Renal inflammation tended to persist after infection had disappeared. 3) Vascular disease and hypertension did not develop; however, the lesions were of insufficient severity to produce azotemia.

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Purification of Hyaluronidase from *Clostridium perfringens.** (22158)

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Although several investigators(1-4) have prepared highly purified hyaluronidase from bovine testicular tissue, bacterial hyaluronidases have not usually been purified to a comparable degree. Most authors(5) have used either simple filtrates of the bacteria or crude $(NH_4)_2SO_4$ precipitates. The notable exception was the discovery by Rogers(6) that crude precipitates from ammonium sulphate treatment of filtrates of *Clostridium perfringens*, Type A, Strain 107, could be absorbed on aluminum oxide at pH 4.6 and eluted at pH 7.0 to give a 16-fold increase in potency of hyaluronidase preparations. This method is difficult to reproduce and Rogers was not always able to get a sharp separation with his preparations. For this reason we initiated an alternate method for purification of the bacterial hyaluronidases. This report deals with the preparation of highly purified hyaluronidase from *Cl. perfringens, culture 2278.*

Methods. The hyaluronidase unit was based on the unit of testicular hyaluronidase previously reported(7). Due to instability of the bacterial filtrates at pH 5.5, the enzyme was incubated with hyaluronic acid at pH 7.0. Fig. 1 shows that turbidimetric curves coincided in the range of 2 to 5 units. Fractions for assay were diluted with a buffered solution of 0.1% bovine serum albumin(8) until they fell within these limits. Nitrogen content of various fractions was determined by micro-Kjeldahl digestion with selenium-sulfuric acid followed by nesslerization. Three strains of *Clostridium perfringens* were studied. A lyophilized stock strain, #47-E-2, Walter Reed Army Institute of Research collection, represented the so-called laboratory strains. It had been held in dried state since 1944, but there were no records of its source. A second culture, #4209, isolated in 1951,

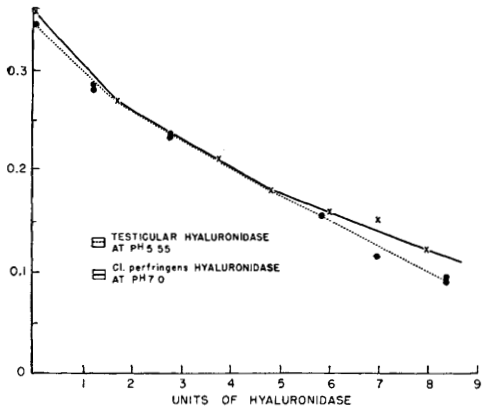

FIG. 1. Comparison of relationship of optical density to *Cl. perfringens* hyaluronidase and testicular hyaluronidase.

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from a gangrenous war wound represented a strain of known pathogenicity. It was isolated and lyophilized at the 406th Medical General Laboratory (Tokyo) approximately one year prior to these studies. A third strain, #2278, had been isolated from the gangrenous wound of a patient at Walter Reed Army Hospital in 1952. This culture had been maintained on blood agar plates in an anaerobic atmosphere and transferred at monthly intervals.

Results. Choice of strain and medium. Considering the nutritional selectivity of *Clostridia* 3 types of culture media, at pH 7.6, were studied to obtain the greatest amount of bacterial filtrate: Difco brain heart infusion broth containing 1.5% yeast extract, Bernheimer's synthetic medium, and trypticase lactose iron broth. Starter cultures for mass inoculation were prepared by seeding 1 liter of each of these media with 10 ml of thioglycollate broth from the blood agar plates on which the various strains had been initially recovered from stock. Good growth was obtained after 16 hours incubation at 35°C. One liter quantities of the 3 selective media were prepared in the usual manner. Excess oxygen was displaced by boiling and cooling to approximately 40°C immediately prior to adding 10 ml of thioglycollate broth starter culture. The 1 liter flasks were next sealed with parafilm and incubated at 35°C for 18 hours, after which the cultures were centrifuged. Supernatants were adjusted to pH 9.0 with 1N sodium hydroxide and sterilized by Seitz filtration.

Hyaluronidase yields by strains of *Clostridia* employed were widely divergent. All strains failed to grow in Bernheimer's synthetic medium. Fair growth was obtained in trypticase lactose iron broth, but hyaluronidase yield was not satisfactory. The failure of both media to support good growth of *Clostridium perfringens* was unexpected. Best results were obtained in brain heart infusion broth with 1.5% yeast extract added. The hyaluronidase yield in this medium was 10 times that obtained in trypticase lactose iron broth and gave values of 0, 60 and 100 units/ml of culture filtrate for strains #47-E-2, #4209 and #2278 respectively. Within the small number of strains tested, there appeared to be a correlation between length of time these cultures have been held in the dried state and their ability to produce hyaluronidase. Culture #47-E-2, in the dried state for more than 10 years, failed to produce the enzyme, although it grew well. Culture #4209, lyophilized in 1951, produced small amounts of hyaluronidase. On the other hand, culture #2278, which had never been lyophilized, produced by far the most enzyme. This culture was used for subsequent preparations of purified hyaluronidase.

Preparation of purified hyaluronidase. Preliminary attempts to fractionate the bacterial filtrate at pH 7.6 were completely unsuccessful. The enzyme appeared to be stable in the filtrate at this pH for only 72 hours and then was rapidly destroyed. Precipitation with 0.7 ammonium sulphate or with ethanol appeared to hasten destruction of the enzyme. Stabilization of the original culture filtrates, and the first crude fractions, was necessary. Since instability of the hyaluronidase might be due to proteolysis by other enzymes in the culture filtrates, the effect of pH on stability of the hyaluronidase at 0°C was investigated, using the culture filtrates and the 0.7 saturated ammonium sulphate precipitate buffered to several pH values with and without ethanol at 0.3 mole fractions. The 0.3 mole fraction of ethanol was selected to represent maximum concentration of ethanol anticipated in subsequent experiments. Below pH 7.0 destruction of the enzymes was exceedingly rapid. At pH 7.0 in the buffer, the enzyme showed a minor loss in 1 hour but was completely destroyed in 24 hours. In ethanol it was completely lost. Between pH 8.6 and 9.5 the enzyme remained indefinitely stable in either buffer or buffer containing ethanol. At lower hydrogen ion concentrations only the buffered enzyme remained stable. Consequently, the cultures of *Cl. perfringens* were immediately adjusted to pH 9.0 after the bulk of organisms had been removed by centrifugation at 4000 rpm and supernatant clarified by

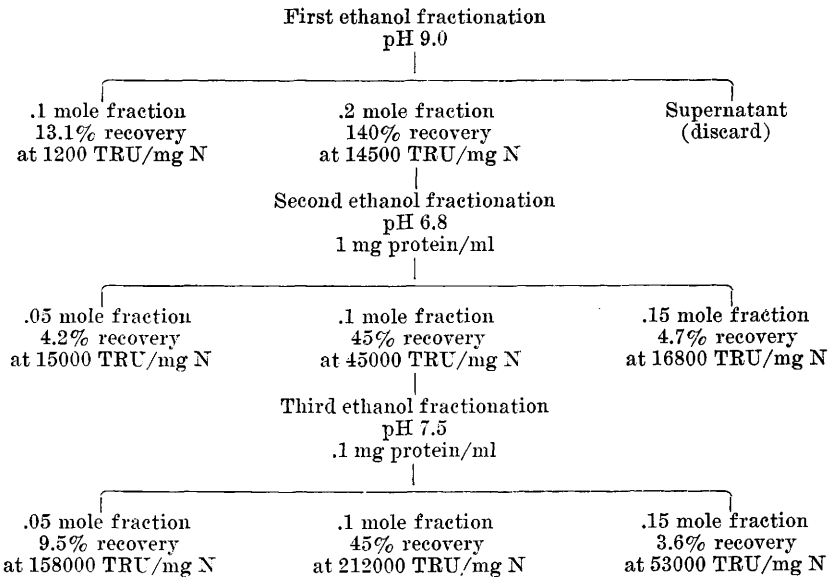

FIG. 2. Ethanol fractionation of hyaluronidase from *Cl. perfringens*.

Seitz filtration. Solid ammonium sulphate was added to the clear filtrate to 0.3 saturation. The suspension was allowed to stand 24 hours at 4°C. The inactive precipitate was removed by centrifugation and discarded. Ammonium sulphate was added to the supernate to 0.7 saturation, and the suspension was again allowed to equilibrate for 24 hours at 4°C. The active precipitate was removed by centrifugation and dissolved in one-fifth the original volume with 0.02 molar veronal buffer, pH 9.0, containing 0.45% NaCl.

The ethanol fractionations were conducted as previously described(2) except that suspensions were allowed to equilibrate for 24 hours after addition of each increment of ethanol and precipitates removed in refrigerated centrifuge, −5°C, at 4000 rpm. Application of this technic to testicular hyaluronidase yielded 15 to 25% recovery of the crude enzyme and a purified preparation assaying 160000 TRU/mg N. Typical results of the first ethanol fractionation are shown in Fig. 2. The recovery figures are based on the hyaluronidase content of 3.5 l of culture filtrate which contained 100 TRU/ml at an index of purification of 200 TRU/mg N. The ammonium sulphate precipitation in combination with the first ethanol fractionation therefore caused a 70-fold purification of the bacterial hyaluronidase. Recovery in the dialyzed 0.1 and 0.2 mole fractions of 153% of the total enzyme, can be partly explained by the increased stability of these fractions at pH 7.0 where the assay was conducted. The apparent anomaly may also be due to removal of inhibitors present in the original culture filtrate. As this stage the 0.2 mole fraction was stable indefinitely at pH 7.3 in buffered solution and was stable for at least 72 hours at pH 7.0.

Previous work suggested that the greatest purification could be achieved by fractionating next at a low pH. Consequently, pH 6.8 was chosen for the second ethanol precipitation. The 0.2 mole fraction from the first ethanol was dialyzed, and concentrated phosphate buffer containing NaCl was added to 0.02 M phosphate, pH 7.3 containing 0.45% NaCl. The solution was diluted with buffer to 1 mg protein/ml, and the pH adjusted to 6.8. Fig. 2 shows destruction of the enzyme at this pH since only 54% was recovered. However, the 0.1 mole fraction contained 45% of the original enzyme 3 times purer.

The third ethanol fractionation was conducted at pH 7.5. The 0.1 mole fraction from the second ethanol treatment was dis-

solved in the 0.02 M phosphate buffer pH 7.3, diluted to 0.1 mg protein/ml and pH was adjusted to 7.5. It is believed that the 4-5 fold increase in purification shown in Fig. 2, is due more to the very dilute solution of protein fractionated rather than change in pH. As in the first ethanol fractionation over 100% recovery of the enzyme being fractionated was obtained. This increase could not be attributed to increased stability of the enzyme at assay pH of 7.0, because the enzyme was stable at this pH for at least 3 weeks after the second ethanol fractionation. It seems more likely to be due to removal of inhibitors during the purification procedure.

An apparent over-all recovery of 45% was obtained by this procedure and the enzyme was purified 1000-fold. However, it is obvious from the data that removal of inhibitors during purification may account for part of this high yield. In small scale experiments, it appeared that if the second ethanol was conducted at pH 7.0 instead of 6.8 even higher yields could be obtained.

Rogers(6,9) does not mention the loss of *Cl. perfringens* hyaluronidase below pH 7.0. In fact, we may assume that this did not occur with his strain since it was absorbed on aluminum oxide at pH 4.6 (a pH which would have caused immediate destruction of hyaluronidase preparations obtained in these laboratories). The marked difference in stability of these two hyaluronidase preparations cannot be explained at present. Conditions of growth, as well as strains, permit many possibilities. However, it is suggested that two hyaluronidase disaccharases may be present in these organisms. Consequently, the two laboratories may not have been dealing with the same enzyme.

Summary. A procedure has been detailed for a 1000-fold purification of a bacterial hyaluronidase from *Cl. perfringens,* strain 2278. Initial crude fractionation with ammonium sulphate followed by three ethanol fractionations at pH 9.0, 6.8 and 7.5 respectively have yielded hyaluronidase fractions containing 200000 TRU/mg N with recovery of 45% of the crude enzyme.

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Production of Impending Hepatic Coma by a Carbonic Anhydrase Inhibitor, Diamox.*† (22159)

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Impending hepatic coma, characterized by confusion and the typical "flapping" tremor, has been described previously in alcoholics with cirrhosis given oral nitrogenous substances—ammonium cation exchange resins, ammonium salts, urea, and increments in dietary protein(1-3). The onset of this syndrome is here described in similar patients following oral administration of a carbonic anhydrase

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