was not detected in one of the 2 control urines. The significance of this finding is discussed, and a procedure is described by which this peptide can be separated essentially free of other hydroxyproline contaminants.

We are indebted to the physicians of the Medical Service for permission to study these patients.

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Diffusion of Penicillinase from *Staphylococcus aureus*.* (27241)

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There is conflicting evidence as to whether or not penicillinase produced by *Staphylococcus aureus* leaves the cell. Favoring the production of some extracellular enzyme are reports of penicillinase activity in filtrates (1,2) and supernatant fluids(3,4) of broth cultures of staphylococci and its diffusion from colonies of such organisms grown on agar(5). On the other hand, other workers have failed to demonstrate penicillinase in filtrates of staphylococcal cultures passed through Seitz, Mandler, Berkefeld or sintered glass filters(3,6,7) and the presence of bacterial cells in the supernatant fluids of centrifuged cultures was not definitely excluded(2, 7). The evidence from agar diffusion data is likewise inadequate because the findings could also be interpreted as indicating that penicillin diffuses to the staphylococcal cells where it is destroyed.

This report presents more convincing evidence from agar diffusion and filtration experiments that staphylococcal penicillinase does indeed leave the cell.

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Agar diffusion. Petri plates, 100 mm in diameter, containing 15 ml of 2% nutrient agar that had been allowed to dry, were marked off into quadrants. A point near the center of each quadrant was touched lightly with the tip of platinum wire that had been dipped into a broth culture of *Staph. aureus* and the plates were incubated at 37°C. The age of the broth cultures varied from 6 hours to 7 days and time of incubation from 12 to 24 hours in different experiments. The resulting colonies with their surrounding and underlying agar were each cut out with a piece of 6-7 mm glass tubing used like a cooky-cutter and aspirated with the aid of a rubber bulb attached to the upper, cotton plugged end. The agar plates with the punched out holes were then incubated at 37°C for another 24 hours to detect any contaminants or any staphylococci that were not removed by this procedure. Plates showing

any bacterial growth were discarded.

Sterile plates thus prepared were each flooded with 1 ml of a 1:50 dilution of a standardized supension of *Sarcina lutea* to which was added 0.15 μg of penicillin G to provide a concentration of approximately 0.01 μg/ml of agar if evenly diffused. The plates were reincubated at 30°C and observed for growth of satellite colonies around the punched out holes. The strain of *S. lutea* was completely inhibited by 0.005 μg/ml of penicillin G and the occurrence of growth was taken to indicate that the local concentration of penicillin had fallen well below that concentration.

Fig. 1 shows a ring of colonies of *S. lutea* around the punched out hole on the right but none around the one on the left; the former is the site where a colony of a known penicillin-resistant, penicillinase-producing strain of *Staph. aureus,* H-2, had grown, whereas the latter is the site of growth of Staph. 209P

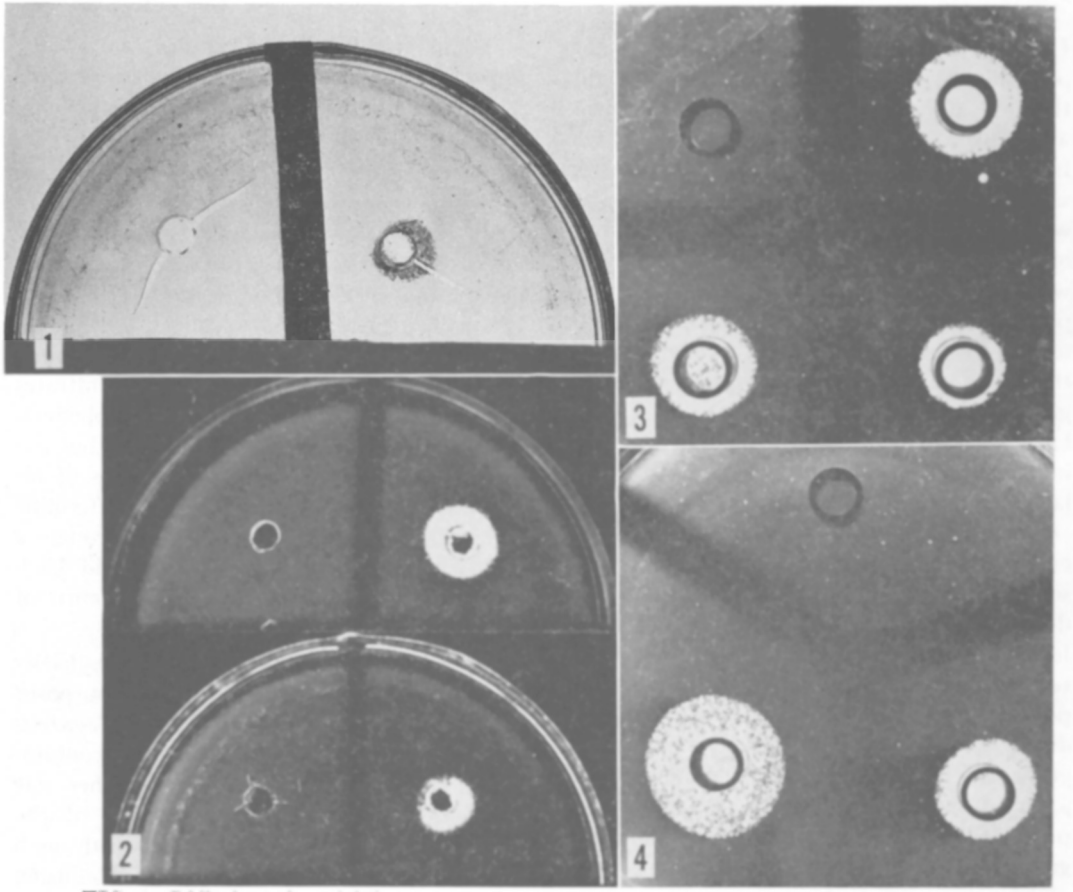

FIG. 1. Diffusion of penicillinase through agar. *Left:* Site from which colony of Staph. 209P (non-penicillinase producer) was removed. *Right:* Site from which colony of Staph. H-2 (penicillinase producer) had been punched out. The halo represents growth of *Sarcina lutea.*

FIG. 2. Diffusion of penicillinase through agar. The plates were refrigerated after growth of the staphylococcus colonies and before they were punched out with the surrounding agar. The halos represent growth of *S. lutea. Left upper:* Staph. 209P (non-penicillinase producer). *Right upper:* Staph. H-2 (penicillinase producer). *Left lower:* Staph. J (non-penicillinase producer). *Right lower:* Staph. 60-1 (penicillinase producer).

FIG. 3. Penicillinase activity in whole culture and in filtrates of Staph. 60-1. *Upper left:* Sterile broth. *Upper right:* Whole culture. *Lower left:* Millipore filtrate. *Lower right:* Same, filtered twice. Only the control broth shows no satellite colonies of *S. lutea.*

FIG. 4. Penicillinase activity of phage-lysate of Staph. 60-1. *Top:* Control broth. *Left:* Sterile, phage-lysed Staph. 60-1. *Right:* Same sterile, phage-lysate after filtration through millipore membrane (0.3 μ pore diameter).

TABLE I. Diffusion of Staphylococcal Penicillinase into Agar.

Strain of *Staph. aureus**	Penicillinase producer	Diffusible penicillinase demonstrated: Without methicillin or refrigeration	Refrigeration method	Addition of methicillin in broth culture
60-1	Yes	Irregularly	Yes†	Yes
H-1	"	No	"	"
H-2	"	Regularly‡	" †	"
60-3	"	No	"	Not tested
60-5	"	"	"	" "
J	No	"	No†	" "
209P	"	" ‡	" †	No

* All were clinical isolates except 209P. † See Fig. 2. ‡ See Fig. 1.

(ATCC 4538P), a penicillin-sensitive, non-penicillinase producing strain. Presumably the ring of colonies of *S. lutea* was growing where penicillinase had diffused into the agar from the staphylococcus colony before the sarcina organisms were seeded on the plate with the penicillin. To be sure, the periphery of the ring may also include a small area where the subinhibitory concentration of penicillin had been reached by diffusion toward the penicillinase in the agar. Using this method, it was possible to demonstrate growth of satellite colonies of *S. lutea* regularly with some known penicillinase-producing strains of *Staph. aureus,* but only irregularly or not at all with others.

These results were first interpreted as indicating that penicillinase leaves the cell of some but not all strains of penicillinase-producing staphylococci. Subsequently it was found that all known penicillinase producers tested could be shown to produce diffusible penicillinase by one of 2 additional procedures: 1. The strain of *Staph. aureus* was grown in broth containing methicillin,† 1.0 μg/ml (a subinhibitory concentration), to promote enzyme formation. 2. Following growth of the staphylococcal colonies, the plates were refrigerated at 4°C for varying periods to permit a longer period of diffusion of the enzyme into the agar from the colonies before they were punched out. Fig. 2 shows for each of 2 strains growth of sarcina around a hole that was punched out after the plate had been refrigerated. Both the amount of penicillinase produced and length of time allowed for its diffusion were thus shown to be important in this method. Results obtained with a few strains are listed in Table I.

Filtration. Like others(3,6,7), we failed in previous experiments(8) to demonstrate penicillinase activity in sterile filtrates of whole cultures, or in filtrates of sterile, phage-lysed preparations of *Staph. aureus* which had passed either Seitz or sintered glass filters having pore diameters of 0.9-1.4 μ. On the other hand, Eriksen(2) was able to detect penicillinase activity in sterile filtrates of cultures that had passed a gradacol membrane with a pore diameter of 0.68 μ, but not in those which had passed other types of filters. It was therefore of interest to determine whether the enzyme would pass through a thin membrane filter that would hold back the organisms and large particulate debris of lysed cells.

Two different preparations of staphylococcal penicillinase were used for this purpose: one was a 7-hour growth of *Staph. aureus* (BCH 60-1) in trypticase soy broth containing methicillin 1.0 μg/ml and the other was a sterile phage-lysed preparation(8) of the same strain. Each of these was passed through millipore filters of pore size 0.3 ± 0.1 μ, using a Swinny adapter on a 5 ml syringe. The filtrates were shown to be bacteriologically sterile.

Penicillinase activity was demonstrated in the filtrate by the Wallmark method(4). Agar containing penicillin G, 0.015 μg/ml, and a 1:100 dilution of a suspension of *S. lutea* was used, metal cylinders, 5 mm outside diameter, being placed on the agar and the various filtrates put into these cylinders. The

† 2,6 dimethoxybenzyl penicillin (Staphcillin, Celbenin).

TABLE II. Penicillinase Activity in Millipore Filtrates* of BCH 60-1.

Material in cylinder	Diameter of zone of growth of sarcina (mm) A	B†
Whole culture (7 hr growth)	15	17 ‡
" " filtered once	13.5	13.5‡
" " " twice	11	11.5‡
Trypticase soy broth (control)	0	0 ‡
Sterile phage lysate	21	17 §
" " " , filtered	15	12 §
Trypticase soy broth (control)	0	0 §

* All filtrations were done through millipore membranes, pore size 0.3 ± 0.1 μ.
† A and B are duplicate experiments.
‡ Shown in Fig. 3.
§ Shown in Fig. 4.

plates were incubated at 30°C for 1-3 days. Growth around the cylinder was taken to indicate penicillinase activity in the filtrate contained within it. With each of these 2 types of sterile filtrates, penicillinase activity was consistently demonstrated by this method. The results of one such experiment are shown in Fig. 3 and 4 and in Table II. The data in the table suggest that some penicillinase activity is lost on the millipore filter since the diameter of the zone of growth of the sarcina produced by the whole culture or by the unfiltered phage lysate was greater than that of the corresponding filtrate in each instance; also the second filtration resulted in a further reduction in size of the zone of growth. No attempt was made to quantitate the penicillinase activity in these studies.

Cellophane dialysis bags were also tested, using whole cultures or phage lysates inside and sterile water or Krebs-Ringer phosphate buffer, pH 7.4, outside. Penicillinase activity could not be demonstrated in the dialysate in any instance.

Discussion. It seems clear from the diffusion experiments that in the agar plates from which all staphylococci had been removed and which had been tested for sterility prior to adding penicillin G, there was no opportunity for contact between penicillin and any enzyme that may be inside or attached to the staphylococcal cell. Had viable staphylococci been left on the agar at the time of penicillin inactivation, as in the experiments of Gots(5), one could not be certain that the enzyme had left the cell, since the possibility that the penicillin had diffused toward the cell and was inactivated there could not be excluded. Moreover, the present studies indicate that active staphylococcal penicillinase can diffuse into agar from bacteria which had not been manipulated, thus eliminating the possibility of mechanical disruption of the cells. However, there is no evidence here to indicate whether the diffusion of the enzyme occurred from living cells or after the death and possibly spontaneous lysis of some of the cells.

The demonstration of penicillinase activity in millipore filtrates of whole cultures of *Staph. aureus* also strongly suggests that penicillinase leaves the cell, but this does not exclude the possibility that some of the cells had been disrupted during the filtration. It is also possible that the enzyme was attached to small particles of cellular debris in the filtrates of the phage lysates. The fact that active enzyme passed through a millipore filter of 0.3 μ pore size but not through sintered glass filters with pore diameters of 0.9 to 1.4 μ suggests that the enzyme was adsorbed to the sintered glass.

Millipore filtrates of staphylococcal cultures may provide a useful source of sterile staphylococcal penicillinase for further studies of the enzyme(s).

Summary and conclusions. The conflicting reports in the literature concerning production of extracellular penicillinase by *Staphylococcus aureus* were briefly reviewed and evaluated. Experimental evidence from studies utilizing agar diffusion and millipore filtration was presented from which it is concluded that the enzyme does indeed leave the staphylococcal cell.

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Effect of Hypervitaminosis D on Serum Factors in the Rabbit.* (27242)

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Hypervitaminosis D is usually associated with changes in the levels of calcium or phosphate in the blood serum. It is often tacitly assumed that abnormal levels of these ions in the blood are responsible for the widespread soft tissue calcification associated with this disease state. A study of the calcific lesions in rabbits with hypervitaminosis D, however, revealed that there are characteristic patterns of distribution of these lesions which cannot be explained by this assumption alone and indicated that the lesions were related to local variations in susceptibility of tissue to the effects of excessive Vit. D(1). To clarify the matter, it seemed desirable to study the state of the calcium and phosphate in the serum of these animals. An electrophoretic study of the serum was also done to determine if there was a change in the serum protein pattern. Inasmuch as elevations of serum muco-proteins have been described in rat hypervitaminosis D(2), hexosamine levels were also determined.

Materials and methods. Three groups of animals were studied. In the first 2, male New Zealand white rabbits weighing between 5 and 6½ lb were fed a stock diet (Rockland rabbit ration containing 0.82 g calcium, 0.35 g phosphorus, and 21 units of Vit. D per 100 g of feed) and given water *ad libitum*. Control blood samples were drawn by cardiac puncture twice at weekly intervals. Twenty-five animals (Group A) were then injected intramuscularly with 100,000 units of Vit. D_2 (Viosterol, U.S.P., 1,000,000 units per ml in corn oil) twice weekly for 4 weeks with blood samples taken at weekly intervals. As additional controls, 16 rabbits (Group B) were treated identically, except that they were injected with equal volumes of corn oil. It was difficult to withdraw enough blood to do all chemical determinations on a single blood sample, so that not all determinations were done on each animal. Nine rabbits of similar size and sex (Group C) were injected with equal doses of Vit. D, but at more widely spaced intervals (once or twice every 4 weeks over a period of several months). These animals were on a mildly hypercholesterolemic dietary regime (stock diet plus 0.6% cholesterol in corn oil)(3). Calcium was measured by a flame photometric method, ultrafiltrable calcium by a modification of the method of Toribara *et al.*(4), inorganic phosphorus by the method of Fiske and Subbarow(5), hexosamine by a method described by Winzler (6) and citric acid by the method of Beutler and Yeh(7), the samples being treated with sulfuric acid and bromine water to remove interfering substances. Paper electrophoresis was done with a Spinco apparatus using 2 buffers, one a veronal buffer, pH 8.6, and the other the veronal buffer with 400 mg of $CaCl_2$ added per liter. Quantitation was done with a Spinco Analytrol recording densitometer.

Results. The normal control levels of total calcium and citric acid were high and variable in rabbit serum, with values ranging between 12.0 and 17.3 mg % for total calcium and between 5.08 and 14.05 mg % for citric acid.

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