signs points to greater involvement of the left ventricle. However, neither gross nor microscopic pathology was evident in hearts of sacrificed animals. Early appearance and late disappearance of signs did not parallel changes in body weight.

Summary. Electrocardiographic changes in cats with acute thiamine deficiency fall into two categories: (1) disorders of rate, regularity, and impulse formation, which yield promptly to thiamine treatment; (2) changes in the ventricular complex, which respond more slowly to treatment.

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An Electrophoretic Study of Tetanus Toxoid Prepared on Mueller's Peptone-Free Medium.

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Recent advances in the study of the complexity of tetanus toxin have been summarized by Smith[1] and Petrie.[2] Smith compared the toxicity of five different tetanus toxins in mice, guinea pigs, and rabbits. She showed that the ratio of the LD_{50} for the rabbit and mouse and the ratio for the rabbit and guinea pig (weight for weight) may show extraordinary and significant variations, whereas the ratio of the lethal dose for the mouse and guinea pig is more or less constant. Her findings indicate that tetanus toxin contains two or more toxic factors and that the mouse and the guinea pig are relatively more sensitive to one and that the rabbit is relatively more sensitive to the other factor. Petrie studied the variable interactions of tetanus toxins and tetanus antitoxins with the object of throwing light upon the cause of the discrepancies that are met when samples of tetanus antitoxin are assayed in different laboratories. His results indicate that crude tetanus toxins are not homogeneous solutions of toxin and toxoid molecules. Whether they are used as antigens for the hyperimmunization of horses or as test-toxins, crude tetanus toxins are to be regarded as mixtures in varying proportions of the "primary" toxin or toxoid molecule and of an antigenic variant of this which is formed during the later stages of the culture growth; preparations of tetanus serum likewise consist of mixtures of varying proportions of the corresponding antitoxins. Differences in mutual neutralizing affinities of these components in a reacting system account for the discrepancies in the titer of tetanus sera when different test-toxins are used for the assays.

The crude tetanus toxins referred to in these papers were prepared from culture media containing Witte, Riedel, or Difco peptone and beef or horse-meat infusion. The tetanus toxoid studied in the present paper was derived from tetanus toxin, prepared on a medium containing only low molecular nutrient materials. The toxoid could be purified and concentrated without chemical treatment.

Materials and Methods. The tetanus toxin was produced on a peptone-free medium according to the method of Mueller and coworkers.[3] It contained approximately 20,000 guinea pig MLD per cc and had an L^+ of 0.04 cc. The conversion of the hydrolysate tetanus toxin to toxoid was also carried out according to Mueller's procedure.[4] The antigenicity was appraised by injecting 0.4 cc of the toxoid subcutaneously into each of 20 mice. At the end of 10 days, the mice were

[1] Smith, M. L., *Bull. of the Health Organization*, 1942-3, **10**, 104.

[2] Petrie, G. F., *Bull. of the Health Organization*, 1942-3, **10**, 113.

[3] Mueller, J. H., Schoenbach, E. B., Jezukawicz, J. J., and Miller, P. A., *J. Clin. Invest.*, 1943, **22**, 315.

[4] Schoenbach, E. B., Jezukawicz, J. J., and Muller, J. H., *J. Clin. Invest.*, 1943, **22**, 319.

challenged with 20 MLD of tetanus toxin; 70% of the animals survived.

The toxoid was concentrated in the cold room by ultrafiltration through an 8% nitrocellulose membrane formed on a 450 cc Coors porous filter balloon flask. The concentration was 100-fold, since 40 liters of toxoid were reduced to 400 cc. The concentrated toxoid was washed twice with two 400 cc portions of distilled water. After the second washing, the filtrate was almost colorless and still gave a positive test for chlorides. The yellow concentrated solution (400 cc) was divided into two 200 cc portions; one was used for various tests and the other was divided into four 50 cc portions and dried by the lyophile technic.

The undried portion was tested for Lf titer, antigenicity, total nitrogen, and phosphotungstic acid precipitable nitrogen. The concentrated toxoid had an Lf titer of 165 per cc compared to an Lf titer of 1.5 to 2 of the unconcentrated toxoid, indicating that essentially all of the LF units had been concentrated by ultrafiltration. The antigenicity was appraised with the fluid concentrated toxoid and with a portion of the same toxoid converted into alum-precipitated material. The fluid toxoid was diluted with 6 volumes of physiological saline solution and 0.4 cc amounts of the diluted material were injected subcutaneously into each of 20 mice. At the end of 10 days, the mice were challenged with 20 MLD of tetanus toxin; all the animals survived. The alum-precipitated material was injected into 10 guinea pigs, in two 0.05 cc doses spaced three weeks apart. The animals were bled four weeks after the last injection; the blood serum was pooled and tested for antitoxin content. The serum contained 4.0 units of antitoxin per cc. The results of the antigenicity tests on the fluid and alum-precipitated material indicated that the antigenicity of the material not only had not been impaired by the method used for concentrating it but had been remarkably and significantly increased.

The concentrated toxoid contained 0.50 mg of nitrogen per cc of which 74% was precipitable by phosphotungstic acid. This might possibly indicate that the concentrated toxoid had not been completely washed free of low-molecular nitrogen-containing substances. To four test tubes were added 1.0 cc of sodium acetate-acetic acid buffer, ionic strength 0.1, at pH 3.6, 4.0, 4.5, and 4.9 respectively. To each tube was then added 1.5 cc of the concentrated toxoid and 0.5 cc of distilled water. Each tube became opalescent. The tubes were placed in the cold room at 5°C overnight. In the morning a precipitate had settled out in each tube. The tubes were centrifuged; the clear supernatant was saved and assayed for total nitrogen. The total nitrogen in the precipitate was calculated. The results were as follows:

pH	% of original N in precipitate
3.6	12
4.0	24
4.5	24
4.9	12

The precipitate in the pH 4.0 tube was dissolved in Na_2HPO_4-NaH_2PO_4 buffer, ionic strength 0.1, pH 7.0, and tested for Lf units. Only 60% of the original Lf units in the tube were recovered in the precipitate formed at pH 4.0. This might indicate that not all of the substance responsible for flocculation is precipitable at pH 4.0 or that two different substances with different isoelectric points are involved.

Analysis of the Tetanus Toxoid Concentrate by Electrophoresis. The material in one of the bottles containing the equivalent of 50 cc of the concentrate in dry form was reconstituted to the liquid state by the addition of 11 cc of distilled water. The resulting solution, which represented 5 liters of the original unconcentrated toxoid, had an intense yellow color.

Dr. Robert C. Warner, United States Department of Agriculture, Eastern Regional Laboratory, Philadelphia, Pa., kindly consented to do the electrophoretic experiment. Doctor Warner reported that the electrophoretic pattern shows three components. The fastest (marked A on the tracing) is present in very small amount and the other two (B and C) are present in roughly equal quantities. The lower patterns on the tracing were obtained after electrophoresis for 3600 seconds at a field strength of 4.50 volts per cm. The upper

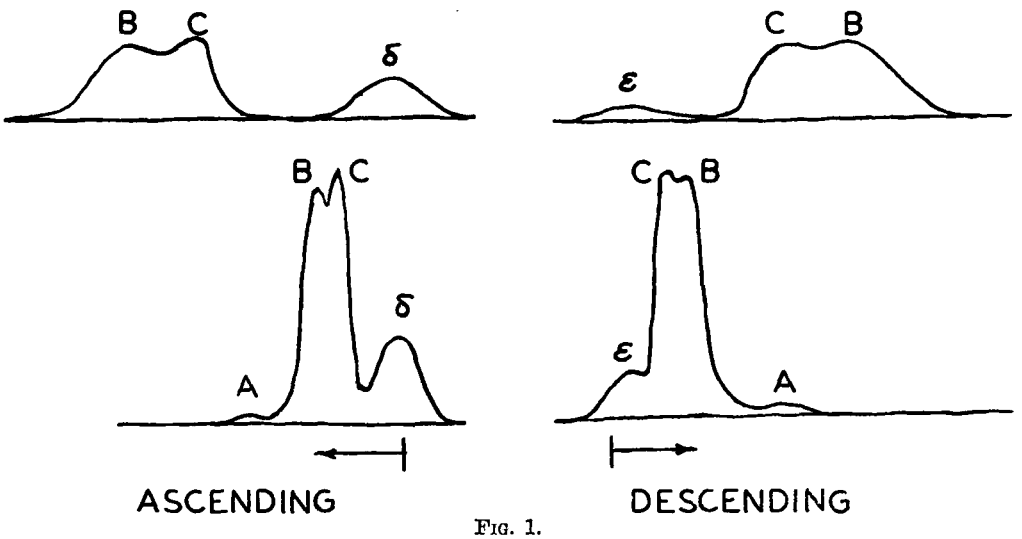

FIG. 1.
The position of the initial boundaries is shown on the tracings by the back end of the arrows at the bottom of each tracing.

patterns were obtained after an additional period of 11,700 seconds at 3.04 volts per cm. The mobility found for component B was 6.5×10^{-5} cm^2 per volt per second and for component C was 4.7×10^{-5} cm^2 per volt per second in a veronal buffer at a pH of 7.79 and ionic strength of 0.1. The only abnormality noticed was a tendency for the boundaries on the ascending side to be somewhat unstable in that the schlieren bands were curved or irregular at their edges. The position of the initial boundary, the direction of migration and the δ and ϵ boundaries are indicated on the tracing.

Doctor Warner found that the yellow color of the solution interfered with the photography to such an extent that it was not possible to obtain suitable prints from the negatives. Doctor Warner made tracings from the negatives which are reproduced in Fig. 1. Incidentally, the yellow pigment present in the solution migrated with component B in both the ascending and descending channels.

Disregarding component A which is present in very small amount, it would appear that in tetanus toxoid prepared on peptone-free medium and concentrated by the method described, are two major components present in about equal amounts. The yellow pigment present in the solution migrates with only one of the two components.

The question naturally arose, are the two components B and C two different molecular forms or factors of the toxoid as postulated by Smith and by Petrie or is one of these the toxoid (protein) and the other a bacterial protein derived from the autolysis of the bacterial cells during the culture growth. It was hoped that when maximum separation of the boundaries had been effected, the contents of the cell compartments could be removed for the determination of antigenicity and serological activity. Unfortunately, Doctor Warner reported that peaks B and C were not sufficiently separated to make it practicable to attempt the isolation of samples of these components from the electrophoresis cell.

Since the direct method was not available to us, we attempted to answer the question by an indirect approach. Tetanus antitoxic globulin, purified and concentrated by an enzyme digest method, was treated with a suspension of washed, live tetanus organisms. The mixture was incubated one and one-half hours at 37°C and then centrifuged. The supernatant was Seitz-filtered. The filtered antitoxin was again treated with washed, live tetanus organisms, incubated, centrifuged, and Seitz filtered. The Lf units of this absorbed antitoxin were determined 3 times against a tetanus toxoid of known Lf titer. It was then possible to calculate how much of the antitoxin should be added to a given amount of the tetanus toxoid concentrate to obtain flocculation at the optimum point.

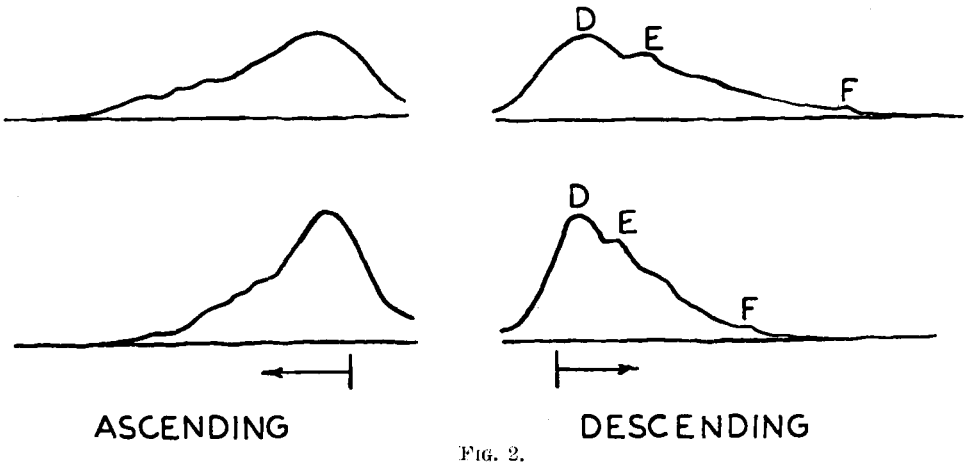

FIG. 2.

The material in another bottle containing the equivalent of 50 cc of the concentrated toxoid in dry form was reconstituted to the liquid state by the addition of 10 cc of distilled water. To 9.5 cc of this (equivalent to 4750 cc of the original toxoid), or approximately 7800 Lf units, were added 7.5 cc of absorbed antitoxin (1020 U/cc). Flocculation occurred within a few minutes. The mixture was incubated at 37°C for 30 minutes and placed in the cold room overnight. The material was then centrifuged and the supernatant was sent to Doctor Warner for a second electrophoretic analysis.

In this experiment the yellow color in the solution was not as intense as in the previous experiment and sufficient contrast was obtained in the photographs to permit prints to be made. Tracings were made from the negative and they are reproduced in Fig. 2. The patterns were obtained after electrophoresis for 8400 seconds (Fig. 2) (lower patterns) and 12,730 seconds (upper patterns) at a field strength of 4.81 volts/cm in a veronal buffer at pH 7.78 and an ionic strength of 0.1.

The patterns show the presence of several components and do not bear any definite relation to the pattern of the original toxoid preparation (Fig. 1). Mobilities have been calculated for three points in the descending pattern, lettered D, E, and F. They were found to be 0.7×10^{-5} cm^2/volt/sec for D; 2.1×10^{-5} for E; and 6.5×10^{-5} for F. Thus, by far the major part of the protein moves with a lower mobility than either peak B or C in the previous experiment. The mobility of the very small peak F, however, corresponds with that of B.

The antitoxin used in the flocculation was a purified and concentrated pseudo-globulin of equine origin and presumably should have been free of bacterial antibodies which are generally attached to the euglobulin fraction. The antitoxin was treated with the live tetanus organisms with the object of removing any anti-tetanic bacterial antibodies if they were present. If this were accomplished, then it might be possible to determine which of the two major components B or C was a bacterial cell derivative if only one of these components reacted with the antitoxin. If both components reacted to form floccules, then neither would be present in the supernatant. The deduction could then be made that neither B nor C was a bacterial cell derivative but both were two different toxoid factors.

In performing the flocculation test, we were well aware that non-reacting proteins present in the antitoxin would be introduced and that these would show up in the supernatant. Antitoxin, in immunologically pure form, has not yet been prepared by methods now in use for fractionating horse plasma. Pappenheimer[5] prepared a diphtheria antitoxic pseudoglobulin which was homogeneous by sedimentation, electrophoresis, and diffusion; yet, only 43.5% of this preparation was specifically precipitable by pure diphtheria toxin.

[5] Pappenheimer, A. M., *J. Bact.*, 1942, **43**, 273.

TABLE I.
Electrophoretic Analysis of Tetanus Toxoid Concentrate.

Before Flocculation	Supernatant after Flocculation
A—present in very small amount	D—u = 0.7 × 10^{-5} cm^2/volt/sec.
B—u = 6.5 × 10^{-5} cm^2/volt/sec. (yellow pigment)	E—u = 2.1 × 10^{-5} cm^2/volt/sec.
C—u = 4.7 × 10^{-5} cm^2/volt/sec.	F—u = 6.5 × 10^{-5} cm^2/volt/sec. (present in very small amount).
B and C present in about equal amounts	

There is every reason to believe that the tetanus antitoxin used in this experiment also contained a large proportion of non-reacting protein. If so, then these would appear in the supernatant liquid recovered from the toxoid-antitoxin floccules. That this actually took place is evidenced by the fact that the electrophoretic patterns on the supernatant show the presence of several components and did not bear any definite relation to the pattern of the original toxoid concentrate.

The mobilities of the various components found in the toxoid concentrate before flocculation and in the supernatant after flocculation are tabulated in Table I.

It will be seen from the table that by far the major part of the protein in the supernatant moves with a lower mobility than either peak B or C in the toxoid concentrate before it was allowed to react with the antitoxin. This indicates that the protein with the lower mobilities in the supernatant came from the antitoxin and very likely represents the non-reacting protein present in the antitoxin. The mobility of F corresponds to that of B but the F component is present in very small amount. It appears, therefore, that all of component C and nearly all of component B reacted with the antitoxin to form floccules, an indication, in our opinion, that neither component B nor C is a bacterial cell derivative but that components B and C may be regarded as two different toxoid molecules.

Work is now in progress whereby tetanus toxin, prepared by a procedure which yields a product relatively pure and with a remarkably high MLD, will be subjected to a similar study.

Summary. Concentrated solutions of tetanus toxoid prepared on a peptone-free medium were examined by electrophoresis in a Tiselius apparatus. The concentrate contained three components—A, present in a very small amount, B and C, present in about equal amounts.

The toxoid concentrate was allowed to react with an absorbed tetanus antitoxic pseudoglobulin, the floccules were separated and the supernatant was examined by electrophoresis. The supernatant contained several components, which bore no definite relation to the pattern of the original toxoid concentrate. By far the major part of the protein in the supernatant moved with a lower mobility than either component B or C. The mobility of F which was present in very small amount corresponded to that of B.

The presence of components D and E in the supernatant is ascribed to the non-reacting protein present in the antitoxin. Since all of component C and nearly all of component B was removed by flocculation, this evidence is taken to indicate that there was present in the toxoid concentrate two different toxoid proteins.

Although this work was done with tetanus toxoid and not with the toxin, consideration of the foregoing statements lends support to the views of Smith and Petrie that tetanus toxin (and by analogy tetanus toxoid) is not a homogeneous solution of toxin or toxoid molecules.

We wish to express our gratitude to Dr. Robert C. Warner of the Eastern Regional Laboratory, Philadelphia, Pa., for performing the electrophoretic experiments and for other valuable assistance.