An Electrophoretic Study of Diphtheria Toxoid Preparations

By P. A. CHARLWOOD

Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent

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It has been found that batches of diphtheria toxoid produced in these laboratories for the immunization of horses exhibit varying degrees of antigenicity. This variation has not been correlated with any variations in the constitution of the medium used for the toxin production or in the conditions of growth of the organism thereon. Since it would be of considerable advantage to possess a method of rapidly assessing the probable antigenic quality of a toxoid preparation prior to its injection into horses, it was thought that an investigation of the electrophoretic behaviour of different batches of toxoid would be worth while, as providing a possible criterion of antigenic potentialities.

Although the results of these investigations did not reveal any consistent differences among groups of toxoid preparations which were known to produce good and poor antigenic responses, a number of points arose which are worth recording. A considerable amount of work has been carried out on the electrophoretic behaviour of the corresponding antibodies (cf. Kekwick & Record, 1941), but data referring to diphtheria antigens are confined to observations by Lundgren, Pappenheimer & Williams (1939), who published a short account of the electrophoretic behaviour of highly purified diphtheria toxin; they did not extend their work to toxoid.

EXPERIMENTAL

Production and preparation of toxoids. Most of the toxoids were prepared from toxins produced on a papain digest of beef medium, except for one produced on a tryptic digest of beef, and another on an acid hydrolysate of casein medium. These preparations had to be concentrated 40–50 times to provide a solution strong enough for the investigation. Concentration was carried out by means of an ultrafilter, and, as dialysis against buffer caused some loss of toxoid through the membrane, the toxoid was thoroughly washed on the ultrafilter with the requisite buffer solution until the refractive indices of the washings and of fresh buffer were indistinguishable within the limits of error of the dipping refractometer. The refractive index of the toxoid preparation was measured at this stage, and dilution with buffer made to a standard difference of 0.00400 between the refractive index n of the protein solution, and n_0 that of the buffer. Mobility measurements were made in solutions of half this strength. The buffer usually employed was a phosphate buffer (pH 8.0, ionic strength $\mu=0.1$). Buffers used in mobility determinations were phosphate buffers, $\mu=0.1$, except for pH values below 6, where acetate buffers were

necessary, and at values above 8, where borate buffers were used.

Electrophoretic technique. Runs were carried out in the Tiselius (1937) apparatus at $+0.5^\circ$. The potential gradient in the U tube was held constant, usually at some value in the range 1.5–3.5 V./cm. The diagonal *schlieren* method (Philpôt, 1938) was employed, using as light source a mercury vapour lamp from which monochromatic light ($\lambda=546\text{ m}\mu$) was isolated by means of a filter. The curves were photographed on Kodak Half-Tone (Wratten) Panchromatic Plates (P 25).

Measurements of pH were made with a double hydrogen electrode, using 0.1N-HCl (pH 1.075) as the standard reference solution. Conductivity measurements on the buffers, necessary for mobility calculations, were made in a Washburn-type conductivity cell, and the distances moved by the components were measured from enlargements (of known magnification) obtained by tracing on graph paper the outlines of the patterns projected from the plates in a photographic enlarger.

A few runs, for the purpose of investigating different fractions, were carried out in the large type of U tube as employed by Blix, Tiselius & Svensson (1941).

RESULTS

Electrophoretic patterns of various diphtheria toxoid preparations

Typical curves are shown in Fig. 1 A–F. These indicate generally two major components, present in rather different relative amounts in the different preparations. A quantitative estimate was not attempted since the separation of the two peaks could not be satisfactorily achieved for this purpose. Examination of the contours of the curves also reveals that, in some cases, these components are themselves undoubtedly composite (e.g. see Fig. 1 A, B). Another feature, most prominent in Fig. 1 E, F, is a sharp spike projecting from the stationary ϵ boundary (for the origin of the ϵ boundary see Longworth & MacInnes, 1939). At one period there was a suggestion that antigenicity might be associated with the material responsible for this peak, but an extension of the observations to a larger number of toxoid preparations (about 30) showed that this could not be correct. The correlation of electrophoretic patterns with the antigenic response in horses was further complicated by the large variation in the responses of different horses to the same batch of toxoid.

The curves shown in Fig. 1 were all obtained from toxoids produced on papain digest media.

Fig. 1. Electrophoretic patterns of typical diphtheria toxoid preparations (see text) in phosphate buffer pH 8, $\mu=0.1$, 2.6 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. All these toxoids were produced from a papain digest of beef medium.

Fig. 2 shows the patterns *A* and *B*, obtained from toxoids produced on an acid hydrolysate of casein and a tryptic digest of beef medium respectively. The latter shows the presence of a new component. Methods of purification similar to those described by Pope & Linggood (1939) and Linggood (1939)

Fig. 2. Electrophoretic patterns of diphtheria toxoid preparations in phosphate buffer pH 8, $\mu=0.1$, 2.8 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. Details of these preparations are mentioned in the text.

were attempted. Treatment of the tryptic digest toxoid with 5% Farnell's *L.S.* carbon (Farnell Carbons Ltd., 1A Conduit Road, S.E. 18) removed most of this new component (see Fig. 2*C*). Treatment with 7% Farnell's *L.S.* carbon followed by precipitation from the filtrate by addition of 15% (v/v) of 5% (w/v) cadmium chloride solution, and subsequent extraction from the precipitate by 2% (w/v)

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ solution gave the product shown in Fig. 2D. The components here appeared to be purer, judging by the regular contours of the peaks.

Treatment of ordinary (papain digest) toxoids with 5% Farnell's *L.S.* or Sutcliffe, Speakman's *P.Z.* charcoal (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) showed that the electrophoretic pattern was scarcely affected.

Ammonium sulphate fractionation of concentrated toxoid produced no significant alteration in the relative amounts of the components present, either in the filtrate or in the precipitate.

A method of purification developed by Dr C. G. Pope, to be described elsewhere, produced a significant alteration in the electrophoretic pattern. A sample of toxoid purified by this method and assaying at 1700 *Lf* doses in each mg. of protein nitrogen gave, at pH 5.5, the pattern shown in Fig. 3. It consists of approximately 80% of slow-moving material together with smaller amounts of faster-moving impurities. This pattern should be compared with that given by an untreated toxoid at the same pH (see Fig. 5).

Fig. 3. Electrophoretic pattern of a purified diphtheria toxoid preparation in acetate buffer pH 5.5, 3.4 V/cm. Exposure 199 min. after starting current. The cathode limb is shown. Migration is from right to left.

Investigation of electrophoretically separated fractions

Runs were carried out in both the ordinary and the large *U* tube, to obtain, if possible, fractions representing one or other of the components present in toxoids. Isolation of fractions was carried out by hydrostatic displacement of the boundaries to the requisite position followed by sectioning off the *U* tube compartments which could be subsequently emptied independently. First, as regards the material responsible for the small, sharp, nearly stationary peak in some toxoids (e.g. see Fig. 1E) solutions containing only this component were isolated, analyzed for total nitrogen, and tested for activity by flocculation. Samples from other sections of the *U* tube were also tested as a check that toxoid activity had not been greatly diminished during the course of the experiments. The strongest solution of the minor component thus obtained gave total N 40.8 $\mu\text{g.}/\text{ml.}$ (allowing for a blank on the buffer), but no *Lf* units could be detected. Thus, this component could not possess activity of more than about 25 *Lf* units/mg. N and would therefore be

able to make only a very small contribution, if any, to the *Lf* units present.

In a similar manner solutions containing the fastest component were isolated and examined. Again, when care was taken to ensure that this component was obtained free from others, no toxoid activity was detectable in it by flocculation testing, although solutions containing nitrogen equivalent to 0.5–1.0% protein were isolated. It would thus appear that the toxoid activity is associated with the slower of the main components.

To test this conclusion a mixture of a toxoid with an equivalent amount of antitoxin (known, from previous electrophoretic work, to contain only β and γ globulins) was flocculated. The floccules were spun off and the supernatant liquid, concentrated and washed with buffer on an ultrafilter, then submitted to electrophoresis. The pattern obtained showed that most of the residual protein consisted of the inactive portion of β , γ globulins; but there was also some material which corresponded with the faster major component of the toxoid. This result was in accord with the experiments just described.

Mobility measurements. Mobility measurements were made over the range of pH values (5.0–9.0) throughout which the toxoids are fairly stable. Calculations were made only for the major components, and only from descending boundary rates (see Longsworth & MacInnes, 1940). Values of the mobilities for both components varied slightly from one preparation to another. Typical results are

Fig. 4. Electrophoretic mobilities of diphtheria toxoid components in the pH range 5.0–9.0. Values are uncorrected for buffer viscosities.

shown in Fig. 4. Each point is the mean of two determinations on the same solution. The curves can be extrapolated to indicate that the isoelectric points are approximately in the region of pH 3.5–4.0, as is known from other data.

Below a pH of about 6 there was a greater tendency for the composite nature of the peaks to be emphasized; thus at pH 5.5 the slower major

Fig. 5. Electrophoretic pattern of a diphtheria toxoid preparation in acetate buffer pH 5.5, 1.7 V./cm. Exposure 335 min. after starting current. The cathode limb is shown. Migration is from right to left.

peak can be seen to possess a very distinct hump, indicative of a boundary due to material which is moving rather more slowly (see Fig. 5).

DISCUSSION

Some discussion of the sharp peak appearing in the electrophoretic pattern of certain toxoid preparations appears to be called for. It is somewhat reminiscent of effects produced by convection and of the β -anomaly (Longworth, Shedlovsky & MacInnes, 1939). Reasons for assuming that it is not due to convection are (a) the detection of non-dialyzable nitrogen in this boundary, (b) the reproducible nature of the phenomenon with a given toxoid. Thus it was found that when a particular toxoid was divided into separate samples and submitted to electrophoresis, the curves obtained either all failed to exhibit this peak or all showed it; and, when present, it was in the same position and moved at the same slow rate. Repeated washing with buffer, on the ultrafilter, did not cause this peak to vanish from the pattern of any toxoid which originally showed it.

An unusual feature is the shape of the mobility curves, the speed of migration attaining a maximum value in each case at about pH 7.0–8.0. Although

the mobilities are uncorrected for buffer viscosities, the corrections would not cause differences large enough to remove the effect of these maxima. A similar effect has recently been observed by Watson, Cromartie, Bloom, Heckly, McGhee & Weissman (1947) in the case of glutamyl polypeptide, etc. However, in their work the effect appeared only in the results obtained from the anode (ascending) limb, which is known to give anomalous results (Longworth & MacInnes, 1940). It appears highly probable that the causes of the maxima are similar in the two cases, due to interaction between the molecules of the substance under examination, or to interaction with the buffer.

SUMMARY

1. Electrophoretic patterns of diphtheria toxoid preparations have been obtained, and typical curves are shown.
2. Most preparations show two major peaks with, in some cases, a slow-moving minor peak. The components represented by the major peaks, judging by their contours, are not quite homogeneous.
3. One preparation, purified chemically, appeared to be about 80% pure.
4. Toxoid activity has been assigned entirely, or almost entirely, to the slower major component.
5. The mobilities of the two major components have been measured over a pH range of 5.0–9.0.
6. Attempts to correlate the electrophoretic pattern with antigenicity have so far failed.

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