantitative Method for Study of Phenolsulfonphthalein (PSP) Concentration by Surviving Renal Slices.* (19202)

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The prevailing method(1) for studying the transport of phenolsulfonphthalein (PSP) by renal tubular cells *in vitro* is a visual one. The concentrations of PSP, substrates or inhibitors are varied and the ratio of concentrations of any two of these variables required to produce an all or none effect (visible concentration or no concentration) is noted by microscopic examination. The technic employed by Cross and Taggart(2) to investigate the tubular transport of p-aminohippurate (PAH) by surviving renal cortical slices of the rabbit suggested that a similar procedure might be used in *in vitro* studies on the renal tubular transport of PSP, providing a satisfactory method were available for the quantitative estimation of this substance in

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