THE LOCALIZATION OF *STAPHYLOCOCCUS AUREUS* IN MICE BY WHOLE-ANIMAL RADIOAUTOGRAPHY

P. F. BONVENTRE, PH.D.,* AND J. G. IMHOFF

From the Department of Microbiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio

The complexities of host-parasite interactions make it extremely difficult to evaluate the factors which determine the outcome of infectious processes. Most investigators have approached this problem by constructing a mosaic of isolated bits of information gleaned by simplifying experimental models and applying them usually to an *in vitro* situation. This has yielded a considerable advance in our knowledge of infection but there still remain enormous gaps in understanding the fundamental mechanisms of pathogenesis. Consequently, any new approach to the study of host-parasite interaction deserves consideration.

Radioautography has received only limited application as a method for studying experimentally induced infection. Autoradiographic images of whole-body sections provide obvious advantages since they represent a comprehensive picture of the distribution of isotopically labeled materials at any time following parenteral injection. Previous autoradiographic investigations have dealt primarily with the localization *in vivo* of antibiotic agents and drugs.[1–5] The feasibility of employing whole-animal radioautography as a means of following the distribution and localization of pathogenic bacteria in experimentally infected hosts has been developed more recently.[6–8] The latter investigations showed that bacteria could be labeled with appropriate isotopes so that autoradiograms of a high degree of clarity and resolution were obtained.

One of the major difficulties in interpreting the whole-animal radioautographs is in distinguishing between radioactivity due to intact organisms and that caused by metabolic or degradation products. The dilution of radioactivity as a consequence of multiplication *in vivo* is also a complicating factor. These difficulties prompted an investigation of the extent to which radioactivity as seen by whole-animal radioautography could be correlated with the number of viable organisms found in the organs and tissues of mice following the parenteral ad-

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ministration of ^{35}S-labeled *Staphylococcus aureus*. In addition, the effects of penicillin and immune serum on the distribution patterns *in vivo* were examined.

Material and Methods

Labeling of Staphylococcus aureus. S. aureus (strain M-240, Welwood) was grown in the synthetic medium described by Mergenhagen [9] to which was added 0.1 per cent vitamin free casamino acids (Difco Laboratories, Detroit, Mich.). The organisms did not incorporate ^{35}S when supplied as Na_2SO_4; a high degree of labeling was achieved, however, with ^{35}S-DL-methionine (Volk Chemical Co., Skokie, Ill.) The amino acid was dissolved in glass distilled water and added to the growth medium so that the final radioactivity was 5 μc per ml. Cultures were incubated at 37° C for 18 hours on a reciprocal shaker (120 strokes per minute) and the cells harvested by centrifugation. All loosely bound radioactivity was removed by washing the cells in a gelatin-buffered saline mixture several times or until the radioactivity in the supernatant fluid reached a constant low level. The labeled staphylococci were then suspended in physiologic saline before parenteral injection into mice. The final concentration of organisms was approximately 10^9 per ml and the radioactivity of the cell suspension which varied, was measured in a gas flow counter.

Animals. Swiss-Webster mice (Hamilton Laboratories, Hamilton, Ohio) of either sex (20 to 25 g) were used in all experiments.

Whole-body Sectioning of Mice. The method used to obtain whole-animal sections of mice was originally described by Ulberg in 1954.[10] A modification of this technique was employed in these studies. Briefly described, the essential elements of the apparatus and procedure were as follows: at desired intervals after inoculation of the ^{35}S-labeled bacteria, the animals were sacrificed under ether anesthesia by immersion in a dry-ice-ethanol mixture at −70° C. Thin layers of wet cotton were placed on the cross-hatched surface of an aluminum mounting block and the animal was frozen to the block in a tray of crushed dry ice. The bond was strengthened by packing and freezing wet cotton between the lateral surface of the animal and the aluminum block. Finally a 2 per cent solution of carboxymethylcellulose (Hercules Powder Co., Wilmington, Del.) was brushed over the entire animal to provide an inert matrix which reduced the tendency of whole-animal sections to crumble.

The frozen animal was sectioned with a mechanically driven sledge microtome housed in a low temperature chamber maintained at −5° C. The whole-animal sections were obtained by placing a strip of acetic acid base tape #810 (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) on the cut surface of the animal. As the sledge was driven under the cutting knife, the section which adhered firmly to the adhesive was lifted off. Sections were dehydrated under vacuum while still frozen so that translocation of radioactive materials did not occur. Sections used in this investigation were cut at thicknesses between 50 and 60 μ.

Autoradiography. The dehydrated sections were placed in apposition to radiosensitive x-ray film and clamped together securely and placed in a light tight container. Exposure of the film to the section was carried out in a dessicator at room temperature and under increased carbon dioxide tension. Two types of film were employed in these studies: Kodak No-Screen (Eastman Kodak Co., Rochester, N.Y.), and Industrex Type D (Kodak Ltd., England). These films possess identical photographic specifications but the English made counterpart gave far superior results. In our hands, Industrex Type D film did not show a nonspecific blackening as was the case with the Kodak No-Screen film. The artifact was due apparently to a chemical or physical reaction between the adhesive binding the section and the film emulsion and did not represent true foci of radioactivity in the whole-animal section. As a result, in most of our later experiments the Industrex Type D film was used exclusively. The films were developed after a suitable exposure period (be-

tween 1 and 4 weeks) using standard photographic procedures. Since the dehydrated sections were susceptible to staining, the developed autoradiograms could be compared with their corresponding sections stained with hematoxylin and eosin (Figs. 1 and 2). This insured a positive identification of organs and tissues which contained areas of radioactivity. Details of the sectioning and autoradiographic technique have been described previously.[8]

Enumeration of Viable Staphylococci in Tissues. Tissues were excised, weighed and rinsed in sterile buffered saline. They were then homogenized individually with a standard quantity of cold buffered saline in tissue homogenizers. Duplicate samples were then plated on trypticase soy agar and incubated at 37° C for 2 days before counting. The number of viable bacteria was expressed as the number per ml of homogenate. Mice employed for the determination of viable bacteria were not the same animals from which the sections and autoradiograms were prepared.

Staphylococcal Antiserum. Antiserum was prepared in adult New Zealand rabbits by administering 1 ml of a standardized, heat-killed vaccine prepared from *S. aureus* grown in trypticase soy broth. Alternate injections were given intravenously and with adjuvant intramuscularly every 5 days. Rabbits were bled by cardiac puncture 2 weeks after the sixth injection. The agglutination titer of the serum after immunization was 1:80.

Experimental Results

Localization of Staphylococci in Mice given Intravenous and Intraperitoneal Inoculations. Initial experiments were carried out to establish the normal distribution and localization patterns. A group of 18 animals received a staphylococcal suspension of relatively high activity (380,000 counts per minute per 0.1 ml). Nine mice were given intravenous injections of 0.25 ml of the suspension and 9 received 0.5 ml intraperitoneally. Three animals in each group were sacrificed at 1 minute, 1 hour and at 20 hours after injection. Autoradiograms were then prepared from the whole-body sections. The areas of blackening on the film corresponded to sites of deposition of the labeled staphylococci *in vivo*. The localization of bacteria following intravenous injection (Figs. 3, 4 and 5) demonstrated the rapid disappearance of staphylococci from the circulation and their accumulation in the lung, liver and spleen. The distribution pattern as seen in autoradiographs substantiated results obtained by classic bacteriologic methods. The pattern encountered in autoradiograms of the animals challenged intraperitoneally (Figs. 6, 7 and 8) were somewhat more difficult to evaluate. It was clear that the organisms were spread very quickly throughout the peritoneum. After 1 hour a slight accumulation appeared in the liver and spleen but foci with most intense radioactivity were arranged in a clumplike fashion on the external surfaces of the viscera. This clumping *in vivo* has been described recently and was found to be a property associated with the virulence of *S. aureus*.[11] Twenty hours after injection, radioactivity was more evenly distributed within the liver, spleen, kidney and intestine, but the foci of greatest intensity still appeared on the surfaces. Unlike the early distribution pattern, some activity was ap-

parent above the diaphragm and in the lung. No radioactivity could be detected in the heart or the central nervous system. The blackenings observed at the periphery of the film and occasionally diffusely in autoradiograms were not truly indications of activity but were artifacts produced when the Kodak No-Screen film was employed. These nonspecific patterns were not observed in the later autoradiograms produced on Industrex Type D film.

Localization of Staphylococci in Untreated and Penicillin Treated Mice. A major difficulty in the interpretation of autoradiograms is in the distinction between radioactivity due to viable bacteria and that resulting from nonviable organisms or metabolic products. One of the methods employed to clarify this was to compare whole-body autoradiograms of penicillin treated and untreated mice. These results were in turn correlated with "viable counts" of staphylococci in animals treated in the same fashion. Twelve mice were treated with 5,000 units of penicillin G (The Upjohn Co., Kalamazoo, Mich.) 30 minutes prior to challenge with the ^{35}S-labeled staphylococci, and daily thereafter until sacrifice. Half of the animals were challenged intravenously and the other half intraperitoneally. Another group of 12 untreated mice which served as controls was divided in half and challenged with the staphylococci in the same manner as the penicillin treated animals. The radioactivity of the bacterial suspension used in this experiment was 129,000 counts per minute per 0.1 ml and each animal received 0.5 ml. Two animals from each of the 4 groups were sacrificed at 5, 24 and 72 hours after injection of *S. aureus*. The distribution of radioactivity in the autoradiograms prepared from these animals was then compared. The distribution in the penicillin treated and untreated mice 24 hours after challenge intraperitoneally was similar but not identical (Figs. 9 and 10). Despite the similarity of the distribution patterns the intensity of blackening was reduced in the penicillin treated animals. This would suggest a dissemination of labeled compounds liberated from the staphylococci by the bactericidal action of the antibiotic. The same conclusions concerning the dissemination of labeled products could be drawn from autoradiograms prepared in the two groups of animals challenged intravenously. In the untreated animals (Fig. 12) the radioactivity after 72 hours was confined primarily to elements of the reticuloendothelial system. The penicillin treated animals (Fig. 11), however, showed a considerable quantity of radioactivity spread throughout the internal organs, skin and hair.

Viable Staphylococci in Penicillin Treated and Untreated Mice. Sixteen mice were challenged intravenously with 3.2×10^9 organisms. The staphylococcal suspension was the same as that employed for the

penicillin experiments described above. Half of the animals were treated with 5,000 units of penicillin G, 30 minutes before challenge and daily thereafter until sacrificed. The other half served as controls. Two animals were sacrificed from each group daily and the numbers of viable *S. aureus* in tissue homogenates of the lung, liver, spleen and kidney were determined among the treated and untreated mice at 1, 3, 5 and 7 days after infection (Table I).

During the first 3 days of infection, the numbers of viable staphylococci in the organs of penicillin treated and untreated animals were not significantly different. Thereafter, however, the staphylococci in the kidneys of untreated animals increased rapidly, while the number in the antibiotic treated mice was reduced to a very low level. The multiplication of *S. aureus* in the kidney of the mouse results in typical abscess formation which is usually the cause of death in this species.[12,13] It is clear that the extensive intrarenal bacterial multiplication could not be detected in autoradiograms. This exemplifies the major limitation of the autoradiographic method. Labeling of the organism with ^{35}S-methionine results primarily in the incorporation of the labeled amino acid into the proteins of the cell. The level to which this incorporation occurs depends for the most part on the quantity of the labeled precursor of protein present in the growth menstruum. The use of very large amounts of isotopically labeled amino acids is economically prohibitive and thus a compromise must be achieved. Once the bacteria have been labeled sufficiently so that they can produce satisfactory autoradiograms any multiplication which takes place results in a relative dilution so that the radioactivity per bacterium diminishes with each division. A point is reached ultimately where the specific activity is too low to yield an autoradiographic image.

Autoradiographic Localization of Staphylococci in Mice passively protected with Antiserum. Another treatment which may alter the course of infection is the presence of specific antiserum. The effect of staphylococcal antiserum on the localization and distribution pattern therefore was also studied. A group of 4 mice were given 0.5 ml *S. aureus* rabbit antiserum 30 minutes prior to bacterial challenge. The radioactivity of the staphylococcal suspension was approximately 400,000 counts per minute per 0.1 ml. Two mice were sacrificed at 3 hours and the other two 20 hours after infection. The localization of radioactivity in these passively protected animals was restricted solely to the peritoneal cavity (Fig. 13). On the other hand, mice unprotected with staphylococcal antiserum, showed a significant accumulation of radioactivity in all internal organs (Figs. 7 and 8). The absence of radioactivity in the organs of the serum treated animal suggested that in this circum-

TABLE I

NUMBERS OF VIABLE ORGANISMS IN THE ORGANS OF PENICILLIN TREATED AND UNTREATED MICE FOLLOWING INTRAVENOUS INJECTION OF STAPHYLOCOCCUS AUREUS

Days post challenge	Staphylococci per ml homogenate *							
	Lung		Liver		Spleen		Kidney	
	Treated †	Control §	Treated	Control	Treated	Control	Treated	Control
1	3.0×10^5	7.0×10^5	2.0×10^7	2.0×10^7	3.5×10^6	1.3×10^6	7.5×10^4	1.0×10^5
3	3.0×10^3	3.9×10^3	2.8×10^4	2.6×10^4	1.9×10^5	1.2×10^5	2.7×10^5	2.7×10^5
5	1.4×10^1	5.7×10^2	2.7×10^3	4.0×10^3	2.0×10^2	1.0×10^4	$< 10^2$	1.3×10^7
7	1.9×10^1	2.3×10^2	1.1×10^1	2.3×10^3	9.0×10^0	1.6×10^3	2.0×10^0	1.5×10^7

* Average number obtained from 2 animals in each group at the designated time interval after infection. The figures are corrected for variations in the weights of the organs from animal to animal.

† Treated mice—received penicillin G (5,000 units) 30 minutes prior to challenge and 5,000 units daily thereafter.

§ Control—normal mice which received no antibiotic therapy.

stance the staphylococci were contained within the peritoneum. An *in vivo* antigen-antibody reaction would likely result in exactly such a situation. The whole-animal autoradiograms in this case therefore presented an accurate description of the *in vivo* distribution of *S. aureus*.

Discussion

The experiments described were designed to assess the limitations of whole-animal radioautography in depicting the course of an experimentally induced infection. It had been shown previously that autoradiograms of remarkable clarity and resolution could be prepared from whole-body sections of mice infected with pathogenic agents labeled with a suitable isotope.[6-8] The problems inherent in the method, however, could not be ignored. Upon introduction of the infectious agent parenterally, the host defense mechanisms (humoral and cellular) immediately come into play and destroy a fraction of the bacterial population. In such a situation a distinction must be made between the radioactivity related to viable bacteria and their labeled degradation products. Since the infectious process is a dynamic one, moreover, with concommitant destruction *and* multiplication of bacteria in different anatomic locations, the dilution of radioactivity per bacterium as a consequence of multiplication *in vivo* introduces another difficulty in interpretation. An attempt was made to determine the extent to which of these problems might limit the application of radioautography to the study of infection. The course of the infection was altered by using penicillin or specific staphylococcal antiserum to ascertain if the changes in the infectious process brought about by such treatments might be observed by changes in the localization patterns established by radioautography.

The whole-body radioautographic method gives a comprehensive picture of distribution and localization of *S. aureus* in a single animal at any time post-infection. This obvious advantage over classic bacteriologic methods must be tempered by the radioactivity dilution problem and the non-quantitative nature of the information radioautography provides. It is conceivable that the method could be modified so that results could be measured quantitatively. Since the degree of blackening in a specific region is directly proportional to radioactivity,[14,15] it would be possible to obtain semi-quantitative data by densitometry. Using a known series of radioactive standards, calibration curves could be obtained so that the numbers of isotopically labeled bacteria within a specific organ or anatomic region might be estimated.[16] This presupposes that intensity of radioactivity observed and the numbers of viable bacteria are proportional. It is clear from the data obtained in the

present investigations that such a relationship does not always hold.

The distribution pattern in mice after the intravenous injection of labeled *S. aureus* is a valid one. The very rapid sequestration of bacteria from the circulation by the reticuloendothelial system, their accumulation within the liver, spleen and lung and their absence from the central nervous system are all shown clearly. It should be noted that the autoradiograms depict conditions *in vivo* which obtain within several hours after inoculation. In this relatively short period of time the factors already discussed have not yet become operative to the extent that they would complicate the interpretation of the autoradiographic images.

The observations made with the antibiotic treated mice provide an insight into both the strength and the limitation of the method. The inhibition of bacterial multiplication by penicillin and subsequent liberation of labeled products can be visualized effectively by autoradiography. This was made evident by the accumulation of radioactivity in the skin and hair, and to a lesser degree in the central nervous system. The method in this experimental situation, provides a static morphologic representation of the transient events occurring in the infected host. When, however, a focus of infection is established, as was the case in the kidneys of untreated mice, and the organisms begin to multiply at a rapid rate, the dilution of radioactivity per bacterium results in autoradiographic distribution patterns which are not consistent with the numbers of viable bacteria actually present. This obvious disadvantage must be kept in mind when using the technique. It may be that this could be obviated by making certain that the initial radioactive label of the pathogen was of such a high specific activity that autoradiographic images would be produced even after significant bacterial multiplication.

The effect of antigen-antibody reaction *in vivo* was also seen clearly in the autoradiograms. Animals treated with antiserum before intraperitoneal challenge did not accumulate radioactivity in any of the internal organs as in the unprotected animals; it was confined primarily to the peritoneal cavity. It would appear that in this case, the autoradiographic method provided information which would not have been readily obtainable by classic bacteriologic methods. If viable bacteria had been enumerated by plating methods, the staphylococci present on the surfaces of the liver, spleen and kidney would have been counted. As a result, an erroneous conclusion that the pathogens were actually present within these organs might have been made.

The results of this investigation suggest that the whole-body autoradiographic technique can be used successfully in the study of infectious processes. The conclusions drawn from the autoradiograms, however, must be made with full cognizance of the difficulties and

limitations of the method. It appears that the early events following infection are most faithfully reproduced and it may be that the method would be more profitably employed for short-term experiments.

In conclusion, mention should be made of the possibility that whole-animal sections might be useful in investigations not concerned with radioautography. For example, they may be used to advantage in immunofluorescence and histochemical studies. In addition, it is conceivable that the whole-body sectioning technique may offer advantage in other experimental pursuits not obtainable by conventional histologic methods.[8,10]

Summary

Whole-body radioautography was assessed as an experimental method for the study of infectious processes. The technique was designed primarily for obtaining whole-animal sections of small laboratory animals and following the distribution and localization of radioactive materials by radioautography. In the present investigation, *S. aureus* infections in mice were studied by this method. The disposition of ^{35}S-labeled staphylococci injected intravenously and intraperitoneally was evaluated radioautographically in untreated mice and under several experimental conditions known to alter the course of infection. These included penicillin therapy and passive protection with staphylococcal antiserum. The information obtained was compared with that obtained by using conventional cultural methods to determine the number of viable bacteria in the tissues of the infected animals. The autoradiograms clearly showed the rapid disappearance of intravenously injected bacteria from the blood stream and their accumulation in the lung, liver and spleen. It also showed the dissemination of by-products liberated by the bactericidal action of penicillin. The immobilization of staphylococci within the peritoneum caused by the presence of specific antibody was also made apparent. The autoradiograms, however, did not reveal the extensive staphylococcal multiplication in the kidneys which took place between the third and seventh days post-injection. This resulted from the dilution of the isotope during bacterial division.

It is concluded that whole-animal radioautography can be most profitably employed in the study of infections during the early stages of disease before extensive bacterial destruction or multiplication *in vivo* has taken place. The advantages and disadvantages of the method are also discussed.

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LEGENDS FOR FIGURES

FIG. 1. Whole-animal sagittal section of mouse infected with *Staphylococcus aureus* and treated with penicillin. Ehrlich's acid hematoxylin-eosin stain. *ca* × 2.

FIG. 2. Whole-body autoradiogram prepared from the sagittal section shown in Figure 1. The animal was sacrificed 3 days after the intravenous injection of ^{35}S-labeled *Staphylococcus aureus*. Total radioactivity of bacterial suspension injected was approximately 6.5×10^6 cpm. *ca* × 2.

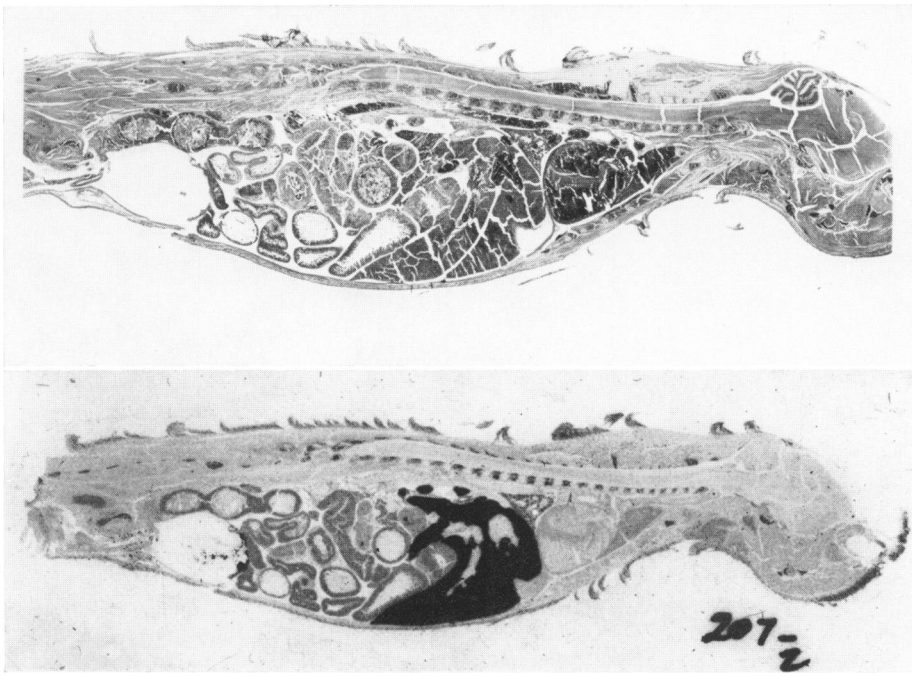

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FIG. 3. Autoradiogram prepared from a sagittal section of a mouse given an intravenous injection of ^{35}S-labeled *Staphylococcus aureus*. The radioactivity of bacterial suspension injected was approximately 9.5×10^5 cpm. Animal was sacrificed 1 minute after bacterial challenge. Actual size.

FIG. 4. Autoradiogram prepared from a sagittal section of a mouse given an intravenous injection of ^{35}S-labeled *Staphylococcus aureus*. Radioactivity of bacterial suspension injected was approximately 9.5×10^5 cpm. Animal was sacrificed 1 hour after bacterial challenge. Actual size.

FIG. 5. Autoradiogram prepared from a sagittal section of a mouse given an intravenous injection of ^{35}S-labeled *Staphylococcus aureus*. Radioactivity of the bacterial suspension injected was approximately 9.5×10^5 cpm. Animal was sacrificed 20 hours after challenge. Actual size.

FIG. 6. Autoradiogram prepared from a sagittal section of a mouse given intraperitoneal injection of ^{35}S-labeled *Staphylococcus aureus*. The radioactivity of the bacterial suspension injected was approximately 1.9×10^6 cpm. Animal was sacrificed 1 minute after bacterial challenge. Actual size.

FIG. 7. Autoradiogram prepared from a sagittal section of a mouse given an intraperitoneal injection of ^{35}S-labeled *Staphylococcus aureus*. The radioactivity of the bacterial suspension injected was approximately 1.9×10^6 cpm. Animal was sacrificed 1 hour after challenge. Actual size.

FIG. 8. Autoradiogram prepared from a sagittal section of a mouse given an intraperitoneal injection of ^{35}S-labeled *Staphylococcus aureus*. The radioactivity of the bacterial suspension injected was approximately 1.9×10^6 cpm. Animal was sacrificed 20 hours after challenge. Actual size.

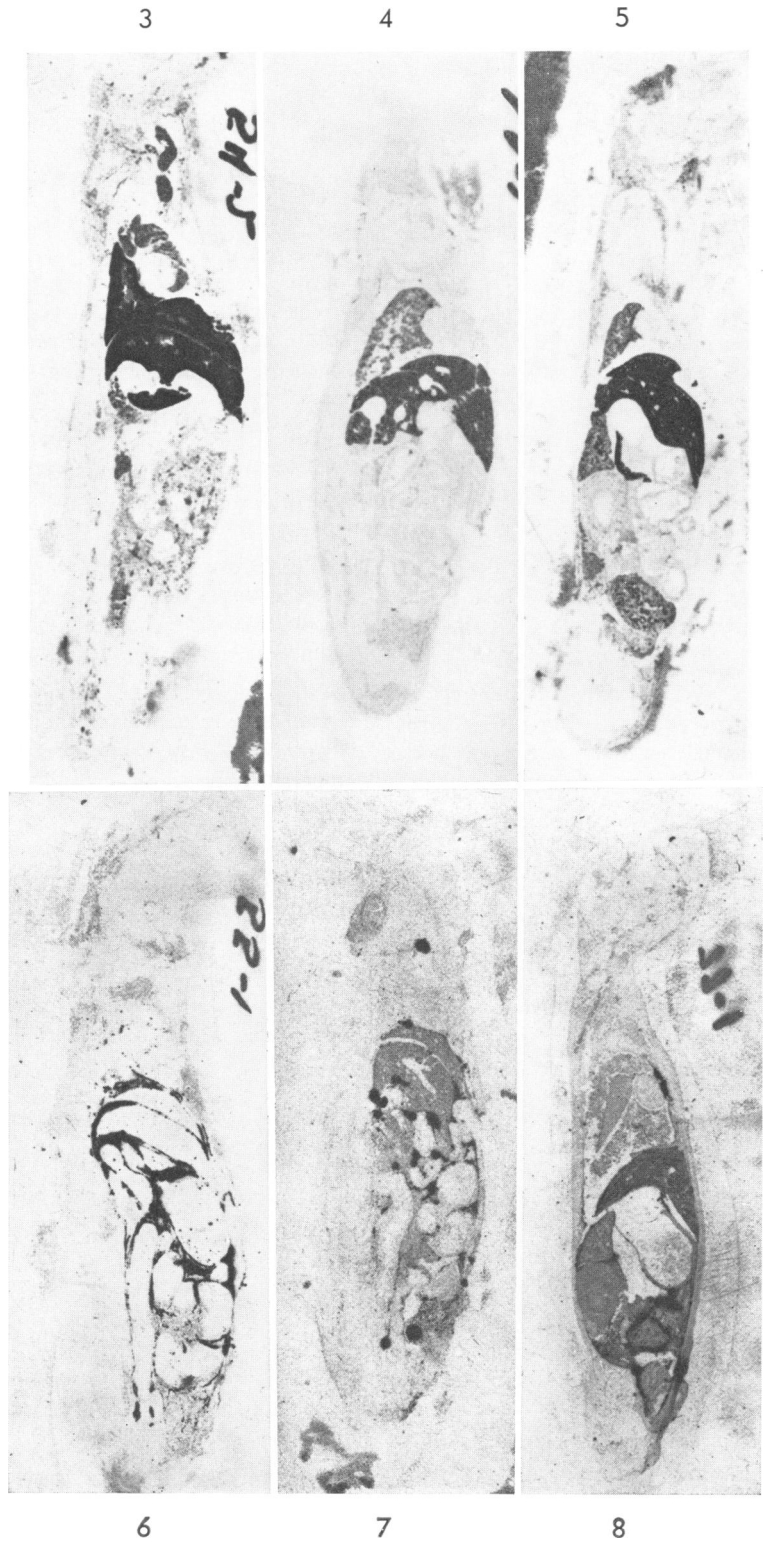
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FIG. 9. Whole-body autoradiogram of a penicillin treated animal given an intraperitoneal injection of ^{35}S-labeled *Staphylococcus aureus* and sacrificed 24 hours later. Radioactivity of the bacterial suspension injected was approximately 6×10^5 cpm. The autoradiogram was prepared from the frontal section shown in Figure 14. *ca* × 1.5.

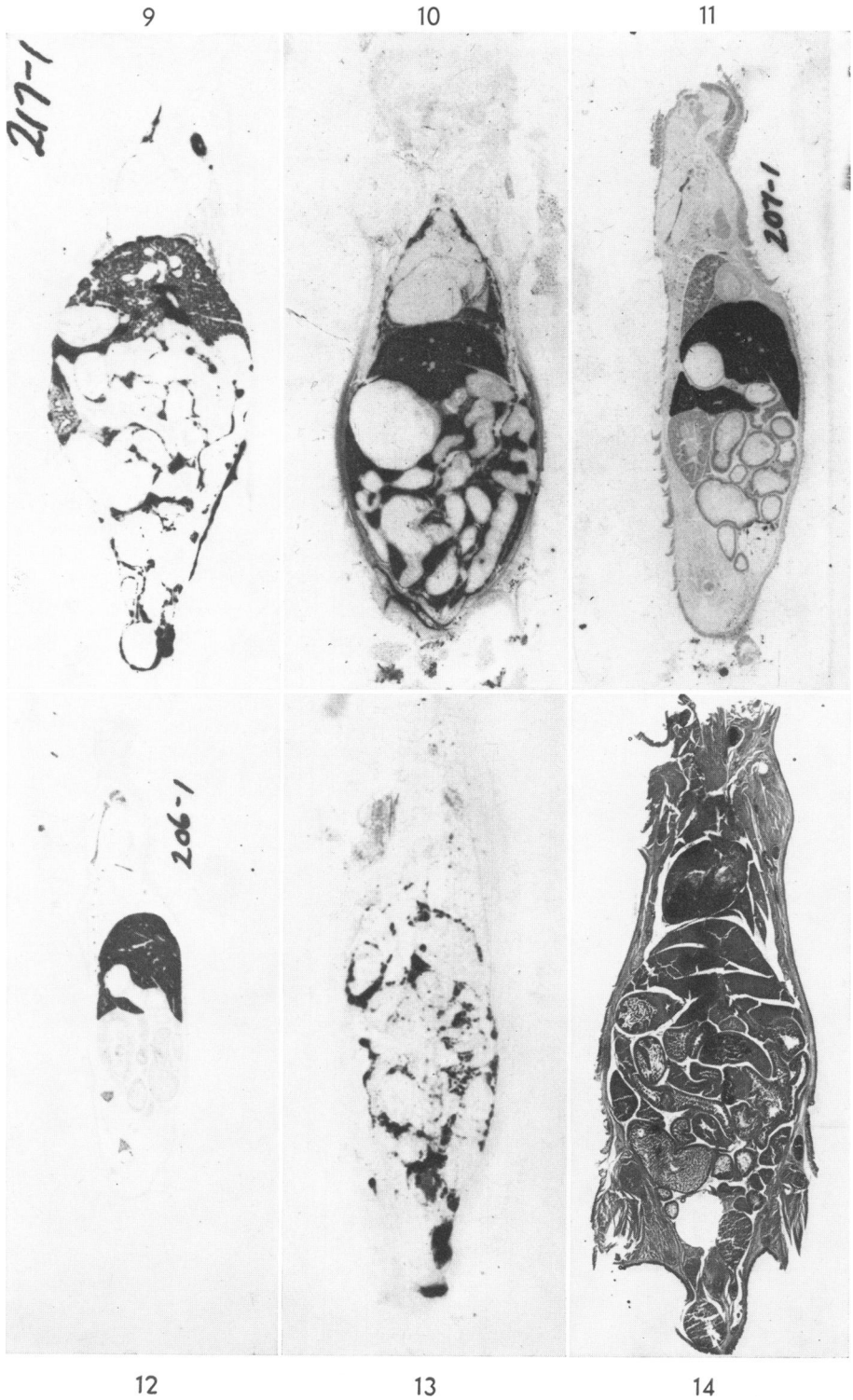
FIG. 10. Whole-body autoradiogram of an untreated (control) animal given an intraperitoneal injection of ^{35}S-labeled *Staphylococcus aureus* and sacrificed 24 hours later. Radioactivity of the bacterial suspension injected was approximately 6×10^5 cpm. Autoradiogram prepared from a frontal section. *ca* × 1.5.

FIG. 11. Whole-body autoradiogram of a penicillin treated animal given an intravenous injection of ^{35}S-labeled *Staphylococcus aureus* and sacrificed 72 hours later. Radioactivity of bacterial suspension injected was approximately 6×10^5 cpm. The autoradiogram was prepared from a sagittal section. Actual size.

FIG. 12. Whole-body autoradiogram of an untreated (control) animal given an intravenous injection of ^{35}S-labeled *Staphylococcus aureus* and sacrificed 72 hours later. Radioactivity of bacterial suspension injected was approximately 6×10^5 cpm. The autoradiogram was prepared from a saggital section. Actual size.

FIG. 13. Whole-body autoradiogram of a mouse protected with *Staphylococcus aureus* rabbit antiserum (0.5 ml intraperitoneally), challenged with ^{35}S-labeled *Staphylococcus aureus* (*ca* 1.9×10^6 cpm) 30 minutes later, and sacrificed 20 hours after challenge. Autoradiogram was prepared from a saggital section. Actual size.

FIG. 14. Whole-animal frontal section of mouse from which the autoradiogram shown in Figure 9 was prepared. The section was stained with Ehrlich's acid hematoxylin-eosin. *ca* × 1.5.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