munization. Individual titers were higher for AL/N mice than for the other strains, perhaps due to their non-ascitic state at time of immunization. Determination of precipitating antibody nitrogen in the ascitic fluid of ovalbumin-immunized mice indicated that early levels of antibody nitrogen as high as 105 γ/ml did not produce a positive PCA reaction sooner than 11 days post-immunization. The need for further studies on the characterization of antibodies in time of their appearance is indicated.

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Received April 4, 1962. P.S.E.B.M., 1962, v110.

Serologic Typing of *Listeria monocytogenes* by Gel Diffusion Using Thermostable Antigens. (27686)

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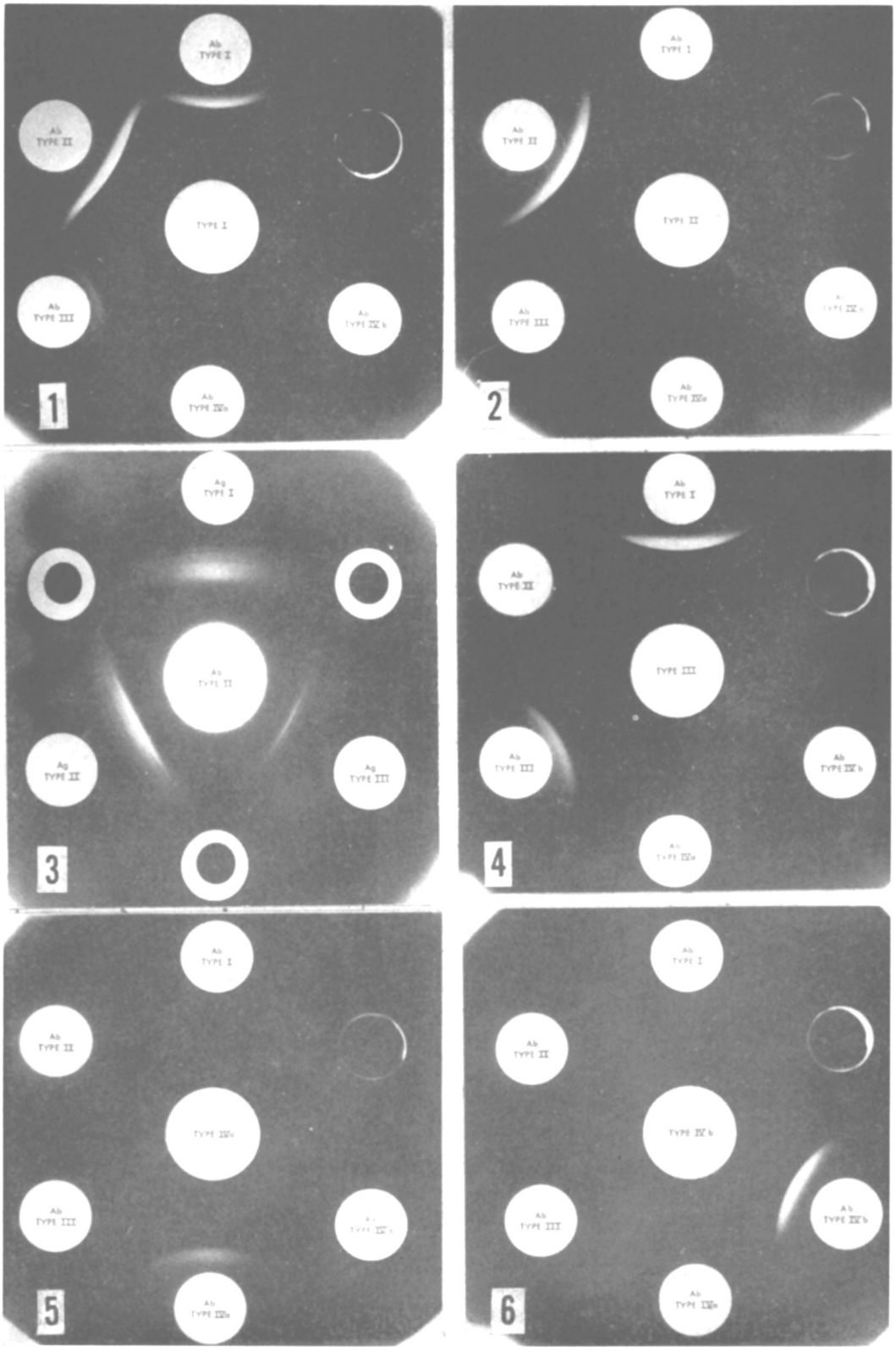
Listeria monocytogenes presents a complex antigenic structure composed of both flagellar and somatic antigens. Early investigators (1-7) classified the organisms serologically into 2 types based on animal origin (*i.e.,* rodent or ruminant). Paterson(8,9) demonstrated the presence of somatic O-antigens and flagellar H-antigens, and on the basis of their somatic and flagellar response distinguished 4 serologic types (*i.e.,* I, II, III, and IV), the type having no relation to its origin. Seeliger(10) confirmed the work of Paterson, further distinguishing 2 subtypes in type IV strains, designating them IVa and IVb.

The use of the typing scheme based on O- and H-antigenic factors requires specially absorbed sera. Too often the results can be erroneous if not accomplished by trained workers. Seeliger(10) has pointed out that precipitin and gel diffusion technics, although requiring high-titer serum, present fewer cross reactions than are observed in agglutination tests if extracts of the organisms are used. Recently Smith and Metzger(11) demonstrated capsule production in *L. monocytogenes.* This investigation was made to determine the antigenic components of *L. monocytogenes* by gel diffusion, taking into consideration the thermostable O-antigens as well as those possibly produced by an enhanced capsule.

Materials and methods. Immune sera. Strains ATCC 4428(I) and Seeliger's strains 5348(II), 5105(III), 5214(IVa), and 1071/53(IVb) were subcultured twice on trypticase soy agar containing 10% rabbit serum and 5% glucose to enhance capsule production (SGTSA)(11). Ten flasks, containing 100 ml of trypticase soy broth plus 10% serum and 5% glucose (SGTSB) were then inoculated from the SGTSA and incubated for 18 hours at 37°C in a rotator incubator. The cultures were centrifuged in the cold at 2,000 rpm, and the supernate discarded. The sediments of each type were pooled and sus-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pended in 25 ml of phosphate-buffered saline, pH 7.2. The suspensions were heated in flowing steam for 1 hour. The steamed sediments were injected intravenously into pairs of mature albino rabbits on alternate days, at dosages of 0.5, 1.0, 1.0, 1.5, 2.0, and 2.0 ml. The animals were bled 7 days after the last injection.

Acid extract. Test organisms were passed once on SGTSA and inoculated into 2 flasks of SGTSB containing 100 ml of medium each. After incubation for 18 hours at 37°C on the rotator incubator, cultures were centrifuged at 4°C, 2,000 rpm, and supernatants discarded. The pooled sediment was extracted essentially according to Lancefield(12) as follows: 5 ml 1/20 N HCl in saline was added and the mixture placed in boiling water for 10 minutes, rapidly cooled, and the supernatant collected by centrifugation. The supernate was then neutralized with NaOH (pH 7.0) and recentrifuged. This extract served as a crude antigen for gel diffusion and precipitin studies.

Precipitin and gel diffusion tests. Precipitin reactions were obtained in standard precipitin tubes containing equal volumes of acid extract and sera. The test materials were incubated at 37°C for 1 hour and then at room temperature for an equal time before reading. Gel diffusions were made with 0.85% purified agar cut with a Shamden cutter. The crude acid extract was then placed in the center well, the specific immune sera in the outer wells, and these materials placed in a moist chamber for 48 hours at 25°C.

Sixty strains of *Listeria monocytogenes* of human and animal origin were used. The collection consisted of standard stock strains as well as recent isolates. They were obtained from cases contributed for consultation, from the American Type Culture Collection, or through the generosity of Dr. M. L. Gray and Dr. H. P. R. Seeliger.

Results. With the precipitin test it was not possible to separate all of the strains tested into specific types. Types II, IVa, and IVb gave specific reactions, whereas types I and III could not be differentiated. With the gel diffusion test it was possible to separate all strains into 5 serologic types. In all instances the types as determined by gel diffusion correspond with the strain types found by conventional methods(10).

Therefore the nomenclature used for somatic "O" and flagellar "H" antigen typing is retained for the gel diffusion method to prevent confusion in the literature (*i.e.*, types I, II, III, IVa, and IVb). Acid extracts of type I organisms give dense bands with type I and type II antiserum and a very light band against type III antiserum, presenting a distinctive pattern (Fig. 1). Fig. 2 shows a monospecific response of type II acid extracts to type II antiserum. Type II antiserum prepared in some rabbits was not monospecific. The acid extracts of types I and III, diluted 1:10 with saline, react, producing multiple bands (Fig. 3). Type III acid extracts (Fig. 4), share a common antigen with type I but produce a typical pattern for type III organisms. Acid extracts of IVa and IVb give a monospecific response (Fig. 5 and 6).

Discussion. As evidenced by gel diffusion, *L. monocytogenes* contains a complex system of thermostable antigens. Not all serotypes could be differentiated by the precipitin test, but gel diffusion provided specific serotyping that separated the strains tested into groups similar to those obtained by using somatic "O" and flagellar "H" antigens. Using Paterson's(9) antigenic scheme, types I and II can be differentiated only on a flagellar response, as they share the same somatic anti-

FIG. 1. Acid extract of type I organism, showing bands reacting with type I and type II antisera.

FIG. 2. Type II acid extract, showing monospecific response to type II antiserum.

FIG. 3. Type II antiserum in the center well shows reaction with its specific acid extract (II) as well as producing multiple bands with types I and III acid extracts.

FIG. 4. Acid extract of type III reacts with its homologous antiserum, sharing a common antigen with type I antiserum.

FIG. 5. Type IVa acid extract, exhibiting a monospecific response.

FIG. 6. Type IVb acid extract, exhibiting a monospecific response.

gens; by gel diffusion, however, the patterns can be readily differentiated. There also appears to be a close relationship between types I and III, since the acid extract of type III reacts by gel diffusion not only with its homologous antiserum, but also with type I antiserum. The acid extract of type II organisms reacts only with its homologous antiserum. Seeliger(10) indicates a close relationship between types IVa and IVb, but no cross reactions are apparent by gel diffusion in our studies.

Robbins and Griffin(13) found that factors I, II, IV and V were the thermostable O-antigens; with these factors, however, the organisms could not be serotyped. It is felt that other factors are present in the antigenic mosaic. With the enhancement of the capsule, more factors appear to be made available for immunization. The antiserum produced with this antigen, when tested against acid extracts of capsular-enhanced organisms, resolves the classification of *L. monocytogenes* into 5 types. These serotypes coincide directly with the serotypes described by Paterson(9) and Seeliger(10) on the basis of the response of both the somatic "O" and flagellar "H" antigens.

In preparation of the immune serum care must be taken to determine its specificity. As pointed out by Robbins and Griffin(14), there is a variability in the response to individual antigenic components. This has been our finding, especially in production of serum monospecific to type II organisms. On one occasion the same antigen injected into a pair of albino rabbits gave a monospecific response in one animal, while in the other it provided a serum of a broader spectrum. Caution must also be used in selection of the strains used to prepare the immune sera. Fifteen strains in addition to our 60 were used to prepare typing antisera. Ten of these strains were recent isolates, and 5 were stock strains, carried on agar slants. The recent isolates gave correlative results, but the stock strains did not possess all of their antigens; duplicate lyophilized cultures of the stock strains, kept at 4°C, did, however, contain all antigenic factors.

The typing method presented is of a simplified nature so as to be usable by facilities other than specialized typing centers. Tedious absorption technics are not involved, nor are the problems of spontaneous agglutination or clumping encountered as with absorbed-factor sera.

The reinforced medium used not only enhances capsule production but also provides an enriched medium for increased growth and furnishes an adequate amount of antigenic material.

The serologic findings on the 60 strains investigated again confirm the findings of Paterson(9) and Seeliger(10) that the serotype has no relation to host species nor is it associated with a particular geographic origin.

Summary. A procedure is described by which acid extracts of capsular-enhanced *L. monocytogenes* are serotyped by gel diffusion. Sixty strains were placed in 5 serotypes that coincide with the serotypes obtained by conventional methods using somatic "O" and flagellar "H" factor sera. The procedure has been simplified so that it can be efficiently utilized by facilities other than specialized typing centers.

We are indebted to Dr. Milton M. Gray for confirming serotypes of cultures and advice during this investigation.

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Received May 29, 1962. P.S.E.B.M., 1962, v110.