bacterium isolated from tobacco plant roots was identified as a member of the genus Arthrobacter. The organism had the ability to convert nicotine to 6-hydroxynicotine. Maximum concentration of 6-hydroxynicotine detectable in the culture medium was approximately 50% of initial nicotine concentration on a molar basis. The identity of the product was established by its melting point, and the melting point of its picrate, its specific rotation, elementary analysis, and ultraviolet and infrared absorption spectra.

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Synergism Between Staphylococci and Proteus in Mixed Infection.* (26881)

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Synergism among bacteria in mixed infection is not a new concept although carefully studied cases of specific synergism *in vivo* appear to be relatively sparse in the literature. Even with the current emphasis on staphylococcal infection few studies on synergism between staphylococci and other organisms have been reported(1-3). It was recently noted in this laboratory that mice receiving mixed infections of *Staphylococcus aureus* and *Proteus vulgaris* died rapidly with fulminant septicemia although neither of these organisms alone appeared to have any deleterious effect on the animals when injected in comparable numbers and by the same routes.

This report presents the preliminary results of an investigation of this phenomenon.

Materials and methods. All mice† used in these experiments were young adult Webster-Swiss females weighing from 17 to 20 g. Prior to use in specific experiments the animals were held in large cages in an isolated stock-room. No evidence of spontaneous infectious disease in the stock animals has been observed even when they have been held up to 3 months in these quarters. (These older mice were not used for the reported experiments). The standard diet was Purina rat chow pellets and water supplied *ad lib.* For infection experiments the mice were transferred to steel box-type cages. Inoculation of these animals was carried out in a room separate from the stock-room where the test mice were then held for observation until experiments were terminated.

The staphylococci‡ used in these observations were isolated from human infections. Both the staphylococci and gram-negative bacteria used were routinely maintained at room temperature on Trypticase-Soy agar slants. Liquid cultures were grown in Trypticase-Soy broth at 37° C under fully aerobic

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conditions. In all cases the inocula were dilutions of 18 hour cultures and were of remarkably uniform titer regardless of the strain of staphylococci employed. As determined by the usual method of dilution and plate-counting the range of 18 hour titers for the staphylococci was 1.6-2.0 $\times$ 10^8, and for the proteus was 1.6-1.8 $\times$ 10^9. Any alterations from these standard procedures will be noted.

Except where indicated, inoculation of the mice was accomplished without anesthesia by intraperitoneal (I.P.) injection using tuberculin syringes and 5/8 inch #22 disposable needles. Intravenous (I.V.) injections were made into the dorsal or lateral tail veins using 5/8 inch #27 needles.

Results. 1. *Original Observations on Synergism.* The initial observations on this phenomenon indicated that there was an unquestionable increase in deaths of those mice inoculated with both staphylococci and proteus over those inoculated with either alone, even when the doses were adjusted so that the total number of organisms injected was the same in mixed infection as in the pure-culture inoculations. It has been stated above that neither staphylococci nor proteus had deleterious effects on the mice, but this is true only when some qualification is placed on the size of the inoculum, since deaths were regularly observed if a sufficient dose of an undiluted proteus culture was injected. The course of disease produced by such injections, however, was entirely different from that observed following the mixed inoculations. The former was much like that seen with a large inoculum of any broth-grown enteric organism and was characterized by marked diarrhea, dehydration, and prostration of the animals beginning within several hours of the injection. Furthermore this same phenomenon could be produced (although to a lesser extent) by injection of sterile culture filtrate. However, the same number of proteus when washed with sterile saline and resuspended at an equivalent titer was not uniformly lethal. From these observations it was concluded that death of the mice following injection of undiluted proteus was due to accumulated toxic products in the broth rather than to inherent pathogenicity of the organisms themselves. In contradistinction, the disease produced by mixed inoculation was slower, did not involve severe diarrhea and terminated with marked septicemia, and death. When autopsied these animals showed invasion of virtually all tissues by the infecting agents, predominantly the proteus. In most cases death occurred within 36 hours after injection although occasionally some mice have survived as long as 48 hours. In the cases where mice have been held longer than 48 hours no additional deaths have been observed for as long as 30 days; thus the standard time for the following observations was 48 hours with all experiments terminated at that point.

Since it is known that gram-negative bacterial endotoxins injected into mice with, or prior to, an experimental bacterial infection appear to enhance the infectious process(4), the initial working hypothesis of the staphylococcus-proteus interaction was that endotoxin from the proteus acted to depress the resistance of the mice to the staphylococci. It shortly became apparent that this was not the case but that the reverse was true. Table I presents supporting data for this conclusion, suggested by the results of using various combinations of live and heat-killed cultures. Also included in this table are observations made with partially purified extracts of staphylococci. These data were obtained using commercially available§ "Staphylococcus lipopolysaccharide," (Difco #0350) although equal success has been obtained using similar materials prepared in this laboratory. These were extracts from "agar-washings" of plate-grown staphylococci prepared by a modification(5) of the Boivin trichloroacetic acid (TCA) procedure for extracting bacterial lipopolysaccharides(6).

The pooled data presented in the first table represent number of deaths per total number of mice tested in a series of experiments. For these studies the dose of proteus was 0.1 ml of a 1:10 dilution of the 18 hour culture, (ca 10^7 bacteria). Both live and killed staphylococci were injected at a dose of 0.2

§ Difco Laboratories, Detroit, Mich.

TABLE I. Synergistic Effect of *S. aureus* and *P. vulgaris* in Mixed Infection: Mouse Mortality at 48 Hours.

	Live staph. culture (0.2 ml, I.P.)	Heat-killed staph. culture (0.2 ml, I.P.)	Difco staph. extract (200 μg)	Controls (no staph.)
Live proteus culture (1:10 dil'n—0.1 ml)	19/20*	12/20	17/20	0/20
Heat-killed proteus culture (1:10 dil'n—0.1 ml)	1/15	0/15	not tested	0/15
Controls (no proteus)	0/15	0/15	0/15	—

* No. dead/Total No. inj.; scored after 48 hr.

ml of an undiluted 18 hour culture. When "lipopolysaccharides" were substituted for live or heat-killed staphylococci the I.P. dose was 200 μg.

From these data it can be seen that the staphylococcus-proteus combination was lethal for mice only when viable proteus was present, although live or heat-killed staphylococci and their TCA extracts were all capable of acting in combination with the proteus. Thus, the phenomenon seems to represent something other than enhancement of infection by gram-negative endotoxins.

2. *Effect of injection route.* Since the mechanism of the interaction did not appear to be that originally proposed, it was thought that the lipopolysaccharide-containing extract from the staphylococci might conceivably act in a physical way much as hog gastric mucin is believed to enhance the virulence of certain bacteria. The long list of such infection promoting agents includes a large number of high molecular weight materials including some bacterial polysaccharides(7). The most direct way to test the action of the staphylococcal material in this respect seemed simply to deliver it by a different route from the proteus challenge. Consequently experiments were done in which the proteus was injected I.P. but the staphylococci or staphylococcal-extract was injected I.V. For these observations doses of materials chosen were the same as those previously used. The results (Table II), demonstrate that even when injected by an entirely different route from the challenge infection the staphylococcal extracts or staphylococci were capable of potentiating the virulence of the proteus but in themselves had no apparent toxicity.

TABLE II. Effect of Injection Route on *S. aureus* *P. vulgaris* Interaction in Mice.

	Live staph. culture		Difco staph. extract	
	I.P.*	I.V.*	I.P.*	I.V.*
Proteus culture*	10/10†	3/10	7/10	6/10
Controls (no proteus)	0/10	0/5	0/10	1/5

* Same doses as in Table I.
† No. dead/Total No. inj.; scored after 48 hr.

3. *Effects of other staphylococcal strains.* Since all of the above observations had been confined to a single coagulase-positive strain of *S. aureus,* it was of interest to determine whether this action was a general property of other staphylococci or unique to the strain under observation. Ten coagulase-positive strains including *S. aureus,* phage type 80/81, as well as 5 different coagulase-negative *S. epidermidis* isolates have been tested. Table III includes only the results of 3 each of the *S. aureus* and *S. epidermidis* strains. The results with the rest of the strains were exactly comparable to those presented. While there was some variation in efficacy of the various *S. aureus* strains tested, all showed some effect in this system. On the other hand, none of the *S. epidermidis* strains tested has shown significant effect in enhancing the virulence of the proteus challenge.

4. *Effects of staphylococci on other gram-negative infections.* A previous report has indicated a synergistic effect between staphylococci and pseudomonas in mixed infection (3). It was therefore of interest to observe whether this phenomenon was applicable to other genera of the gram-negative enteric organisms. The preliminary results obtained will be reported elsewhere. It may be noted here that in the small numbers of mice examined other gram-negative bacteria such as

TABLE III. Effect of Other Staphylococcus Strains in Mixed Infection with *Proteus vulgaris*; Mouse Mortality at 48 Hr.

	Coagulase positive			Coagulase negative		
	80/81	11096	11462	11305	10827	7196J
*P. vulgaris**	11/15	13/15	9/15	0/15	0/15	1/15
Controls	0/10	0/10	0/10	0/10	0/10	0/10

* 1:10 dil'n of live culture; 0.1 ml—I.P.

E. coli, A. aerogenes, and *Pseudomonas* seem to share in the phenomenon.

Discussion. On the basis of the above data it seems evident that under certain circumstances, staphylococci can so enhance the virulence of otherwise relatively innocuous microorganisms as to cause rapid death of the host. Less clear, however, is the importance of this phenomenon in natural infection and further investigation of the time relationships and the quantitative aspects of the interaction must be made.

The second important question concerns the mechanism of this event. The effects of gram-negative bacterial endotoxin previously mentioned are well documented in a variety of test infections, and in many ways the staphylococcal extracts used in these studies resemble this closely. Higginbotham and Bass(8) have recently reported that similar extracts of *S. aureus* show other properties of endotoxin such as tumor necrosis and preparation for the dermal Shwartzman reaction. Without further data, however, one cannot equate the staphylococcal material with true "bacterial endotoxin."

Finally, the marked difference between coagulase-positive and coagulase-negative negative staphylococci as to their ability to enhance the virulence of a proteus challenge in mice is worthy of additional mention. The number of different strains tested is not sufficient to generalize about this as a further property of "virulent" or "pathogenic" staphylococci although the correlation has so far been excellent.

Summary. Evidence has been presented indicating that *in vivo* synergism exists between *Staphylococcus aureus* and *Proteus vulgaris* when these organisms are injected into Webster-Swiss mice. By using combinations of live and heat-killed cultures of these organisms as well as extracts of the staphylococcus it has been demonstrated that the apparent lethal infectious agent in this combination is proteus and that some product elaborated by the staphylococcus is responsible for the enhancement of virulence of the proteus challenge. Although the mechanism of this interaction is not understood, it has been suggested that in this phenomenon the staphylococcal product acts in a manner similar to that of gram-negative bacterial endotoxins. Furthermore, this material is extractable by procedures used for extraction of such endotoxins. It has been shown also that the *Staphylococcus epidermidis* strains so far examined do not appear to have the same infection-enhancing properties as the coagulase-positive *S. aureus* strains which have been tested.

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