2. Hochstein, P., Ernster, L., Biochem. Biophys. Res. Comm., 1963, v12, 388.
3. Hochstein, P., Nordenbrand, K., Emster, L., Biochem. Biophys. Res. Comm., 1964, v14, 323.
4. Deutsch, H. F., Kline, B. E., Rusch, H. P., J. Biol. Chem., 1941, v141, 529.
5. Radsma, W., Van Groningen, H. E. M., Acta Physiol. Pharmacol., Neerlandica, 1959, v8, 364.
6. Ottolenghi, A., Bronheim, F., Wilbur, K. M., Arch. Biochem. Biophys., 1955, v56, 157.
7. Tappel, A. L., Zalkin, H., Arch. Biochem. Biophys., 1959, v80, 333.
8. Hoffsten, P. E., Hunter, F. E., Jr., Gebicki, J. M., Weinstein, J., Biochem. Biophys. Res. Comm., 1962, v7, 276.
9. Hunter, F. E., Jr., Gebicki, J. M., Hoffsten, P. E., Weinstein, J., Scott, A., J. Biol. Chem., 1963, v238, 828.
10. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Guerra, F., Weinstein, J., Schneider, A., Schuty, B., Fink, J., Ford, L., Smith, E., J. Biol. Chem., 1964, v239, 604, 614, 622.
11. Tappel, A. L., Brown, W. D., Zalkin, H., Maier, V. P., J. Amer. Oil Chem. Soc., 1961, v38, 5.
12. Donman, S. K., J. Biol. Chem., 1950, v182, 415.
13. Shuster, C. W., Proc. Soc. Exp. Biol. and Med., 1955, v90, 423.
14. Wolfson, N., Wilbur, K. M., Bernheim, F., Exp. Cell Res., 1956, v10, 556.
15. Thiele, E. H., Huff, J. W., Arch. Biochem. Biophys., 1960, v88, 203, 208.
16. Bernheim, F., Ottolenghi, A., Wilbur, K. M., Radiation Research, 1956, v4, 132.
17. Kohn, H. I., Liversedge, M. J., Pharmacol. Exp. Therap., 1944, v82, 292.

Received November 2, 1964. P.S.E.B.M., 1965, v118.

An Electrophoretic Study of the Neurotoxin(s) of Nine Strains of *Clostridium tetani.* (29941)

M. CAROLYN HARDEGREE* AND LEWIS W. WANNAMAKER†
Department of Pediatrics, University of Minnesota Medical School, Minneapolis

The observations of investigators working with *Cl. tetani* have frequently suggested the existence of biological differences in the toxins produced by different strains of this organism(1-6).

After Pillemer *et al*(7) crystallized the neurotoxin of the Harvard strain, little attention was given to the possibility that more than one neurotoxin might be produced until Largier(8) reexamined the concept of qualitative differences among the toxins produced by different strains of *Cl. tetani.* In a comparison of 2 strains, he found no differences in physical or immunological properties.

The present study was designed to examine a larger number of strains from a variety of sources for possible qualitative differences. Starch zone electrophoresis was used to examine the electrophoretic mobility of the neurotoxins of 9 strains of *Cl. tetani.*

Materials and methods. The 9 strains of *Cl. tetani* used were: Massachusetts C_2 (Mass. State Biological Laboratories); Harvard (Dept. of Public Health, State of Michigan); F/3 (Connaught Laboratories, Toronto, Canada); Tulloch II #127 (NIH); Wellcome G.S. #761 (South African Inst. of Medical Research); 301; 43/50-51 (Statens Seruminstitut, Copenhagen, Denmark); CN 1339 (T61B) (Wellcome Research Laboratory, Beckenham, England); Pease TP (Lederle Laboratories).

The Massachusetts strain was grown in the medium described by Latham, Bent and Levine(9). For all other strains the medium developed by Mueller and Miller was used(10). To obtain a filtrate containing only those macromolecules produced by *Cl. tetani* the cellophane bag technique described by Polson and Sterne(11) was employed. A bag of Visking dialysis tubing containing 100 ml of saline was attached to the glass apparatus described by Koch and Kaplan

* Trainee, U.S.P.H.S. Training Grant in Research Pediatrics, T1 HD 53. Present address: Division of Biologics Standards, Nat. Inst. Health, Bethesda, Md.

† Career Investigator, American Heart Assn., Professor of Pediatrics.

(12) and immersed in 1000 ml of the selected medium. After the unit had been autoclaved, an inoculum of 10 ml of a 24-48-hour culture in thioglycollate medium was added to the saline-filled bag. The toxin containing supernate was harvested by centrifugation after a 7- to 11-day incubation period at 37°C. Albumin 0.1% (W/V), was added to prevent spontaneous toxoiding of the toxin and the supernate was filtered through a Coors porcelain filter (#3). The preparation was concentrated by ultrafiltration under vacuum in a collodion bag (50 cm Hg). The concentrated preparation was dialyzed against the phosphate buffer to be used in electrophoresis. All processing was in a cold room (4°C).

Zone electrophoresis with potato starch (Mallinckrodt) as a supporting medium was done by the method of Kunkel and Slater (13). In all experiments phosphate buffer, pH 6.2, $\mu = 0.1$, was used. The starch block was a 39 × 6.6 × 0.6 cm pad contained in a plastic tray. One-tenth ml of the concentrate was applied to the starch pad 16 cm from the cathode. A direct current with a potential difference of 400 volts was applied for 16 hours. After electrophoresis the block was sectioned into 1 cm segments and elutions made with 2 ml of the phosphate buffer.

Protein determinations were performed by a modification of the Kunkel and Tiselius adaptation of the Folin-Ciocalteau phenol reaction(14).

Inbred Swiss strain mice, 6 to 8 weeks old, were used for the detection of neurotoxin. A 0.2 ml aliquot of the eluate of the starch segment was injected subcutaneously into the right hind leg. The signs noted were recorded every 24 hours for a period of 10 days. In the majority of these experiments every third segment of the block was examined. These experiments were repeated at least once for each strain.

Results. The symptoms of tetanus in the mouse are distinctive enough for objective recording. After inoculation of toxin into the right hind leg, a scoliosis with the major curvature to the left develops, followed by spastic paralysis of the injected limb. The curvature disappears as the left side becomes paralyzed. Later a dorsal thoracic hump is evident and the animals are in respiratory distress. At this point the mouse may not be able to right himself and he convulses upon stimulation. Death rapidly follows this severe state. In a highly toxic preparation the initial stages may not be observed and death will occur within the first 24 hours after injection.

In the present studies the degree of toxicity of the eluates was judged by the rapidity of appearance of the various signs of tetanus intoxication. Attempts to determine the location of the peak or peaks of toxicity by serial dilution of the eluates in saline or albumin were unsuccessful, apparently due to the instability of the toxin. An isolated death occurring 3 to 4 days after injection of the eluate without preceding symptoms was not considered to be a result of a toxic fraction.

The distributions of toxicity on zone electrophoresis for the nine strains studied are illustrated in Fig. 1. There was always marked toxicity near the point of application but this was apparently not due to prevention of migration by adherence of the toxin to the starch as migration readily occurred in a water gradient. For all 9 strains the area of maximum toxicity always appeared to be within a few centimeters of the point of application, although some variation in the extent of the area showing neurotoxicity was found from one block to the next, even with the same strain. This variation in the overall distribution of the neurotoxic activity from strain to strain and within a strain may be in part the result of application of unequal concentrations of the toxic filtrate to the block.

Some suggestion of a second peak of neurotoxicity was found in the patterns of 2 of the strains (301 and Harvard) examined, but these were not clearly resolved nor always reproducible in repeat experiments. The late death without preceding symptoms in relationship to fraction #13 of the Wellcome GS 761 is probably not due to neurotoxicity and therefore cannot be taken as evidence of a secondary peak in the pattern of this strain (Fig. 1).

The location of maximal toxicity did not correspond with a definite or consistent peak in the protein pattern in any of the experi-

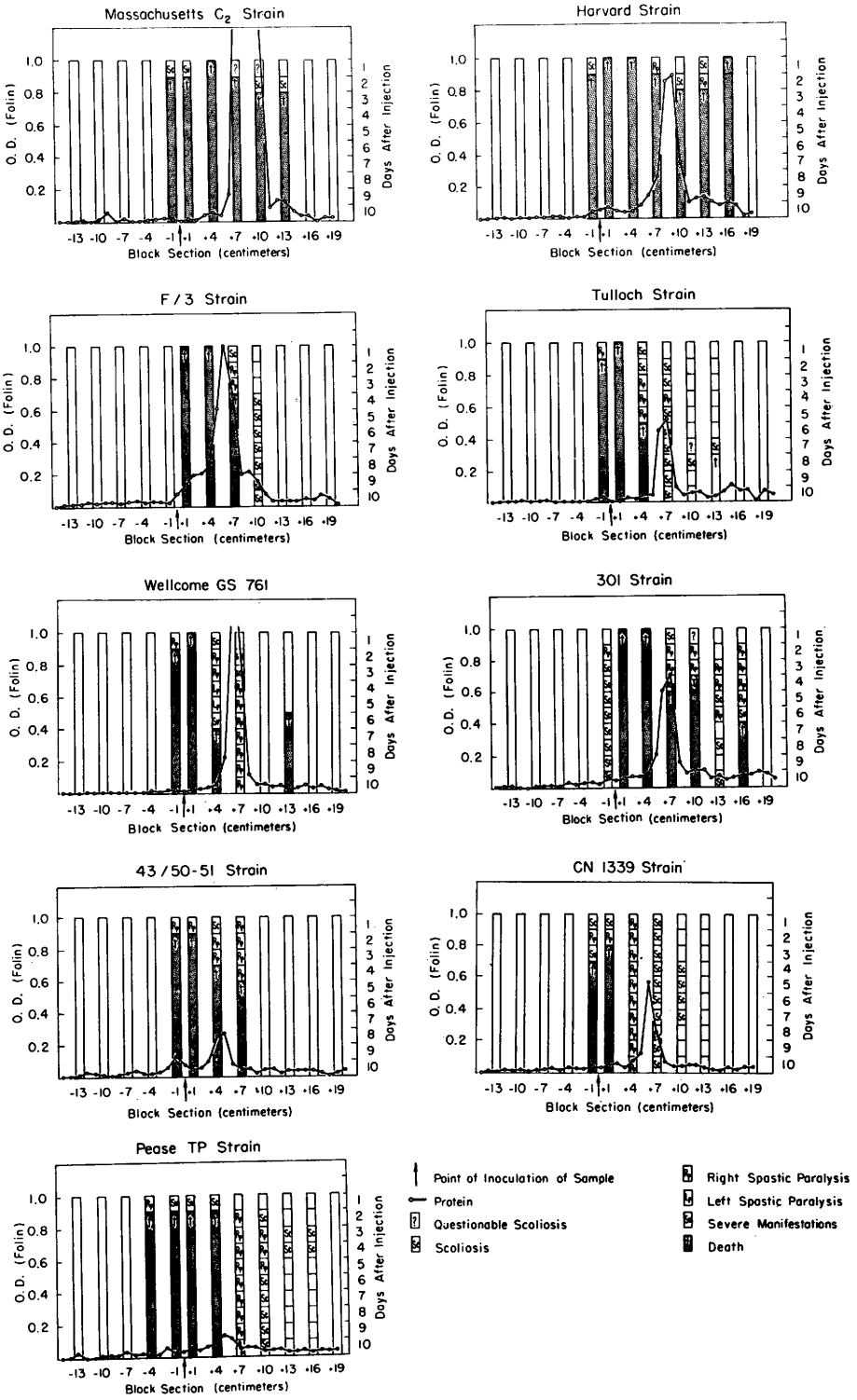

FIG. 1. Starch zone electrophoretic patterns from concentrated supernates of 9 strains of *C. tetani*.

ments (Fig. 1). Indeed, in areas of maximum toxicity there was often little protein. This probably reflects the comparative insensitivity of methods of protein determination as compared with biological methods for detecting toxins and indicates a high toxicity per unit of protein. The large protein peak apparent in all experiments represents albumin, which varies in amount according to the initial volume of fluid within the culture bag prior to concentration. In general, the albumin migrates somewhat more rapidly toward the anode than does the toxin. In some experiments the toxin appears in a position corresponding to a shoulder on the albumin peak, but there is no clear indication of migration or complexing of toxin with albumin. It is apparent from the animal studies that toxin does exist in these areas of increased protein concentration, but usually in lesser quantity.

Discussion. Even after the successful World War II immunization program, it was suggested by Miles(15) that a large number of toxins from different international sources should be selected for use as challenge toxins in testing toxoids and antitoxins. However, it would appear that the need for such a complex testing program would depend on finding that the various toxins are not identical in their antigenic and toxic effects. Zone electrophoresis provides one means of comparison and possible separation of these various tetanospasmins.

Maximal toxicity for the mouse, as judged by the rapidity of development of symptoms and death, shows a similar electrophoretic migration of the neurotoxins for each of the 9 strains of *Cl. tetani* examined. The variations observed appear to be no more than those which may be found in comparing electrophoretic patterns on the same strain done at different times. In those patterns where the albumin marker migrated somewhat less rapidly toward the anode, the area of maximal toxicity was also located less close to the anode. Since electrophoresis does not discriminate between these strains in regard to the location of maximal neurotoxicity for the mouse, these findings are consistent with the view that a predominant toxin is shared by all of the strains examined and are in agreement with those of Largier(8), who compared 2 of the 9 strains included here. However, the rather wide area of total distribution of toxicity, especially for certain strains, and the occasional evidence of a secondary peak of toxicity with some strains, suggests the possible existence of secondary toxins which have not been fully resolved by the techniques used here. Evidence for a secondary toxin, which has a peripheral rather than a central effect, has been recently published (21).

A review of the experimental literature and reported experience with immunization and prophylaxis would suggest that if strains of *Cl. tetani* produce more than one neurotoxin, these toxins, or at least a major toxin, are shared by different strains although quantitative differences in the amounts produced by different strains may exist. Tulloch(1) found that one antitoxin neutralized toxin of all types, although his studies indicated that monotypical antitoxic serum protected more adequately against infection with the homologous strain.

The use of other indicator species in addition to the mouse might have revealed a different pattern of migration of the toxic moiety or a second tetanospasmin as suggested by Llewellyn Smith(2). The existence of species differences in susceptibility to tetanus has long been documented(2,3), but the basis for these differences has not been adequately explained. Rowson(16) has postulated that species variations in susceptibility to the same toxin may be dependent upon a difference in capacity of the brain tissue from different species to combine with low concentrations of toxin. However, Van Heyningen(17) has found that tetanus toxin is readily fixed by grey matter from the chicken, a species which is extremely resistant to tetanus toxin. Species variations in susceptibility to preparations of toxin from different strains(2,4,5) are more difficult to explain but may be related to postulated qualitative differences among neurotoxins.

Based upon the observation that serum potentiates the toxin produced by some strains more than that produced by others, Traub *et al*(6) suggested a qualitative difference in toxins. It is possible that some of

the other antigens known to be present in *Cl. tetani* filtrates(18) are also stabilized by the serum and contribute to the pathogenicity of the tetanospasm or possess similar neurotoxic properties. Differences in the antigens produced by the various strains of *Cl. tetani* have been demonstrated by Illytovich(19) by the use of gel diffusion techniques, but the identity of the antigens which vary from strain to strain has not been established.

The findings of Turpin and Raynaud(20) also suggest either qualitative differences among tetanus toxins or the existence of toxin aggregates with different physical properties. Using a different strain than the one used by Pillemer, they obtained a toxin preparation with an ultracentrifugal sedimentation constant which was approximately twice that obtained by Pillemer. Depolymerization was demonstrated under the influence of cysteine but it is not stated whether the depolymerized component retained its toxicity. The existence of toxin aggregates which may be present under certain conditions is a possible explanation for the suggestive evidence of secondary peaks of toxicity in some of the electrophoretic patterns reported here.

Summary. Examination of the toxic filtrates from 9 strains of *Cl. tetani* by starch zone electrophoresis revealed a similar migration of the area of maximal neurotoxicity for the mouse. These observations are consistent with the view that a predominant neurotoxin is shared by all strains of this organism. The findings do not exclude the possibility of the existence of additional, perhaps minor, neurotoxins which may differ from strain to strain and which may account for the discrepancies reported in the behavior of toxic filtrates from different sources.

This research was supported by a Mead Johnson Grant for Pediatric Research.

1. Tulloch, W. J., J. Hyg., 1919, v18, 103.
2. Llwellyn Smith, M., Bull. Health Org., League of Nations, 1943, v10, 104.
3. Ipsen, J., Bull. Health Org., League of Nations, 1940, v9, 452.
4. Friedemann, U., Hollander, A., J. Immunol., 1943, v47, 23.
5. Friedemann, U., Hollander, A. Traub, F. B., J. Immunol., 1946, v52, 247.
6. Traub, F. B., Hollander, A., and Friedemann, U., J. Bact., 1946, v52, 169.
7. Pillemer, L., Wittler, R. G., Burrell, J. I., Grossberg, D. B., J. Exp. Med., 1948, v88, 205.
8. Largier, J. F., J. Immunol., 1956, v76, 393.
9. Latham, W. C., Bent, D. F., Levine, L., Appl. Microbiol., 1962, v10, 146.
10. Mueller, J. H., Miller, P. A., J. Bact., 1954, v67, 271.
11. Polson, A., Sterne, M., Nature, 1946, v158, 238.
12. Koch, W., Kaplan, D., J. Immunol., 1953, v70, 1.
13. Kunkel, H. G., Slater, R. J., Proc. Soc. Exp. Biol. and Med., 1952, v80, 42.
14. Kunkel, H. C., Tiselius, A., J. Gen. Physiol., 1952, v35, 89.
15. Miles, A. A., Bull. World Health Org., 1949, v2, 59.
16. Rowson, K. E. K., J. Gen. Microbiol., 1961, v25, 315.
17. Van Heyningen, W. E., J. Gen. Microbiol., 1959, v20, 291.
18. Bjorklund, B., Berengo, A., Acta Path. Microbiol. Scand., 1954, v34, 79.
19. Illytovich, A. H. U., J. Microbiol. Epidemiol. Immunobiol., 1961, v32, 482.
20. Turpin, A. Raynaud, M., Ann. Institute Pasteur, 1959, v97, 718.
21. Feigen, G. A., Peterson, N. S., Hofmann, W. W., Genther, G. H., Van Heyningen, W. E., J. Gen. Microbiol., 1963, v33, 489.

Received November 4, 1964. P.S.E.B.M., 1965, v118.