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Temperature Effects on Botulinum A Toxin.* (24033)

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The physical and chemical properties of botulinum toxin(1,3,7,12,13,15,16,18,19, 20) and its physiological action(2,4,5,6,17) have been studied extensively because of the interest in the agent's high toxicity. Burgen *et al.*(4) found that toxin heated in a water bath at 100° for 15 minutes lost its ability to produce paralysis, and Ambache(2) showed that boiling it for 2 minutes destroyed its toxicity. In this report, more detailed data on the effect of temperature on type A botulinum toxin are presented.

Materials and methods. Five toxin preparations, supplied by Fort Detrick, Frederick, Md., were used. Two were partially purified and 3 had been crystallized. The initial studies were performed with the partially purified samples, designated S-1 and S-2. Inactivations at various temperatures and pH values were done with a crystalline sample labelled WA-19. The effect of heating on ultracentrifuge sedimentation patterns was determined with the remaining 2 crystalline samples. Toxicity was assayed in the goldfish, *Carassius auratus*, kept in aquaria at room temperature before and after inoculation. The technic has been described(5). The end points were calculated by modification(10) of the method of Reed and Muench (14) on the basis of mortality ratios observed after 36 hours. Thermal inactivations were carried out in a thermostatic water bath with temperature fluctuations of less than 0.1°. Since preliminary experiments showed that the toxin inactivated rapidly, the diluent, 0.1 M phosphate buffer at pH 6.9 containing 0.2% gelatin, was heated a few degrees above the desired temperature before mixing with the toxin, so that the mixture of toxin and diluent came to the inactivation temperature immediately. This eliminated the necessity of waiting for the mixture to come to thermal equilibrium before the first sample could be taken. Samples were removed from the reaction mixture at subsequent time intervals and tested in 10 goldfish. In this way, the effect of heating on the 50% lethal dose was determined.

Results. Preliminary experiments with

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the crude samples, S-1 and S-2, were performed at 50°. The first experiment showed that the toxin inactivated rapidly at first, but, after about 10 minutes, the inactivation rate slowed down. When the difference between LD_{50} at zero time and LD_{50} at time of heating, t, was plotted against time of heating, a curve was obtained which indicated the presence of at least 2 toxic components in the sample. The inactivation curves for both partially purified preparations were similar when heated under the same conditions, but the data were somewhat more scattered for S-2. Later work showed that endpoints obtained with goldfish were temperature dependent(5). Therefore, part of the data scatter was due to fluctuations in room temperature. Preparations S-1 and S-2 contained impurities, and it was believed that perhaps these impurities were toxic and might be one of the components seen in the inactivation curve. A crystalline preparation, WA-19, was obtained and the experiments were repeated. The inactivation curve produced by this more highly purified material had the same shape as the curves for the less pure preparations. WA-19 was inactivated at other temperatures. These temperatures were from 40° to 60° in 5° intervals. The results for 40°, 50° and 60° are shown in Fig. 1, and it can be seen that the toxin does not inactivate after heating for ½ hour at 40°. Other curves in Fig. 1 represent studies at 50° and 60°, and indicate a minimum of 2 components. These 2 components have different rates of thermal inactivation and the rates of each component were temperature dependent. By extrapolating the limb of the inactivation curve produced by the more slowly inactivating toxin to the zero time ordinate, it was found that this component constituted about 5% of the toxicity of the preparation. About 95% of the preparation inactivated rapidly. The data at 45° were too scattered to analyze, and those at 55°, while scattered, were entirely consistent with the interpretation of a 2-component system.

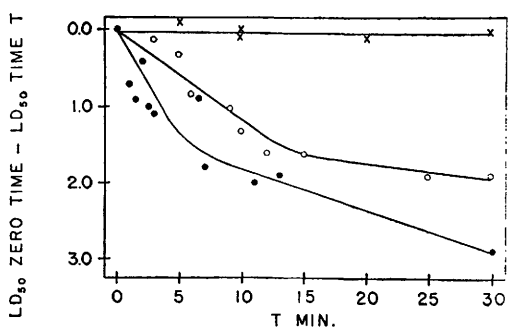
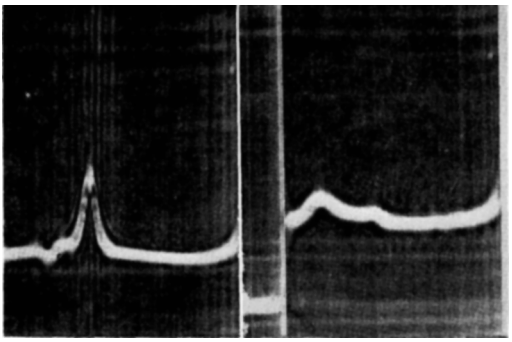


FIG. 1. Thermal inactivation curves for botulinum A toxin preparation WA-19. × = 40°; ○ = 50°; ● = 60°.

LD_{50} means logarithm to base 10 of the extent to which a toxin solution must be diluted to give 50% mortality when 0.1 ml is inoculated into each fish. Ordinates represent differences between logarithms of these dilutions for materials heated zero time and the times indicated on abscissa. It is possible to convert LD_{50} to the basis of No. of LD_{50}'s/ml or to the basis of No. of LD_{50}'s/mg nitrogen. However, such a conversion would not affect the difference between logarithms shown on ordinates. To convert the ordinates in Fig. 1 to logarithm of fractions of surviving toxicity, it is necessary only to change the sign of the difference between logarithms from plus to minus.

Since the presence of impurities would not account for the second toxic component, the nature of the 2 constituents was investigated. It has been shown that the toxic principle and the hemagglutinin of botulinum A toxin are separate entities(8,9,11). The hemagglutinin of WA-19 was removed by repeated absorption with chicken red blood cells. Parallel inactivation experiments on samples containing hemagglutinin and those not containing hemagglutinin were carried out. The end points, after heating at 50° for various times, were nearly identical, showing that hemagglutinin was not one of the components. The finding of Lamanna and Lowenthal(9) that hemagglutinin thermally inactivates more slowly than the toxic principle was confirmed in these experiments. The possibility that gelatin in the buffer might be affecting the shape of the curve was investigated, but the shape of the inactivation curve was the same in the presence and in the absence of gelatin.

The ultracentrifuge studies of Wagman and Bateman(19,20) and Wagman(18) on dissociation of botulinum toxin at high pH and ionic strength suggested the possibility that one of the effects of heat might be the dissociation of the toxin particle. Two small samples of toxin in acetate buffer at pH 3.8 were obtained and were run in the ultracentrifuge. Each produced a single sharp



A B

FIG. 2. Sedimentation diagrams for botulinum A toxin before and after heating. A. Toxin in 0.1 molar phosphate buffer at pH 6.9. B. Same sample after heating at 60° for 2 min.

peak. These samples were dialyzed against 0.1 M phosphate buffer pH 6.9 and the centrifuge analysis repeated. At pH 6.9, the sedimentation diagram showed 2 peaks, a small one which sedimented more slowly than a large peak which sedimented at the usual rate. Portions of these samples were heated for several minutes at the various temperatures used in inactivation experiments and again were run in the ultracentrifuge. After heating, the fast boundary became more diffuse indicating a possible increase in its inhomogeneity, and, in some cases, the slow boundary disappeared. Definite evidence for dissociation was obtained when the sample was heated at 60° for 2 minutes. When the sample was treated this way, the slow peak increased in area while the fast peak decreased in area and became more diffuse. The results are shown in Fig. 2.

Discussion. The results of these studies can be interpreted if it is assumed that the botulinum toxin particle can dissociate into 2 entities which have different thermal inactivation rates, and if it is further assumed that this dissociation can be produced by heating the particle. Wagman and Bateman (19,20) and Wagman(18) have presented evidence that the toxin molecule can dissociate into small toxic fragments when the ionic strength and pH of solvent buffer are increased. A similar experiment of ours confirmed their results. Changes produced in sedimentation diagrams of the toxin after it is heated, especially the diagram obtained after heating for 2 minutes at 60°, provide a basis for the second assumption.

The physical nature of these dissociation products might be postulated in at least 2 ways. Both of them might be fragments of the original particle or one might be the intact molecule and the other might be small dissociated parts of a molecule. The evidence does not permit the selection of one of these alternatives over the other.

Although the sedimentation diagram shows 2 components at pH 6.9, there is no evidence of 2 components when the toxin is inactivated at 40°. Either the components have the same inactivation rates at 40°, or the loss in toxicity is too small to be detected by the assay technic. The technic can detect about 2-fold differences in toxicity(5).

Summary. 1) Thermal inactivation of botulinum A toxin was studied. The LD_{50} was found for samples heated for various times at 40°, 50° and 60° by assaying samples in goldfish. Inactivation curves were plotted by plotting the difference between the LD_{50} at zero time, and the LD_{50} at time of treatment, t, against time of heating. At 40°, no inactivation occurred after heating one hour. At 50° and 60°, the inactivation curves were characteristic of a system which contained a minimum of 2 components which inactivated at different rates. Hemagglutinin was not one of these components and gelatin in the buffer did not affect the shape of the inactivation curve. 2) Sedimentation diagrams taken before and after heating at inactivation temperatures suggested that the toxin might be dissociating to form 2 particles having different properties. The data can be explained on the basis of this hypothesis.

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Variation in Virulence of Poliovirus. III. On Type III Virus by Plaque Method.* (24034)

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In a previous paper(1) experimental variation in mouse virulence of type III (Leon) poliovirus grown in tissue culture (TC) was reported and the influence of host cell and maintenance medium on viral virulence was demonstrated. The experiments have been extended, using the plaque technic. A mixed virus population and one purified by terminal dilution and by the plaque method were studied in regard to (a) distribution of virulent and avirulent progeny, and (b) reversibility of avirulent phase under influence of virulence enhancing media. The question as to whether the avirulent variant failed to multiply in the host or whether it multiplied without causing paralysis was also studied. The results are here reported.

Materials and methods have been described, (1) except for the following additions or modifications. The *plaque technic* of Dulbecco and Vogt(2,3) as modified by Hsiung and Melnick(4,5) was followed. Rhesus monkey kidney tissue cultures (MKTC) grown for approximately one week in Melnick's medium (0.5% lactalbumin hydrolysate in Hanks' salt solution with 2% calf serum), in 2-oz flat "prescription" bottles, were used. After removing the medium, each bottle was inoculated with 0.2 ml of virus dilution of known titer and incubated at 35°C for 45 minutes. Excess of inoculum was removed and the cells washed once with Hanks' salt solution. Approximately 4.5 ml of melted agar overlay (formula below) were added to each bottle. After agar had solidified, the bottles were turned over and incubated at 35°C for 3-4 days before plaque counts were made. *Agar overlay* was prepared as follows: Melted, sterilized 3% agar (Difco "Noble") in distilled water was mixed with equal portion of nutrient medium consisting of Earle's saline (10 x concentrated without either phenol red or $NaHCO_3$), 18.0 ml; calf serum, 4.5 ml; $NaHCO_3$, 5.4 ml of a 7.5% stock solution; neutral red (1:1000), 3 ml; antibiotics (penicillin 20,000 units/ml, streptomycin 10,000 mu/ml), 2 ml; and distilled water sufficient to make total volume of 90 ml. This mixture then contained 2.25 g of NaHCO/liter. *Virulence test in mice.* For assay of viral virulence, in addition to direct inoculation of TC fluid into mice as previously reported(1), the following procedure was followed: Well separated plaques were harvested with platinum loop, often from those bottles containing only

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