Localization of Antigens by Autoradiography.* (26248)

H. F. CHENG,† M. DICKS, R. H. SHELLHAMER,‡ E. S. BROWN, A. N. ROBERTS AND F. HAUROWITZ

Department of Chemistry, Indiana University, Bloomington

The object of this investigation was to determine the localization of injected radioactive antigens by means of autoradiography. Deposition of isotopically labeled proteins in various tissues and subcellular fractions had been reported from this laboratory(1-5) and from other laboratories(6-8). The methods used in these previous studies involved tissue homogenization and subsequent fractional centrifugation. With such manipulations, it was difficult to exclude transfer of labeled protein antigens from one to another intracellular fraction. Ambiguities of this type are avoided by using colored azoproteins which can be detected histologically in the tissue sections (9-11), or by investigating the tissues of animals injected with isotopically labeled antigens using autoradiographic technics(7,8,12). It appears that, hitherto, no attempts have been made to evaluate the autoradiographic findings on intracellular localization of injected antigens by comparison with the distribution and level of radioactive antigen in the organs of the same animals. Such experiments are described in this paper.

Materials and methods. Preparation and injection of antigens. Heavily or lightly labeled S^{35}-sulfanilazo-BGG was prepared by coupling 1.0 g BGG (Pentex Inc., Kankakee, Ill.) with 0.1 or 0.01 g respectively, of diazotized S^{35}-sulfanilic acid(2,3,7). Internally labeled S^{35}-rabbit serum albumin (S^{35}-RbSA) was obtained by injecting a rabbit with the hydrolysate of S^{35}-yeast protein and isolating the albumin fraction of the blood serum as described earlier(13). A single dose of 10 to 13 mg of the heavily labeled azoproteins or of the internally labeled rabbit serum albumin was injected intravenously into mice. The lightly labeled azoproteins were injected subcutaneously into the foot pads of rabbits. The animals were killed by exsanguination from the cervical vessels. The organs of some of the injected rabbits and mice were homogenized and protein powders were prepared(13) for measurement of tissue radioactivity. In some of the experiments, liver and spleen homogenates were fractionated by differential centrifugation(1,13).

Histological procedure. Tissues were removed from the animals immediately after death, fixed in neutralized 10% formalin solution for 12 to 24 hours, washed with water, dehydrated, cleared with methylsalicylate and embedded in Fisher Tissuemat M. P. 56-58.5°. Sections of 3 to 7 μ in thickness were mounted on slides coated with gelatin-potassium alum solution and then decerated. Some sections were stained with Harris hematoxylin or Feulgen-fast green prior to autoradiography. These slides were then dipped for 20 seconds into 1% celloidin solution, allowing all excess celloidin to drain off. After drying, the celloidin layer was hardened by treatment with 80% ethanol for 1 minute, coated with gelatin and dried. Some sections were stained with Giemsa following development of the autoradiographs. Kodak Autoradiographic Permeable Base Safety Stripping Film was used in some of the experiments; in other studies, Kodak Nuclear Track Type NTB3 emulsion, obtained through the courtesy of Eastman Kodak Co., Rochester, N. Y., was employed. The slides and the liquid emulsion were warmed up to 37° in the dark room. Two or 3 drops of the emulsion were spread over the tissue section with a fine brush and the slide placed for about ½ minute on the slide warmer at 37°. After

* Support of this work by grants of U. S. Public Health Service and Nat. Science Foundation to one of us (F.H.) and by contracts of Indiana Univ. with U. S. Atomic Energy Comm. and Office of Naval Research is gratefully acknowledged. We are also greatly indebted to Dr. R. R. Ruth for advice and cooperation. The following abbreviations are used: BGG = bovine γ-globulin, RbSA = rabbit serum albumin.

† Fellow of China-America Foundation 1955-56.

‡ Dept. of Anatomy, Indiana Univ.

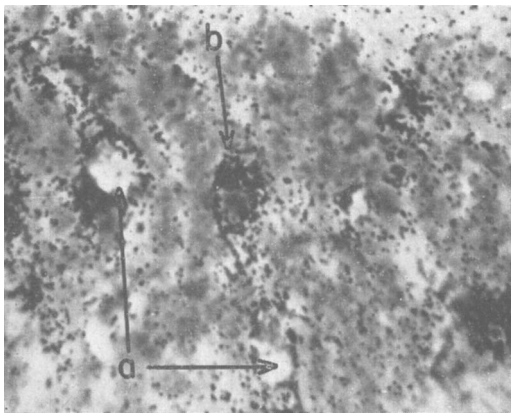

FIG. 1. Liver section of Mouse 13 which was inj. i.v. with 12.5 mg (225 × 10^6 cpm) of S^{35}-azo-BGG and killed after 6.5 hr. Section was stained with Giemsa and covered with stripping film. Radioactive material (a) in sinusoids and (b) in Kupffer cells. Nucleus of the Kupffer cell is free of silver grains. Exposed 21 days. × 700.

drying at room temperature, slides were kept in a light-proof desiccator over calcium chloride at 5°C. Exposure times with the wet emulsion were approximately one-fifth of those required for stripping film which varied from a few days to several months.

Results. Localization of heavily substituted S^{35}-sulfanilazo bovine γ-globulin. Mice No. 13, 15 and 16 were killed 6.5 hours, 5 and 10 days, respectively, after intravenous injection of the antigen. Tissue sections were prepared from liver, spleen and femoral bone marrow and studied with autoradiography. Examination of the liver sections revealed silver granules concentrated (a) in the cytoplasm of Kupffer cells, forming a zone around the nucleus of each cell, and (b) in the endothelium of the sinusoids (Fig. 1). Much less radioactivity was found in the hepatic parenchymal cells. These cells also displayed more silver granules over the cytoplasm than over the nuclei. In autoradiographs of the spleen of injected animals, most of the activity was found in the red pulp, confirming earlier reports by Ingraham(7). Autoradiographs of the bone marrow sections showed the greatest number of grains to be around the islets of adipose tissue. Unequivocal identification of some cell types which contained radioactive material was impossible. However, very little activity was present in the lymphocytes.

Table I shows that the protein-bound radioactivity was concentrated in the liver and spleen, later also in kidney, whereas its concentration was low in lung, muscle and in blood. Differential centrifugation of the liver homogenate revealed that the mitochondrial protein had the highest specific activity (Table I). Similar results were obtained earlier after injection of rabbits with heavily labeled azoproteins(2).

Localization of lightly substituted, externally labeled antigens. The lightly labeled S^{35}-sulfanilazo-BGG was purified by dialysis against distilled water and precipitated by alum(14). After resuspension in isotonic saline solution, 0.3 to 0.4 ml of the suspension containing 13 to 16 mg of the antigen were injected into each of the 2 hind foot pads of 3 rabbits, No. 300, 308 and 310. The protein-bound radioactive material was present in all tissues examined (Table II). Its distribution was not very different from that found after intravenous injection(3). Elimination of the injected antigen from the blood was determined in Rabbit No. 310 by taking small samples of blood from the ear vein each day. The proteins were precipitated by trichloroacetic acid and their radio-

TABLE I. Distribution of Protein-Bound Radioactivity† after Intravenous Injection of Sulfanilazo-Bovine γ-Globulin into Mouse No. 15 and 16.

	Mouse No. 15		Mouse No. 16	
	(cpm/mg) × 10^{-3}	RSpA*	(cpm/mg) × 10^{-3}	RSpA*
Organs:				
Liver	39.7	2.4	7.1	.55
Spleen	18.2	1.1	3.7	.29
Lung	7.0	.42	.73	.057
Heart	3.0	.18	.53	.042
Blood	4.4	.26	.69	.054
Kidney	10.6	.64	5.8	.45
Liver fractions:				
Nuclear	28.1	—	8.0	—
Mitochondrial	73.3	—	15.1	—
Microsomal	26.3	—	4.7	—
Supernatant	29.0	—	5.2	—

* RSpA = Relative Specific activity = $\frac{\text{Counts/min. in 100 mg organ protein}}{\text{Counts/min. inj./g animal}}$.

Weights of mouse 15 and 16 were 38 and 25 g, inj. counts/min. 63 and 32 × 10^6, respectively.

† Methods used for determination of protein-bound radioactivity in organs and liver fractions have been described(1).

TABLE II. Distribution in Rabbits of Bovine γ-Globulin Labeled by Traces of Diazotized S^{35}-Sulfanilic Acid after Injection into Foot Pads.

Rabbit No.	300		308		310	
Day killed	2		4		6	
Wt (g)	1820		2770		2940	
Inj. protein (mg)	26		28		32	
Inj. cpm × 10^{-6}	25.5		30.6		23.8	
Urine†	56%		83%		90%	
Protein-bound radioactivity:	cpm/mg	RSpA*	cpm/mg	RspA*	cpm/mg	RSpA*
Liver	20.0	1.43	7.6	.83	1.45	.17
Spleen	23.1	1.66	8.2	.89	3.88	.44
Lung	185.0	13.2	34.7	3.8	1.02	.12
Heart	38.1	2.7	12.2	1.3	.34	.039
Bone marrow	112.0	8.0	14.1	1.5	4.4	.50
Kidneys	35.2	2.5	74.0	7.8	1.7	.20
Blood serum	1010	72	60.3	6.6	3.8	.44
Muscle	—	—	1.2	.12	.22	.025

* RSpA = Relative specific activity (see Table I).
† Total urinary excretion from time of inj. to time of death, in % of total inj. activity.

activity measured as described earlier(13). The specific activity of the blood proteins, 2, 3, 4, 5 and 6 days after injection was 133, 98, 75, 10.6 and 4.7 cpm/mg, respectively. The thymus had a specific activity of 1.03 cpm/mg. Less than one per cent of the urinary activity (Table II) was precipitable by trichloroacetic acid. Blood sera of Rabbits 300 and 308, killed 2 and 4 days after injection of the antigen, were free of precipitins, whereas the serum of Rabbit No. 310, killed 6 days after injection, showed a weak but distinct precipitin reaction when mixed with the 10-fold volume of a 0.03% or 0.01% antigen solution. Precipitin formation in this animal is also demonstrated by the steep drop in circulating radioactive protein from 75 cpm/mg on the 4th day to 10.6 cpm/mg on fifth day after injection. Fig. 2 shows that most of the radioactive material in Rabbit No. 300 is concentrated in eosinophilic macrophages of the regional lymph node, whereas the lymphocytes contain little of the radioactivity. No increase in plasma cells was observed in sections of the lymph nodes.

Localization of internally labeled RbSA (S^{35}). Rabbit serum albumin containing S^{35}-amino acids(13) was intravenously injected into mice, and distribution of the internally labeled antigen in the spleen was examined. Autoradiographs of mouse spleen sections showed that some of the radioactivity was ex-

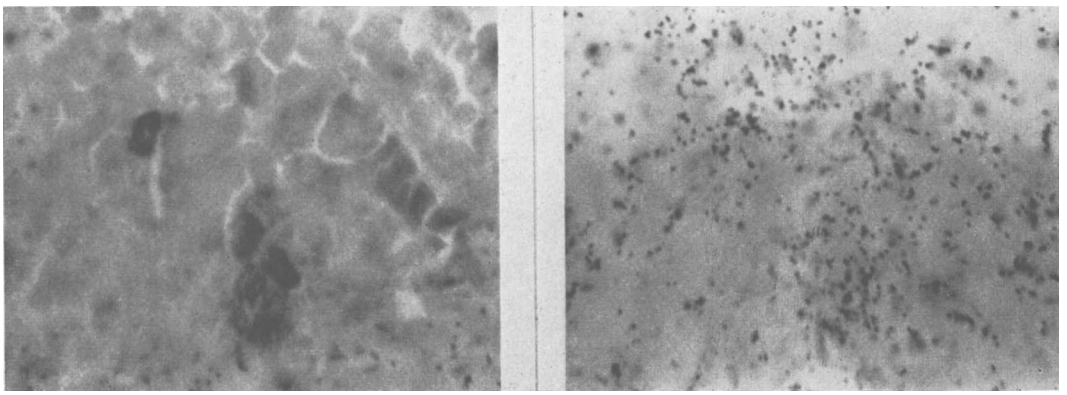

FIG. 2. Popliteal lymph node section of Rabbit 300 which was killed 2 days after inj. of BGG-S^{35} into hind foot pad. Section was stained with hematoxylin-eosin and coated with photographic emulsion. Photomicrographs with focus on cells (left) and on emulsion (right). Most of activity is concentrated in cytoplasm around nuclei of eosinophilic macrophages. Twenty-eight day exposure. × 700.

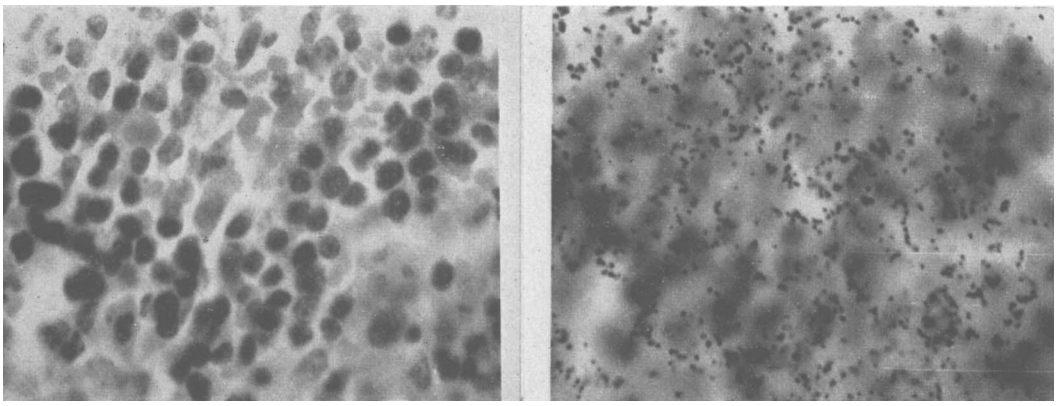

FIG. 3. Spleen section of Mouse 20 which was inj. i.v. with RbSA (S^{35}) and killed after 3 days. Section was stained with Feulgen-Fast green and coated with wet emulsion. Photomicrographs with focus on cells (left) and on photographic emulsion (right). Radioactive material is intracytoplasmic, with no intranuclear activity evident. Seventy-four day exposure. × 700.

tracellular, while tracks of silver granules were found over the cytoplasm of lymphoid cells (Fig. 3). All cell nuclei remained essentially free of radioactive material.

Discussion. Using externally and internally labeled proteins, we had demonstrated earlier that both the external as well as the internal label persist as protein-bound material in tissues of rabbits for many months following intravenous injections of the protein antigens(2,5,13). This was particularly true for tissues of the reticuloendothelial system. However, the method employed in these previous studies did not indicate the cell types in which the antigens were concentrated nor sites of intracellular localization of the antigens. In the present paper, an attempt has been made to answer these questions using autoradiographic studies of animal tissues removed a few hours or days after injection of the labeled protein antigens.

We found that intravenously injected antigens were concentrated in the Kupffer cells of the mouse liver and in the red pulp of the mouse spleen. Antigens injected into the foot pads of rabbits were localized in the eosinophilic macrophages of the popliteal lymph nodes. In all these experiments, the bulk of the radioactive material was found in the cytoplasm or adsorbed to the cell membranes, whereas nuclei remained almost free of radioactivity (Fig. 1, 2 and 3). These findings agree with analogous results obtained in experiments using colored azoproteins(9-11) and with our earlier findings in fractions of homogenized tissues. The antigens used in all our experiments were water-soluble proteins. Previous investigations of liver and spleen homogenates demonstrated, however, that most of the radioactivity resided in the cytoplasmic granules of the liver and spleen, and not in the supernatant fraction which contains the soluble substances (1,2,7). From these observations and from the results of recovery experiments(15), we concluded that the antigen is converted to some insoluble material by combination with antibodies or by nonspecific combination with other cellular constituents such as lipids, nucleic acids or mucopolysaccharides. McMaster and Kruse(16) have drawn similar conclusions, since they were not able to detect reactive antigen in organs of injected animals with direct methods, although indirect methods clearly indicated the presence of antigen. Garvey and Campbell(17) assumed that the low serological activity of tissue-bound antigens was due to degradation of the antigen and presence of antigen fragments which were bound more or less firmly to RNA or other constituents of the cells. Our results are reconcilable with either of these views.

Summary. Analyses of the distribution of isotopically labeled antigens in the animal were combined with autoradiography of the tissues. After intravenous injection of heavily substituted S^{35}-sulfanilazo-bovine γ-globulin into mice, we found protein-bound

S^{35} in the cytoplasm of the Kupffer cells of the liver. Similarly, activity of injected biosynthetically labeled S^{35}-RbSA was concentrated in the cytoplasm of lymphoid cells of the spleen, particularly in the red pulp. After injection of lightly labeled S^{35}-sulfanilazo-bovine γ-globulin into the foot pads of rabbits, the radioactive material was concentrated in the cytoplasm of the eosinophilic macrophages of the popliteal lymph nodes. In all these cell types, the protein-bound S^{35} was either present within the cytoplasm or adsorbed to the cellular membrane. Little intranuclear radioactivity was evident.

1. Haurowitz, F., Crampton, C. F., *J. Immunol.*, 1952, v68, 73.

2. Crampton, C. F., Reller, H. H., Haurowitz, F., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 448.

3. ———, *J. Immunol.*, 1953, v71, 319.

4. Crampton, C. F., Haurowitz, F., *ibid.*, 1952, v69, 457.

5. Friedberg, W., Walter, H., Haurowitz, F., *ibid.*, 1955, v75, 315.

6. Warren, S., Dixon, F. J., *Am. J. Med. Sci.*, 1948, v216, 136.

7. Ingraham, J. S., *J. Infect. Dis.*, 1951, v89, 109, 117.

8. Libby, L., Madison, C. R., *J. Immunol.*, 1947, v55, 15.

9. Sabin, F. R., *J. Exp. Med.*, 1939, v70, 67.

10. Robertson, J. S., *Austral. J. Biol. a. Med. Sci.*, 1952, v30, 59.

11. Latta, H., Gitlin, D., Janeway, Ch. A., *A.M.A. Arch. Path.*, 1951, v51, 260.

12. Gavosto, F., Ficq, A., *Nature*, 1953, v172, 406.

13. Walter, H., Haurowitz, F., Fleischer, S., Lietze, A., Cheng, H. F., Turner, J., Friedberg, W., *J. Biol. Chem.*, 1957, v224, 107.

14. Heidelberger, M., Kendall, F. E., *J. Exp. Med.*, 1935, v62, 697.

15. Hawkins, J. D., Haurowitz, F., *Biochem. J.*, 1959, v72, 5p.

16. McMaster, P. D., Kruse, H., *J. Exp. Med.*, 1951, v94, 323; 1954, v100, 341.

17. Garvey, J., Campbell, D. H., *J. Immunol.*, 1956, v76, 36.

Received October 31, 1960. P.S.E.B.M., 1961, v106.

Necrotizing Effects of *Staphylococcus aureus* Extract on Mouse Sarcoma 180.* (26249)

E. MIHICH AND E. NETER

Depts. of Experimental Therapeutics and Medicine C, Roswell Park Memorial Institute and Statler Research Laboratories, Children's Hospital, Buffalo, N. Y.

In susceptible hosts endotoxins of gram-negative bacteria cause a variety of biological effects, such as fever, local and systemic Shwartzman reactions, necrosis of experimental tumors, as well as alterations of nonspecific resistance, antibody formation, and dermal reactivity to epinephrine(1-5). The lipopolysaccharide moiety of the bacterial endotoxins seems to be responsible for the majority or all of these biological effects. In contrast to the extensive data reported on endotoxins from gram-negative bacteria, only limited information is available on similar biological effects by products from gram-positive microorganisms(3,6,7).

Recently it was shown that a trichloracetic acid extract from *Staphylococcus aureus* strain D alters dermal reactivity of rabbits to epinephrine(8). This effect was comparable to that observed with endotoxins of gram-negative bacteria(5) and the lipid A component of *E. coli* endotoxin(9). In view of this observation, a study of the possible tumor-necrotizing effect of the same staphylococcal extract was undertaken. As reported herein, this extract causes extensive hemorrhagic necrosis of mouse sarcoma 180.

Materials and methods. Two strains (D and 5550) of coagulase positive *Staphylococcus aureus* were used for preparation of extracts, carried out as previously described (8). The extracts were prepared at Difco Laboratories, Detroit, and obtained through

* This investigation was supported in part by research grants from Nat. Cancer Inst. and Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.