# SOME EFFECTS OF FIVE PROTEOLYTIC ENZYMES ON RABIES VIRUS

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by

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# I. INTRODUCTION

The routine laboratory methods employed for the diagnosis of rabies, in most instances, consists of attempting to demonstrate the presence of Negri bodies by microscopic examination of stained impression smears prepared from infected brain tissue. If Negri bodies are demonstrated in such preparations, a diagnosis of rabies is reported and quite frequently no further diagnostic procedures are performed. In instances where Negri bodies cannot be demonstrated, a common procedure is to attempt to isolate the virus by intracerebral inoculation of supernates of either 10% or 20% suspensions of infected brain tissue into laboratory animals. Laboratory animals which are inoculated with such suspensions or their supernates may not receive a lethal dose of virus if the titre of the virus in the brain tissue is extremely low. It is not inconceivable that a positive case of rabies could remain undetected by the methods employed for the routine laboratory diagnosis of rabies.

A possible solution to the problem encountered in attempting to isolate rabies virus from brain tissue containing a low titre of virus would be to include greater quantities of the brain tissue in the suspensions employed for animal inoculation. However, the use of greater quantities of tissue in the preparation of such suspensions is not entirely satis-

factory. A tissue suspension of greater concentration than 20% is frequently lethal to mice when inoculated intracerebrally. Such suspensions are not usually lethal when inoculated intraperitoneally or intramuscularly. The incubation period for rabies is frequently longer when the virus is inoculated by the intraperitoneal and intramuscular routes. For this reason the intracerebral route with its shorter incubation period is preferred for routine laboratory diagnostic procedures.

Supernates prepared from suspensions containing large quantities of infected brain tissue of low titre could possibly be employed for animal inoculation. However, the sediment of brain tissue and tissue debris which is precipitated when a tissue suspension containing rabies virus is centrifuged, contains a large percentage of the available virus. This can be easily demonstrated by re-grinding the sediment; re-suspending it in a suitable diluent, and titrating the resultant supernate. At a centrifugation speed of 1,000 rpm for 45 minutes it has been reported that as much as 90% of the free virus in a suspension can be precipitated with the tissue particles. With increased centrifugation speeds or increased time required at a given speed necessary to obtain suitable supernates from suspensions containing large concentrations of infected brain tissue, a proportional decrease in the amount of free virus in the supernate can be expected.

A means of liberating additional quantities of virus from infected brain tissue, in addition to the amount liberated solely by mechanical maceration, would offer a possible solution to the problems associated with the isolation of rabies virus from brain tissue of low titre. Proteolysis of mechanically macerated infected brain tissue could possibly liberate additional quantities of live virus. A number of proteolytic enzymes are known which do not attack living cells or most native proteins. The employment of such enzymes in the proteolysis of mechanically macerated infected brain tissue should, theoretically, result in proteolysis of the nerve cells without affecting the virus.

The action of various enzymes of animal and vegetable origin on rabies virus has been studied by various investigators. In every instance these investigations were limited to determining whether or not a given enzyme inactivated the virus. In those instances where the enzymes employed did not inactivate the virus, no efforts were made to determine if the enzymes had liberated additional quantities of virus. No investigations have been reported in which the more recently discovered fungal and bacterial proteases have been studied for their effect on rabies virus.

The purpose of this study is to determine the effect of a selected group of proteolytic enzymes on rabies virus with particular emphasis on fungal and bacterial proteases. This

evaluation should ascertain the potentialities of employing proteolytic enzymatic digestion of mechanically macerated rabies infected brain tissue as a means of rabies virus elution.

## II. REVIEW OF PERTINENT LITERATURE

### A. The Effect of Proteolytic Enzymes on Rabies Virus

Remlinger (41) reported in 1905 that a, "thick emulsion of fixed rabies virus," when placed in "viscus" sacks and inserted in the peritoneal cavity of dogs or rabbits, was inactivated within twenty-four hours. He repeated his basic experiment, removing the sacks from the peritoneal cavity every hour. By animal inoculation he was able to demonstrate that attenuation of the virus is perceptible after one hour. After six hours, half the animals inoculated remained unaffected, and by twelve hours the virus was completely attenuated. The possibility that the temperature of the peritoneal cavity, or phagocytosis accounted for the attenuation of the virus was investigated and disproved. Particular note was made of the fact that the attenuation of the virus occurred more rapidly when the emulsion of virus was enclosed in a "viscus" sack within the peritoneal cavity, than when it was injected directly into the cavity. He concluded that, "it remains therefore to incriminate a possible and singularly energetic rabicide of the liquid of the peritoneal cavity."

In 1934, Hirano (23) conducted a series of experiments to determine the effects produced on fixed rabies virus by the enzymes, lipase, trypsin, and diastase. He prepared

various dilutions of virus and enzyme using a phosphate buffered diluent with a pH of 7.4. In a series of animal inoculations, utilizing albino rabbits, it was determined that rabies virus was unaffected by a 1:10,000 dilution of lipase, but was partially attenuated by a 1:8,000 dilution, and completely destroyed by a 1:4,000 dilution. With a five percent dilution of trypsin in 50% glycerol, diluted 1:20, 1:100, and 1:200 with phosphate buffer, he was able to demonstrate that the virus resisted the action of trypsin in dilutions greater than 1:100. Similar results were obtained with a ten percent solution of diastase in 50% glycerol. In dilutions of 1:40 and greater, diastase had no effect on the virus.

In 1934, Jonnesco (26) investigated the natural immunity of some animals to infection with rabies virus. At a later date, he reported that as a result of his earlier research on the problem, he was convinced that, "enzymes appeared to have an important role in the processes of defense against rabies."

In 1936, Jonnesco (27) investigated the action of pancreatic juice on fixed rabies virus. He demonstrated that human pancreatic juice, in maximum dilutions of 1:10, can have a destructive effect on the virus. The degree of the destructive action of the pancreatic juice varied as to the donors from which it was collected. The nature of the destructive action was found to be due, in the major part,

to the action of trypsin. This was demonstrated by the fact that when the trypsin content of pancreatic juice is inactivated by the addition of antitryptic enzymes, obtained from normal human serum, the pancreatic juice is incapable of destroying the virus.

Bailly (2) conducted a series of experiments in 1945, in which he studied the action of pancreatic extract on fixed rabies virus. He concluded that the virulence of the virus is destroyed by an aqueous extract of dog pancreas, after 24 hours of contact at 10 C. The destructive effect of the extract takes place at a pH of 10, and is inhibited by an acid pH or an alkaline pH between 7.0 and 9.0.

In 1947, Remlinger and Bailly (42) studied the effect of fig latex on rabies virus. They subjected a 1:50 emulsion of rabbit brain, infected with a strain of fixed virus, to the proteolytic activity of fig latex. The 1:50 emulsion of infected brain was mixed with 1:10, 1:15, 1:20, and 1:50 dilutions of the latex and incubated for 17 hours at 15 C. Two mice were inoculated, intracerebrally, with each mixture of brain emulsion and dilutions of latex. The eight mice, so inoculated, survived and two mice inoculated with an aqueous suspension of the untreated brain emulsion died. The authors drew attention to the fact that the juice of various species of aloes had produced similar destructive effects on rabies virus.

According to Bodansky, (4, p. 158), the proteolytic enzyme, cathepsin, is present in autolyzed tissue. The possibility that this enzyme may have some effect on rabies virus is suggested by the statements of several authors. Hagan (21) states that putrefaction destroys rabies virus rather slowly. Kelser (29) also states that the virus will resist putrefaction for a long period of time, if protected from other detrimental influences. VanRooyen (56) states that temperature also has an effect on the virulence of rabies virus in autolyzed brain. Virus has been recovered after four months from autolyzed brain kept at 18 C., but only after a few days when the brain was kept at 37 C.

During the preparation of a suspension of rabies virus from infected tissue, Johnson ( 25, p. 287) states that intracellular enzymes are liberated with the virus. These enzymes may have an unfavorable effect on the activity of the virus. He also states that "tissue enzymes liberated from brain material used in the preparation of rabies vaccine undoubtedly have a deleterious effect on the antigenicity of the virus."

### B. The Effect of Heat on Rabies Virus

Babes and Lepp (1) reported in 1889 that rabies street virus was inactivated when subjected to a temperature of 63

to 70 C. for an undisclosed period of time. This virus, after inactivation, possessed no immunizing properties. Another strain of virus which was heated to 60 C. for 12 hours was not inactivated by this treatment. It retained sufficient virulence to cause the death of a dog which had been inoculated, within 14 days.

In 1905, Remlinger (41) reported that an emulsion of fixed rabies virus, which he had maintained for 24 and 48 hour periods in a 39 C. water bath, still exhibited virulence when inoculated into rabbits. He also subjected portions of the same emulsion to 24 and 48 hour periods in a 39 C. oven, and found that the virus still exhibited virulence.

Circa 1907, Lamb and McKendrick (36) conducted a series of experiments on the effect of incubation at 37 C. on 1:200 and 1:1600 dilutions of fixed rabies virus in normal saline. They incubated the virus dilutions for varied periods up to 24 hours, and reported that the virus was partially destroyed after two to four hours, and completely destroyed after 24 hours.

Semple (48) conducted extensive tests on the effect of heat on rabies virus, which were reported in 1911. In one series of experiments he was able to demonstrate that a five percent emulsion of fixed virus, in normal saline solution, was destroyed at a temperature of 50 C. in 15 minutes, but

not at a temperature of 45 C. in the same time. In another series of experiments on the effect of normal saline solution on the virus, he stated that his results had failed to confirm those of Lamb and McKendrick (36). Utilizing animal inoculation tests, he demonstrated that the virulence of a "one percent dilution" of virus in normal saline remained unimpaired when held at room temperature for 11 days, and in one case for 15 days. He repeated this experiment, at which time the virus was incubated at 37 C. rather than room temperature, and concluded that; "the results of this series of experiments prove that dilutions of 1 in 200 rabies virus in normal saline solution are not destroyed in 24 hours at a temperature of 37 C.; and that a dilution of 1 in 1,600 loses nothing of its virulence when kept for four hours at a temperature of 37 C., but has lost a little of its virulence although not destroyed as the end of 24 hours."

Hirano (23) in 1934, in the course of subjecting a strain of fixed rabies virus to proteolytic digestion by lipase, trypsin, and diastase, incubated a 10% suspension of infected brain tissue in normal saline at 37 C. for two to three hour periods. After incubation the virus was allowed to stand at room temperature for various periods of time varying from 17 to 22 hours. He did not report any decrease in the virulence of the virus after incubation.

Tierkel (53), in his presentation before the Panel on

Rabies of the Section on Public Health at the 87th annual meeting of the American Veterinary Medical Assocation in 1950 stated, "in laboratory experiments in Montgomery, suspensions of the virus were killed in four to five days at 37 C. In the last tests, they were killed in three to four days at that temperature. At 45 C., we could kill them in twenty-four hours; at 50 C., in one hour; and at 100 C., in less than two minutes. So it is quite a thermolabile organism."

## III. STANDARD MATERIALS AND APPARATUS

The materials and apparatus described below were routinely employed throughout this problem. Those materials and apparatus which pertain only to a particular investigation are described separately with the methods of procedure employed in that investigation.

### 1. Antibiotic solution

An antibiotic solution prepared with sterile physiological salt solution, containing 12,500 units of penicillin G and 25 mg. of streptomycin sulphate per ml., was routinely used to control bacterial contaminants.

### 2. Experimental animals

a. Rabbits. Ten to 15 pound adult albino rabbits were used as indicated. No preference was shown as to the sex of the rabbits used. All the rabbits were procured from a local source and their strain was unknown.

b. Mice and guinea pigs. The mice and guinea pigs employed in various investigations varied as to sex and strain as described in the description of each investigation.

3. Weights and measurements

a. Fluid measurements. All fluids were measured with TD pyrex, sterile, pipettes of suitable capacity.

b. Solid measurements. All solids were measured by weight. An Ohaus, "Cent-O-Gram", triple beam balance, model CG 311, was used to weigh the specimens.

4. Syringes and syringe needles

Hypodermic syringes, of $\frac{1}{4}$ ml. capacity, were routinely used for the intracerebral inoculation of experimental animals. Twenty-seven gauge, $\frac{1}{4}$ inch, and 25 gauge, 3/4 inch, sterile syringe needles were used for the intracerebral inoculation of mice and rabbits respectively. Hypodermic syringes, of 5 ml. capacity, and 25 gauge, 3/4 inch, sterile syringe needles were routinely used for the intraperitoneal inoculation of mice and rabbits.

5. Mechanical apparatus

a. Blendor. A standard Waring blendor with a stainless steel, covered, small size blendor cup, was routinely used to emulsify all brain tissue.

b. Colorimeter. A Klett-Summerson photoelectric colormeter, model 900.3, was routinely used for making turbidimetric

determinations.

c. Water bath. An electric serological water bath, thermostatically controlled to automatically maintain the temperature to which it was adjusted, was routinely used as indicated in various methods of procedure.

d. Incubator. A standard type of dry heat laboratory incubator was routinely used as indicated in the various methods of procedure. This incubator was electrically operated and thermostatically controlled to constantly maintain the temperature to which it was adjusted.

e. Centrifuge. An International Centrifuge with a horizontal head was routinely employed as indicated in the various methods of procedure.

f. Roller-tube drum. A motor driven roller-tube drum, commonly used for tissue culture, was constructed by the instrument shop of the Physics Department of Iowa State College. This drum holds 120 tubes, 16 x 150 mm. It was geared so that the drum rotated eight times per hour. This apparatus was routinely used as a means of agitating enzyme-brain emulsion preparations while they were being incubated in a dry heat laboratory incubator.

# IV. INVESTIGATIONS

## A. Toxicity Produced in Mice by Intracerebral Inoculation of Various Enzymes

### 1. Materials and apparatus

a. Diluent. Physiological salt solution, prepared by adding 8.5 gm. of sodium chloride to 1,000 ml. of distilled water, was employed as the standard diluent. The solution was prepared in one liter flasks, stoppered, and sterilized by heating in an autoclave for 30 minutes at 15 pounds of steam pressure.

b. Experimental animals. The mice used in the following procedures were obtained from two sources; Stokley-Peterson Farms, (S-P strain) and the Veterinary Physiology Department of Iowa State College, (CFW strain). All of the mice used were 20 gm., albino, swiss mice, and although two different strains were employed, no preference was given as to the strains or sex used.

c. Proteolytic enzymes. Various manufacturers of biological products were requested to furnish samples of suitable proteolytic enzymes. A total of 12 samples were received. Brief descriptions of the products which were submitted are as follows:

(1) Trypsin. Manufactured by General Bio-Chemicals Inc. Harrow (22, p. 100) states that trypsin is derived from the pancreas, and hydrolyzes proteins to peptides and amino acids. It can digest substrates much simpler than proteins. Pure trypsin gradually digests itself at pH 7.0 to 9.0, however the optimal temperature and pH for the proteolytic activity of this enzyme is 37 to 50 C. at pH 7.8 to 8.7.

(2) Bromelain. Manufactured by General Bio-Chemicals Inc. According to Bodansky (4, p. 137) and Harrow (22, p. 100), bromelain is derived from the pineapple and has a proteolytic function similar to trypsin.

(3) Ficin. Manufactured by General Bio-Chemicals Inc. Ficin is a proteolytic enzyme obtained from the latex of certain species of the fig tree. Ficin also contains the enzyme peroxidase. The optimal pH for its proteolytic activity, as measured on gelatin, is 5. (15)

(4) Papain (crystalline). Manufactured by Jensen-Salsbery Laboratories. Harrow (22, p. 100) states that papain is obtained from the milky juice of the melon tree (papaw), and from plant cells in general. It is similar to trypsin in its proteolytic activity.

(5) Chymotrypsin. Manufactured by Nutritional Biochemical Corp. Harrow (22, p. 107) states that chymotryp-

sin is derived from chymotrypsinogen, which is isolated from the beef pancreas. Chymotrypsinogen is converted into a powerful proteolytic enzyme by trypsin, which differs from trypsin in that it clots milk.

(6) "Rhozyme P-11". Manufactured by Rohm & Haas Co. The manufacturer (45) and Reed (39) state that "Rhozyme P-11" is a fungal protease. It is produced from an aqueous extract of the fungus by alcohol precipitation followed by dilution with salt.* Fungal proteases from this organism, the identity of which the manufacturer did not reveal, have never been produced in pure form, and therefore the purity of this enzyme is undetermined. The optimal temperature for its proteolytic activity is 50 C., but satisfactory results are obtained at lower temperatures if the reaction time is extended. The optimal pH range for its proteolytic activity is 6.5 to 7.5.

(7) "Protease 15". Manufactured by Rohm & Haas Co. The manufacturer (44) and Reed (39) state that "Protease 15" is a bacterial protease prepared in an analogous manner to that of "Rhozyme P-11". It is characterized by its ability to hydrolyze proteins. Its action lowers the viscosity of protein solutions and increases the solubility

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*Specific chemical structure of salt not stated by manufacturer.

of the protein material. The action does not proceed as far as amino acids. The optimal temperature for its proteolytic activity is 50 C., but satisfactory results may be obtained at lower temperatures if the time of reaction is increased. The optimal pH for proteolytic activity is 6.5 to 7.5, however satisfactory results may be obtained from pH 5.5 to 8.5.

(8) "Protease (trypsin)". Manufactured by Takamine Laboratory Inc. The manufacturer states (52) that "Protease (trypsin)" contains both proteases and peptidases. The optimum conditions for the action of the two components of this enzyme are different. The optimal pH for its proteolytic activity is 7.5 to 8.5 at a temperature of between 40 and 52 C.

(9) Lysozyme. Prepared by Dr. William P. Switzer of the Veterinary Medical Research Institute, Iowa State College. A review of recent literature prepared by the Research Division of Armour and Co. (43) states that sources of lysozyme are wide spread in nature. This particular preparation was prepared from egg white. The enzyme possesses nucleolytic and mucolytic activity, but does not possess protease, kinase, amylase, lipase or phosphatase activity. Certain bacteria are susceptible to its action. Under various conditions the enzyme is readily destroyed by pepsin and trypsin. It is also inactivated and denatured by mechanical foaming.

(10) "Proteinase A". Manufactured by Sharp & Dohme Division of Merck & Co. Inc. The manufacturer (46) (49) states that "Proteinase A" is a crude preparation produced from the filtrates of a culture of an aerobic soil saprophyte. It possesses proteolytic activity, and degrades casein, denatured hemoglobin, gelatin, fibrin, fibrinogen, and lactalbumin. It does not attack native bovine, pork, or rat collagen. In solution it is inactivated in the presence or absence of a substrate by prolonged incubation at 50 to 60 C. The optimal temperature and pH for its proteolytic activity is 37 C. at pH 7.0. "Proteinase A" was supplied by the manufacturer in 5 ml. vials containing 25,000 units each. The potency of the enzyme is expressed in units determined by manufacturer's assay by a modification of the method of Kunitz.

(11) Pancreatic desoxyribonuclease. Manufactured by Sharp & Dohme Division of Merck & Co. Inc. The manufacturer (50) states that pancreatic desoxyribonuclease is prepared from frozen beef pancreas. It has the capability of rapidly degrading desoxyribonucleoprotein. The action is characterized as a depolymerization, since there is no release of inorganic phosphorus nor does any other readily dialyzable substance occur. The optimal pH for its proteolytic activity is 7.0 to 7.1. The manufacturer supplied the enzyme in 5 ml. vials containing 100,000 units. The potency

of pancreatic desoxyribonuclease is expressed in units as determined by manufacturer's assay based upon the rate at which it reduces the viscosity of thymonucleic acid.

(12) Streptokinase-streptodornase. Manufactured by the Lederle Laboratory Division of the American Cyanamid Co. The manufacturer states (37) that these enzymes are excreted by streptococcal organisms into the culture medium in which they are grown. They are separated from the medium, purified, and filtered before freezing and drying. Streptokinase acts indirectly upon fibrin or fibrinogen by activating a fibrinolytic enzyme in human serum, which then splits fibrin into polypeptides. Streptodornase acts directly upon desoxyribonucleoprotein and desoxyribonucleic acid, which are the main constituents in nuclei. Streptodornase does not act upon nuclei in living cells. The optimal pH for their proteolytic activity is 7.5. The manufacturer supplied the product in 5 ml. vials containing 100,000 units of streptokinase and at least 25,000 units of streptodornase. The potency of the enzymes is expressed in units as determined by the manufacturer's assay, method not stated.

2. Methods of procedure

a. Preparation of inoculum. Serial dilutions were prepared of trypsin, bromelain, ficin, chymotrypsin,

"Rhozyme P-11", "Protease 15", "Protease (trypsin)", and lysozyme. 1.0, 0.5, 0.25, 0.125, 0.1, 0.0625, 0.04687, 0.03125, and 0.01562 grams of each enzyme were suspended in a diluent composed of 1 part antibiotic solution and 9 parts physiological salt solution to make a total volume of 10 ml. Each dilution was mixed by flushing it 10 times with a separate, sterile 5 ml. pipette.

Similar dilutions of papain were prepared, with the exception that three additional dilutions were prepared. These dilutions contained 0.00020, 0.00010, and 0.00005 grams of papain respectively, per 10 ml. of diluent.

Serial dilutions of "Proteinase A" were prepared, in the same manner as above, with the exception that the unit of measurement was units rather than by weight. 25,000; 18,750; 12,500; and 1,560 units of the enzyme were suspended in 10 ml. of diluent.

Pancreatic desoxyribonuclease and streptokinase-streptodornase were suspended full strength, as supplied by the manufacturer, in 10 ml. of diluent. No serial dilutions were prepared of these two products.

b. Animal inoculations. Each enzyme preparation described above, was inoculated into 20 gram, swiss mice. Four mice were used per dilution. Each mouse received 0.03 ml. of inoculum intracerebrally, while under the influence of deep ether anesthesia.

The inoculated mice were observed for a period of 14 days. All deaths occurring during this period were attributed to the toxicity of the inoculum.

c. Controls. Mixtures of the antibiotic solution and physiological salt solution were prepared in 1:1, 1:1.5, 1:2.75, 1:4 and 1:9 proportions. Four, 20 gram swiss mice per dilution were inoculated and observed in a manner identical to that employed in the actual toxicity tests.

### B. Time Required to Degrade Brain Tissue by Digestion with Various Enzymes

1. Materials and apparatus

a. Diluents. Diluent A consisted of sterile physiological salt solution.

Diluent B consisted of a mixture of 9 parts physiological salt solution and 1 part antibiotic solution.

b. Substrate. The brain tissue employed as a substrate in the following determinations was obtained from 10 to 15 pound albino rabbits. The rabbits were sacrificed under deep ether anesthesia, and the fresh brain removed and stored in a sealed, sterile jar at -30 C. until used.

c. Proteolytic enzymes. Only five of the proteolytic enzymes, previously described, were employed. This group was comprised of trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)", and "Proteinase A".

2. Methods of procedure

a. Turbidimetric method employed. A modification of the Klett-Summerson method (31) for the determination of protein in cerebrospinal fluid was employed to determine the amount of time required at 37 C. for maximum digestion of brain tissue by each of the five proteolytic enzymes. The modification consisted of making four colorimeter readings of 1:100 dilutions of the sample at 0 hours, and at various time intervals subsequent to 0 hours. The average of the readings obtained at 0 hours served as the standard, and the average of each of the subsequent readings as the basis for calculation of the percentage of protein degradation.

b. Turbidimetric determinations. Four distinct groups of turbidimetric determinations were made as follows:

(1) Determination of the amount of degradation occurring in suspensions of enzymes in the absence of a substrate. Solutions of each of the following enzymes: trypsin 0.125 gm.; "Rhozyme P-11" 0.1 gm.; "Protease 15" 0.125

gm.; "Protease (trypsin)" 0.125 gm.: and "Proteinase A" 2,500 units; were prepared to a total volume of 10 ml. in diluent A. Each solution was prepared in a sterile, stoppered, test tube, and thoroughly mixed by flushing 10 times with a separate, sterile, 5 ml. pipette.

The control consisted of 10 ml. of diluent A mixed in a manner identical to that of the five enzyme solutions.

Colorimeter readings were made on the control and each of the five enzyme solutions immediately after preparation. These were recorded as the 0 hour readings. The control and samples were then incubated at 37 C. Subsequent colorimeter readings were made after 4 hours incubation, and again at the end of 24 hours incubation.

(2) Determination of the amount of degradation occurring in suspensions of enzymes in the presence of a substrate and antibiotics. A fresh rabbit brain was emulsified in a sterile, chilled, Waring blendor. The emulsified brain was diluted to a 20% suspension, by weight, in diluent B. This suspension constituted the stock substrate.

Standard solutions were made of each of the following enzymes: trypsin 0.25 gm.; "Rhozyme P-11 0.2 gm; "Protease 15" 0.125 gm.; "Protease (trypsin)" 0.25 gm.; and "Proteinase A" 5,000 units; to a total volume of 10 ml. in diluent B. Each standard solution was prepared in a sterile test tube, and throughly mixed by flushing 10 times with a separate,

sterile, 5 ml. pipette.

The samples for turbidimetric determinations were prepared by adding 5 ml. of the 20% brain suspension to 5 ml. quantities of each of the standard solutions of enzymes. Each sample was then mixed in a manner identical to that used above. This procedure resulted in a 10% suspension of brain in each of the five enzyme solutions. The final concentration of each enzyme was identical to that used in the previous group of determinations.

The controls consisted of a 10% brain suspension in diluent B. This suspension was mixed in a manner identical to the test samples, and then divided into two equal parts.

Colorimeter readings were made on the controls and each of the five samples immediately after preparation. These were recorded as the 0 hour readings. One control and the five samples were incubated at 37 C. for 24 hours. The duplicate control was held at 4 C. for a similar period of time. Colorimeter readings were made on the controls and the five samples at the end of this 24 hour period.

(3) <u>Determination of the rate of degradation occurring in suspensions of enzymes in the presence of antibiotics in conjunction with the presence or absence of a substrate.</u> A 20% suspension of fresh rabbit brain in diluent B was prepared in a manner identical to that used in the second group

of determinations above. This suspension constituted the stock substrate.

Standard solutions of each of the five enzymes, in diluent B, were made in a manner identical to that used in the second group of determinations.

Two sets of samples for colorimeter determinations were prepared. One set was prepared of a 10% suspension of brain in each of the five enzyme solutions. This set was prepared in a similar manner and was identical to the samples employed in the second group of determinations. The second set of samples consisted of 5 ml. of each standard solution of enzyme added to 5 ml. of diluent B. This set of samples was identical to those used in the first group of determinations, with the exception that diluent B was employed rather than diluent A.

Two sets of controls were prepared. One set consisted of a 10% suspension of brain in diluent B. This suspension was mixed in a manner identical to the test samples, and then divided into two equal parts. The other set consisted of 20 ml. of diluent B, which was divided into two equal parts.

Colorimeter readings were made on the two sets of controls and samples immediately after preparation. These were recorded as the 0 hour readings. A control and the five samples of each set were incubated at 37 C. for 24 hours.

The duplicate controls for the two sets of samples were held at 4 C. for a similar period of time. Colorimeter readings were made on the two sets of controls and samples at 2, 4, 6, 12 and 24 hour intervals, subsequent to 0 hours.

(4) <u>Determination of the amount of degradation occurring in suspensions of enzymes in, (a) the presence and absence of antibiotics without substrate, and (b) the presence of substrate with or without antibiotics</u>. Two fresh rabbit brains were separately emulsified in sterile, chilled, Waring blendors. One emulsified brain was diluted to a 20% suspension, by weight, in diluent A. The other brain was similarly suspended in diluent B. These two 20% suspensions constituted the stock substrates.

Two sets of standard solutions of each of the five enzymes were prepared in a manner identical to that used in the second and third groups of determinations. One set was prepared using diluent A and the other using diluent B.

Four sets of samples for colorimeter determinations were prepared in a manner similar to that used in the second and third group of determinations, with the following exceptions. The first set consisted of a 10% suspension of brain in diluent A in each of the five enzyme solutions in diluent A. The second set was identical to that used in the second and third groups of determinations and consisted of a 10% suspension of brain in diluent B in each of the five enzyme

solutions in diluent B. The third set was identical to that used in the first group of determinations and consisted of 5 ml. of each of the standard solutions of enzymes in diluent A. The fourth set was identical to that used in the third group of determinations and consisted of 5 ml. of each of the standard solutions of enzymes in diluent B added to 5 ml. of diluent B.

Four sets of controls were prepared. The first and second sets consisted of a 10% suspension of brain in diluents A and B respectively. These suspensions were mixed in a manner identical to the test samples, and then divided into two equal parts per control. The third and fourth set of controls consisted of 20 ml. of diluents A and B respectively, which were each divided into two equal parts.

Colorimeter readings were made on the four sets of controls and samples immediately after preparation. These were recorded as the 0 hour readings. A control and the five samples of each of the four sets were incubated at 37 C. for four hours. The duplicate controls for the four sets of samples were held at 4 C. for a similar period of time. Colorimeter readings were made on the four sets of controls and samples at the end of the four hour incubation period.

c. Calculation of results

(1) Calculations for percentage of decrease in

total protein. With X representing the average of the colorimeter readings at any given hour subsequent to 0 hours, the percentage of decrease in the total protein value for any given sample was calculated by the formula:

$$100 - 100 \times \frac{\text{average of colorimeter readings at X hour}}{\text{average of colorimeter readings at 0 hour}} =$$

the percentage of decrease in total protein value.

(2) Calculations for percentage of decrease in brain protein. The percentage of decrease in brain protein, for any given sample was determined by subtracting the sum of the percentage of decrease in total protein value of the respective enzyme and substrate controls from the percentage of decrease in total protein value of the given sample.

## C. Rabies Virus Elution in the Presence of Five Proteolytic Enzymes

### 1. Materials and apparatus

a. Diluents. Diluent A consisted of sterile physiological salt solution.

Diluent B consisted of a mixture of 9 parts Krebs-Ringer-phosphate solution, pH 7.6, and 1 part antibiotic solution. The Krebs-Ringer solution was prepared in the manner described

by Cohen (11) as modified by Graca (17), as follows:

100 parts of 0.90% NaCl (0.154 M) in distilled water
4 parts of 1.15% KCl (0.154 M) " " "
3 parts of 0.61% $CaCl_2$ (0.11 M) " " "
1 part of 3.82% $MgSO_4 \cdot 7$ HOH (0.154 M) " "

To 90 ml. of the Krebs-Ringer solution, 10 ml. of a phosphate buffer, pH 7.6, was added. The phosphate buffer was prepared by the method described by Umbreit (55).

The components of the Krebs-Ringer-phosphate solution were kept under refrigeration in stock solutions. The required amounts of Krebs-Ringer-phosphate solution, pH 7.6, were prepared fresh from these stock solutions each time they were used. Prior to use the freshly prepared buffer solutions were sterilized by autoclaving for 30 minutes at 15 pounds of steam pressure. After sterilization and immediately prior to use, the pH of the solution was verified with a Coleman pH electrometer, model 3A.

b. Experimental animals

(1) Rabbits. As described in section III.

(2) Mice. The mice used in the following procedures were obtained from the Veterinary Physiology Department of Iowa State College, (CFW strain). All the mice used were 20 gm., albino, swiss mice. No preference was given as

to the sex of the mice used.

c. Proteolytic enzymes. Five of the proteolytic enzymes, previously described, were employed. This group was comprised of trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)", and "Proteinase A".

d. Viruses

(1) Control viruses

(a) National Institutes of Health standard challenge virus #59. This is a fixed rabies virus, and is commonly referred to in the literature as standard challenge virus (CVS). This specimen was received from Dr. Killinger of the Fort Dodge Laboratories, Fort Dodge, Iowa. It is routinely supplied as a 20% mouse-brain suspension in a 2% horse serum and distilled water diluent. This virus will be referred to subsequently as CVS.

(b) Lederle R174BK222AP99 (7-20-54) rabies street virus. This particular strain is commonly referred to as the "New York" strain. It is employed in the potency test for avianized rabies vaccine as challenge virus. It was supplied by Lederle Laboratories to the Fort Dodge Laboratories, as a 20% canine salivary gland suspension in a 10% filtered and inactivated rabbit serum and buffered saline diluent. This virus will subsequently be referred to as the

"N.Y." strain.

(2) Test viruses. Four strains of street rabies virus, isolated from dogs, were obtained from the Virology Section, Fourth Army Area Medical Laboratory, Brooke Army Medical Center, Fort Sam Houston, Texas. The material received consisted of frozen specimens of canine brain tissue. All four of these strains were originally diagnosed as rabies by microscopic examination of impression smears prepared from the canine brain. The Wilhite method as described by Bohls (5) was employed as the straining method for the impression smears. These four strains will subsequently be referred to by the case number originally assigned by the Fourth Army Area Medical Laboratory, and the prefix FAAML. Case histories are as follows:

(a) FAAML 237. A canine head was submitted to the Virology Section on 14 August 1952. There was a history of military personnel having been bitten by the animal prior to its death. The suspected animal had been under the observation of a local civilian veterinarian for several days prior to death and he had made a clinical diagnosis of rabies.

A section of the semilunar ganglion was submitted to the Pathology Section of the laboratory. The pathologist's report stated that there were changes in semilunar ganglion characteristic of rabies.

(b) FAAML 309. A canine head was submitted to the Virology Section on 8 October 1952. There was a history of the suspect animal having attacked another dog and two military personnel at Fort Sam Houston, Texas. In the process of capturing the suspect its skull was fractured and it arrived at the Station Veterinarian's office in a comatose condition. The suspect died within 24 hours after capture, and a clinical diagnosis of rabies was made by the Station Veterinarian.

Negri bodies were observed in impression smears prepared from the animal's brain.

(c) FAAML 363. A canine head was submitted to the Virology Section on 26 November 1952. There was a history of the suspect having bitten a child, and two dogs at Kelly Air Force Base, Texas. The suspect died shortly after the attacks and was dead when it arrived at the Base Veterinarian's office.

Negri bodies were observed in impression smears prepared from the animal's brain.

(d) FAAML 372. A canine head was submitted to the Virology Section on 8 December 1952. There was a history of the suspect having snapped at children, and having attacked four military personnel and other animals at Lackland Air Force Base, Texas. The air policeman who captured

the suspect was bitten. He had to shoot the animal in order to restrain it. The suspect was dead on arrival at the Base Veterinarian's office.

Negri bodies were observed in impression smears prepared from the animal's brain.

e. Sera

(1) Immune serum. Lederle's concentrated anti-rabies serum was utilized as immune serum for serum-virus neutralization tests. Prior to use, the undiluted serum was dialyzed for 48 hours at 4 C. against 100 volumes of diluent A which was replaced at 12 hour intervals. After dialysis the serum was filtered through a Sietz E-K filter. These procedures were instituted to remove the preservative, as recommended by Dr. Koprowski (33).

(2) Normal serum. Normal, filtered (Sietz E-K) equine serum was used as the normal serum for the serum-virus neutralization tests. This serum was prepared by the Department of Veterinary Hygiene of Iowa State College.

2. Methods of procedure

a. Preparation of stock virus material. Each of the canine brains of the four FAAML virus strains were separately emulsified in a chilled Waring blendor. After emulsification

they were each diluted to a 40% suspension, by weight, in diluent B.

The CVS and "N.Y." strains of challenge virus were each diluted, by volume, to a 10% suspension in diluent B.

These suspensions of the four FAAML strains, and the two challenge strains were centrifuged for 15 minutes at approximately 2,000 rpm. After centrifugation, one rabbit per strain was inoculated intracerebrally with 0.1 ml. of supernate. All inoculations were performed under deep ether anesthesia.

The brain was harvested from each rabbit that died subsequent to inoculation. Impression smears were prepared from each brain, at the time it was harvested and microscopic examinations for Negri bodies were made according to the method described by Tierkel (54). All of the impression smears were stained according to Seller's method (47).

The whole brains of these rabbits were stored separately, at -30 C. in a mechanical freezer, and constituted the stock virus for the CVS, "N.Y.", and FAAML strains of virus.

b. Identification of the stock viruses. Each of the rabbit brains, which constituted the stock CVS, "N.Y.", and FAAML strains of virus, were separately emulsified in a sterile, chilled, Waring blendor. One gm. of each emulsion was used to prepare a 20% suspension, by weight, in diluent A of each of the six strains of stock virus. These 20%

suspensions were then subjected to serum-virus neutralization tests, performed according to the method of Koprowski and Johnson (34).

Normal, filtered, undiluted equine serum was employed as the normal serum for all serum-virus neutralization tests performed. Lederle's concentrated anti-rabies serum, dialyzed and filtered as previously described, was employed as the immune serum. Immediately prior to use, the concentrated immune serum was diluted 1:10 with diluent A, as recommended by Burns (6) and Koprowski (33).

The $LD_{50}$ endpoints in all of the serum-virus neutraliation tests performed were calculated by the method of Reed and Muench (40). This method for calculating 50% endpoints was used throughout the entire problem.

The unused portion of each 20% suspension of stock virus was sealed and stored at -30 C., in a mechanical freezer.

c. Attempted elution of stock viruses by proteolytic enzymatic digestion

(1) Enzyme standard solutions. Standard solutions of each of five proteolytic enzymes were prepared in diluent B. Each standard solution was prepared to contain a specific amount, by weight, of a particular enzyme per 9 ml. of diluent, as follows: trypsin 0.125 gm.; "Rhozyme P-11" 0.1 gm.; "Protease 15" 0.125 gm.; "Protease (trypsin)" 0.125 gm.; and

"Proteinase A" 2,500 units. Each of these standard solutions were thoroughly mixed in a Waring blendor.

(2) <u>Digestion of stock virus brain emulsions with proteolytic enzymes</u>. One gm. quantities of each emulsion of the stock viruses, as described for the serum-virus neutralization tests were weighed into each of seven 16 x 150 mm sterile, test tubes. This procedure resulted in seven tubes of each stock virus with each tube containing 1 gm. of emulsified brain. The tubes containing a particular stock virus were identified as to the virus they contained and numbered 1 through 7.

Tubes number 1 through 7 of CVS, "N.Y.", and FAAML strains of stock virus were diluted with the diluents indicated below to form 10% suspensions, by weight, of emulsified brain. These suspensions were throughly mixed by flushing 10 times with a separate sterile 5 ml. pipette.

Tube No. 1 diluent B

Tube No. 2 diluent B

Tube No. 3 trypsin standard solution

Tube No. 4 "Rhozmye P-11" standard solution

Tube No. 5 "Protease 15" standard solution

Tube No. 6 "Protease (trypsin)" standard solution

Tube No. 7 "Proteinase A" standard solution

Tube number 1 of each stock virus suspension was used as the 4 C. temperature control, and tubes numbered 3 through

7 were identified as to the particular enzyme standard solution with which the suspension was prepared. The 4 C. temperature controls for each of the stock virus suspensions were placed in a mechanical refrigerator at 4 C. for a period of 4 hours. The 37 C. temperature controls and the suspensions of each stock virus in the standard solutions of enzymes were incubated at 37 C. in a water bath for 1 hour. After the initial heat transfer period of 1 hour in the water bath, they were placed in a roller-tube drum, and incubated at 37 C. in a dry heat incubator for an additional 3 hour period.

(3) Preparation of inoculum for animal inoculation. At the end of the 4 hour incubation period, as described above, all suspensions of each strain of stock virus were centrifuged for 15 minutes at approximately 2,000 rpm. The supernate of each suspension was transferred to a sterile test tube and properly identified.

Serial ten-fold dilutions, $10^{-2}$ through $10^{-5}$, in diluent B were prepared from each supernate. The supernate recovered from each original suspension was designated as the $10^{-1}$ dilution. These dilutions were prepared by the same method employed in the preparation of serial ten-fold dilutions for the serum virus neutralization tests described by Koprowski and Johnson (34).

(4) Titration, in animals, of serial dilutions of

stock virus brain emulsions subjected to proteolytic enzymatic digestion. For the infectivity titration of the CVS, "N.Y.", and FAAML strains of stock virus, swiss mice, weighing 20 gms. each, were selected. One hundred and forty of these mice were divided into 7 groups of 20 mice each. One group was used for the supernate of each of the temperature controls, and enzyme standard solution suspensions of each stock virus. Each group of 20 mice was further divided into 5 sets of 4 mice each. One set of mice per dilution, was inoculated with each of the ten-fold serial dilutions prepared per strain of stock viruses, as described above. Each mouse received 0.03 ml. of inoculum intracerebrally. All mouse inoculations were performed by the method described by Koprowski (32).

All inoculated mice were observed for a period of 21 days. Deaths occurring after the fifth day following inoculation were considered as rabies deaths. Mice which survived the twenty-first day of observation, but exhibited symptoms of rabies at the end of the twenty-first day were also considered as rabies deaths. The $LD_{50}$ was calculated for the serial dilutions of the supernates of each temperature control and enzyme standard solution suspensions of each strain of stock virus.

## D. Challenge of Mice Which Survived in the Titration of Serial Dilutions of Stock Virus Brain Emulsions Subjected to Proteolytic Enzymatic Digestion

### 1. Materials and apparatus

a. Diluent. A mixture of 9 parts Krebs-Ringer-phosphate solution, pH 7.6, and 1 part antibiotic solution was employed as the standard diluent.

b. Experimental animals. The mice employed as controls in the following procedures were obtained from the Veterinary Physiology Department of Iowa State College, (CFW strain). All the mice used were 20 gm., albino, swiss mice. No preference was given as to the sex of the mice used.

c. Challenge viruses. The rabbit brain emulsions prepared from the first rabbit passage of CVS, "N.Y.", and FAAML 237, 309, 363, and 372 strains of stock virus, and which were employed in the serum-virus neutralization tests were employed as the challenge viruses. Portions of these rabbit brain emulsions of each stock virus had been stored in a mechanical freezer at -30 C. for a period of 35 days prior to being used. The titre and identity of each of these viruses were previously established by the serum-virus neutralization tests.

2. Methods of procedure

a. Preparation of challenge virus suspensions. The titre of the challenge virus suspensions were based on the $LD_{50}$ calculated for each titration of the serial dilutions of the 4 C. temperature controls of the stock viruses as described in the preceeding methods of procedure. Each homologous strain of stock virus rabbit brain emulsion was suspended in that quantity of diluent necessary to contain 4 $LD_{50}$ in each 0.03 ml. of inoculum. Each suspension of homologous challenge virus was thoroughly mixed by flushing 10 times with a separate, sterile 5 ml. pipette.

b. Inoculation of surviving mice with homologous challenge virus. At the conclusion of the 21 day observation period of the titrations of serial dilutions of stock virus brain emulsions, subjected to proteolytic enzymatic digestion, the surviving mice were challenged. All survivors including those of the 4 C. and 37 C. temperature controls were challenged without exception. Each mouse was anesthetized with ether and received 0.03 ml. intracerebrally of the homologous challenge virus suspension.

c. Controls. For each homologous challenge virus suspension employed, six normal mice were inoculated. Each of the six mice were anesthetized with ether and received 0.03 intracerebrally of the challenge virus suspension.

d. Observation and recording of results. Each mouse, survivors and controls, inoculated with a homologous challenge virus suspension was observed for a period of 21 days. Deaths occurring after the fifth day following inoculation were considered as rabies deaths. Mice which survived the twenty-first day of observation, but exhibited symptoms of rabies at the end of the twenty-first day were also considered as rabies deaths.

Results were recorded on the basis of the ratio of deaths to survivors subsequent to challenge inoculations.

## E. Attempts to Immunize Mice against Rabies by a Single Inoculation of a Supernate of CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

1. Materials and apparatus

a. Standard diluent. A mixture of 9 parts Krebs-Ringer-phosphate solution, pH 7.6, and 1 part antibiotic solution was employed as the standard diluent.

b. Experimental animals

(1) Rabbits. As described in section III.

(2) Mice. The mice employed in the following

procedures were obtained from Stokley-Peterson Farms, Madison, Wisconsin, (S-P strain). All the mice used were 20 gm., female, albino, swiss mice.

c. Proteolytic enzymes. "Rhozyme P-11", and "Protease 15" were employed as indicated in the methods of procedure. Both of these proteolytic enzymes have been previously described.

d. Stock virus. A 20% suspension of rabbit brain emulsion from the first rabbit passage of the CVS strain of rabies virus was employed as the stock virus.

e. Control vaccine. Phenolized rabies vaccine of caprine origin, produced by Pitman-Moore Laboratories, serial number 692656, was employed as the control vaccine. This product was originally supplied as a 20% suspension, but was diluted to a 10% suspension with standard diluent for use as the control vaccine.

2. Methods of procedure

a. Second rabbit passage of CVS strain of rabies virus. Two rabbits were anesthetized with ether and inoculated intracerebrally with a 20% suspension of rabbit brain emulsion from the first rabbit passage of the CVS strain of rabies virus. Each rabbit received 0.25 ml. of inoculum.

The rabbits were sacrificed on the seventh day subsequent to inoculation and their brains were harvested and placed in a sterile, chilled, Waring blendor cup. The brains were emulsified to form a pool of stock rabbit brain emulsion. Two gm. quantities of this pooled rabbit brain emulsion were weighed out and transferred to each of seven sterile 16 x 150 mm. test tubes. The tubes were identified as containing the second rabbit passage of the CVS strain of rabies virus.

The contents of two of the seven tubes were diluted to a 20% suspension, by weight, with standard diluent and equally divided among four vials. These 20% suspensions were stored in a mechanical freezer at -30 C. for later use as stock and challenge virus and live virus antigen.

b. Preparation of experimental vaccines

(1) "Rhozyme P-11" vaccine number 1. A standard solution containing 0.2 gm. of "Rhozyme P-11" per 8 ml. of standard diluent was prepared. Two tubes containing 2 gms. each of the rabbit brain emulsion from the second rabbit passage of the CVS strain of rabies virus were diluted to a 20% suspension, by weight, with "Rhozyme P-11" standard solution.

These suspensions were incubated for 1 hour at 50 C. in a water bath. After the initial heat transfer period of 1 hour in the water bath, they were placed in a roller-tube drum and incubated for an additional 3 hour period at 50 C.

in a dry heat incubator.

At the end of the 4 hour incubation period both suspensions were centrifuged for 15 minutes at approximately 2,000 rpm. The supernate of each suspension was transferred to a sterile erlenmeyer flask to form a pool of the supernates. The volume of the pooled supernate was doubled by adding an equal volume of the standard diluent. The diluted, pooled supernate was thoroughly mixed by repeated flushings with a 10 ml. sterile pipette. After mixing the supernate was equally divided among four sterile screw-cap vials. Three vials were labeled as "Rhozyme P-11" vaccine number 1, and one vial was labeled as "Rhozyme P-11" antigen number 1. This material was stored in a mechanical freezer at -30 C.

(2) "Protease 15" vaccine number 1. The procedure described above was repeated with the modification that the enzyme standard solution contained 0.25 gm. of "Protease 15" in place of "Rhozyme P-11". Three vials of the diluted, pooled supernate were labeled as "Protease 15" vaccine number 1 and one vial was labeled as "Protease 15" antigen number 1. This material was stored in a mechanical freezer at -30 C.

c. Test of viability of experimental vaccines. "Rhozyme P-11" vaccine number 1, and "Protease 15" vaccine number 1 were both titrated by intracerebral inoculation in mice to determine if either vaccine contained live virus.

Serial ten-fold dilutions, $10^{-1}$ through $10^{-3}$, in standard diluent were prepared for each vaccine. The undiluted vaccines were designated as the $10^{-1}$ dilutions.

For each vaccine five mice were inoculated per each serial dilution. Each mouse was anesthetized with ether and received 0.03 ml. of inoculum. All mice were observed for a 14 day period after inoculation.

d. Titration, in mice, of serial dilutions of the second rabbit passage of CVS strain of rabies virus. One tube, containing 2 gms., of the rabbit brain emulsion from the second rabbit passage of the CVS strain of rabies virus was diluted to a 10% suspension, by weight, with the standard diluent. This 10% suspension was designated as the $10^{-1}$ dilution and from it serial ten-fold dilutions, $10^{-1}$ through $10^{-8}$, were prepared in the standard diluent. The procedure employed was identical to that described above in the test of viability of the experimental vaccines. That portion of the 10% suspension which was not employed in the serial ten-fold dilutions was labeled and stored in a mechanical freezer at -30 C.

Six mice were inoculated intracerebrally per serial dilution, $10^{-1}$ through $10^{-8}$. Each mouse was anesthetized with ether and received 0.03 ml. of inoculum. Inoculations were commenced with the $10^{-8}$ dilution and proceeded in order to the $10^{-1}$ dilution.

The inoculated mice were observed for 14 days after

inoculation. Deaths occurring after the fifth day following inoculation were considered as rabies deaths. The $LD_{50}$ of each titration was calculated and from this data the number of $LD_{50}$ in 0.03 ml. of undiluted rabbit brain emulsion was calculated and expressed logarithmically.

e. Potency test of experimental vaccines

(1) Attempted immunization of mice with experimental vaccines. Thirty mice were inoculated intraperitoneally per each experimental vaccine. Each mouse was anesthetized with ether and received a single inoculation of 0.5 ml. of experimental vaccine. After inoculation the mice were observed for 14 days.

(2) Vaccine control. Thirty mice were inoculated intraperitoneally with the control vaccine described under materials and apparatus. The procedure followed was identical to that employed with the experimental vaccines.

(3) Preparation of challenge virus dilutions. Using the standard diluent, serial dilutions were prepared of the rabbit brain emulsion from the second rabbit passage of the CVS strain of rabies virus. Based on the number of $LD_{50}$ in 0.03 ml. of undiluted rabbit brain emulsion, as determined in the titration described above, the serial dilutions were prepared to contain approximately 12,443; 9,331; 6,221; 2,110; 1,244; and

124 $LD_{50}$ per 0.03 ml. Each dilution was thoroughly mixed by flushing 10 times with a separate, sterile, 1 ml. pipette.

(4) Challenge of inoculated mice. On the fourteenth day of the post-inoculation period, each group of 30 mice inoculated with experimental and control vaccines, were divided into 6 lots of five mice each. One lot of mice of each group was inoculated intracerebrally per serial dilution of challenge virus as described above. Each mouse was anesthetized with ether and received 0.03 ml. of inoculum.

The challenged mice were observed for an additional period of 14 days. Deaths occurring after the fifth day following inoculation were considered as rabies deaths.

(5) Challenge control. A group of 30 unvaccinated mice was divided into 6 lots of five mice each. One lot of mice was inoculated intracerebrally per each serial dilution of challenge virus. The procedure followed was identical to that employed in the challenge of vaccinated mice.

The challenge control group of mice were observed for a 14 day period. This observation period paralleled that of the challenged mice.

## F. Attempts to Immunize Mice against Rabies by Multiple Inoculations of Vaccines Produced from CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

### 1. Materials and apparatus

a. Stock and challenge viruses. Twenty per cent suspensions of rabbit brain emulsion from the second rabbit passage of the CVS strain of rabies virus were employed as the stock and challenge viruses.

b. Control vaccine. Phenolized rabies vaccine of caprine origin, produced by Pitman-Moore Laboratories, serial number 692,665, was employed as the control vaccine.

c. Apparatus. Sterile mortars and pestles and sterile alundum were employed to emulsify mouse brain material, as indicated in methods of procedure.

### 2. Methods of procedure

a. Third rabbit passage of CVS strain of rabies virus. Six rabbits were anesthetized with ether, and inoculated intracerebrally with a 20% suspension of stock virus. Each rabbit received 0.25 ml. of inoculum.

The rabbits were sacrificed on the seventh day subsequent

to inoculation, and their brains were harvested and placed in a sterile, chilled, Waring blendor cup. The brains were emulsified to form a pool of stock rabbit-brain emulsion. One gm. quantities of this pooled rabbit brain emulsion were weighed out, and transferred to each of 48 sterile, 16 x 150 mm. test tubes. These tubes were identified as containing the third rabbit passage of the CVS strain of rabies virus.

b. Titration, in mice, of serial dilutions of the third rabbit passage of CVS strain of rabies virus. Serial ten-fold dilutions of a 10% suspension of the rabbit brain emulsion from the third rabbit passage of the CVS strain of rabies virus was titrated by intracerebral inoculation into mice. The procedure employed was identical to the procedure used for the titration, in mice, of serial dilutions of the second rabbit passage of the homologous virus. The $LD_{50}$ of the titration was calculated and on the basis of this data the number of $LD_{50}$ in 0.03 ml. of undiluted rabbit brain emulsion was calculated and expressed logarithmically.

c. Preparation of experimental and control vaccines

(1) "Rhozyme P-11" vaccine suspension number 2 and vaccine supernate number 2. A standard solution, containing 0.1 gm. of "Rhozyme P-11" per 9 ml. of standard diluent, was prepared. Sixteen tubes, each containing 1 gm. of the rabbit brain emulsion from the third rabbit passage of the CVS strain

of rabies virus, were diluted to 10% suspensions, by weight, with the "Rhozyme P-11" stock solution.

These suspensions were incubated for 1 hour at 50 C. in a water bath. After the initial period of 1 hour in the water bath, they were transferred to a roller-tube drum and incubated for an additional 3 hour period at 37 C. in a dry heat incubator.

At the end of the 4 hour incubation period, the suspensions were removed from the roller drum, and seven of the tubes were identified as "Rhozyme P-11" vaccine suspension number 2. This material was stored in a mechanical refrigerator at 4 C.

The remaining nine tubes of 10% suspensions were centrifuged for 15 minutes at approximately 2,000 r.p.m. The supernate of each suspension was transferred to a sterile erlenmeyer flask to form a pool of the supernates, and thoroughly mixed by repeated flushings with a 10 ml. sterile pipette. After mixing, the supernate was equally divided among eight sterile screw-cap vials. Seven vials were labeled as "Rhozyme P-11" vaccine supernate number 2, and stored in a mechanical refrigerator at 4 C. One vial was labeled as "Rhozyme P-11" antigen number 2, and stored in a mechanical freezer at - 30 C.

(2) <u>"Protease 15" vaccine suspension number 2 and vaccine supernate number 2</u>. The procedure described above

was repeated employing a standard enzyme solution containing 0.125 gm. of "Protease 15" per 9 ml. of standard diluent 2. Seven tubes each of "Protease 15" vaccine suspension number 2, and "Protease 15" vaccine supernate number 2; and one tube of "Protease 15" antigen number 2, were prepared. The vaccines were stored in a mechanical refrigerator at 4 C., and the antigen was stored in a mechanical freezer at - 30 C.

(3) Heat inactivated CVS vaccine suspension and vaccine supernate. Sixteen tubes each containing 1 gm. of the rabbit brain emulsion from the third rabbit passage of the CVS strain of rabies virus, were diluted to 10% suspensions, by weight, with standard diluent. The same procedures, as outlined above, employed for the production of the experimental enzyme vaccine suspensions and vaccine supernates were used to produce seven tubes of heat inactivated CVS vaccine suspension and seven tubes of vaccine supernate. These vaccines were stored in a mechanical refrigerator at 4 C.

(4) Control vaccine suspension and vaccine supernate. Eighty ml. of the control vaccine, which was originally supplied as a 20% suspension, was diluted with standard diluent to form a 10% suspension. Ten ml. quantities of this 10% suspension were delivered into each of 16 sterile screw-cap vials. Seven of these vials were set aside and labeled as control vaccine suspension.

The remaining nine tubes of 10% suspensions of control vaccine were centrifuged for 15 minutes at approximately 2,000 rpm. The supernate of each suspension was transferred to a sterile erlenmeyer flask to form a pool of the supernates, and thoroughly mixed by repeated flushings with a 10 ml. sterile pipette. After mixing, the supernate was equally divided among seven sterile screw-cap vials, and labeled as control vaccine supernate. These preparations were stored in a mechanical refrigerator at 4 C.

d. Test of viability of experimental vaccines. The six experimental vaccine preparations, described above, were titrated by intracerebral inoculation in mice, to determine if any of the vaccines contained live virus. The method employed was identical to that used to test the viability of "Rhozyme P-11" vaccine number 1, and "Protease 15" vaccine number 1, as previously described.

The brain of each mouse that died on or after the fifth day subsequent to intracerebral inoculation with an experimental vaccine, was harvested. As the brain material from each vaccine viability test was harvested, it was pooled and stored at -30 C. in a mechanical freezer. At the end of the initial 14 day observation period, the various pools of brain material harvested from each of the viability tests were emulsified. A 20% suspension, by weight, was prepared of each emulsion. Each 20% suspension was then diluted to a 1:360

dilution of original mouse brain. This dilution was calculated to contain 100 $LD_{50}$ per 0.03 ml., based upon the results of the titration in mice of the third rabbit passage of the CVS strain of rabies virus.

The 1:360 dilutions, of each suspension, were subjected to the modified serum-virus neutralization test, performed according to the method of Koprowski and Johnson (34). Filtered, undiluted equine serum was employed as the normal serum. A 1:100 dilution of Lederle's concentrated antirabies serum which had been dialyzed and filtered as previously described was employed as the immune serum.

c. Potency test of experimental vaccines

(1) Attempted immunization of mice with experimental and control vaccines. Separate groups of mice were inoculated with each of the six experimental and two control vaccine preparations. The procedure employed was the same as recommended by Habel (20) in his modified test for potency, with the following modifications:

(a) Due to the toxicity of some of the experimental vaccine preparations, each group of mice immunized contained 25 rather than 20 mice as recommended by Habel. At the end of the immunization procedures, only 20 of the survivors in each group of mice were challenged.

(b) The mice received 0.5 ml. of a 10% sus-

pension per intraperitoneal dose of vaccine, rather than 0.25 ml. of a 20% suspension as recommended by Habel.

(2) Challenge of inoculated mice. A 1:79 suspension of the challenge virus was prepared in standard diluent. This dilution was calculated to contain 500 $LD_{50}$ based upon the titration, in mice, of serial dilutions of the second rabbit passage of CVS strain of rabies virus. The method employed in the challenge procedure was the same as recommended by Habel, as indicated previously, with the exception that groups of five control mice received the challenge virus diluted $10^{-4}$, as well as the $10^{-5}$ through $10^{-7}$ dilutions recommended by Habel.

(3) Calculation of results

(a) Challenge results. The results of the challenge of mice inoculated with the six experimental, and two control vaccine preparations were calculated on the basis of the percentage of survivors.

(b) Challenge control. The $LD_{50}$ of the titration of challenge virus in normal mice was calculated and on the basis of this data the number of $LD_{50}$ in 0.03 ml. of undiluted challenge virus was calculated and expressed logarithmically. From this calculation, the actual number of $LD_{50}$ in the 1:79 dilution of challenge virus was determined.

## G. Evaluation of Complement-fixing Antigens Produced from CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

### 1. Materials and apparatus

a. Standard diluent. Physiological salt solution, as described in investigation A, was employed as the standard diluent.

b. Antigens and antigen components

(1) Experimental antigens. Supernates of 10% suspensions of "Rhozyme P-11" vaccines number 1 and 2, and "Protease 15" vaccines number 1 and 2, were employed as experimental antigens. These antigens were designated as "Rhozyme P-11" antigen number 1 and 2, and "Protease 15" antigen number 1 and 2.

(2) Live virus antigens. Supernates of 10% suspensions of rabbit brain emulsion from the second rabbit passages of "N.Y." and CVS strains of rabies virus were employed as live antigens. The diluent employed in the preparation of these live antigens was the same as that employed in the preparation of the experimental antigens. These antigens were designated as "N.Y." live virus antigen and CVS live virus antigen.

(3) Normal rabbit brain antigen. A supernate of a 10% suspension of normal rabbit brain emulsion was employed as the normal rabbit brain antigen. The diluent employed in the preparation of this antigen was the same as that employed in the preparation of the experimental and live virus antigens.

(4) Antigen components. Dilutions were prepared of each of the following standard solutions: Krebs-Ringer-phosphate pH 7.6, antibiotic, "Rhozyme P-11" and "Protease 15". The various dilutions were calculated to contain 2 concentrations of the particular antigen component identical to that of the experimental second control brain antigens. These four preparations were tested to determine the anti-complementary activity of each of the component parts of the antigens. They will subsequently be referred to as antigen components.

c. Homolysin. Filtered (Sietz E-K), undiluted pooled serum obtained from rabbits sensitized to washed, sheep red blood cells, was employed as hemolysin. This material was prepared by the Veterinary Hygiene Department of Iowa State College.

d. Sheep red blood cells. Fresh, citrated, normal sheep blood was used as the source of sheep red blood cells. The cells were washed three times in 3-4 volumes of standard

diluent. After the final washing they were diluted to a final concentration of 3%, by volume, in standard diluent.

e. Complement. Pooled, normal guinea pig serum, obtained from eight guinea pigs, was employed as complement. The pooled complement was dispensed into 5 ml. vials, sealed, and stored in a mechanical freezer at -30 C. until used.

f. Sera

(1) Anti-rabies sera

(a) Equine serum. Dialyzed and filtered (Sietz E-K), Lederle's concentrated anti-rabies serum of equine origin was employed as equine anti-rabies serum.

(b) Rabbit serum. Pooled sera, collected from three rabbits which were immunized against rabies, was employed as the rabbit anti-rabies serum. The rabbits were immunized by six intraperitoneal inoculations of 3 ml. doses of 20%, phenolized rabies vaccine, of caprine origin, serial number 692,668, produced by Pitman-Moore Laboratories. The inoculations were made at 2 day intervals. The sera was collected and pooled on the fourteenth day following the first inoculation.

(2) Normal sera

(a) Equine sera. Normal, filtered (Sietz E-K), equine sera obtained from two apparently normal horses, was

employed as the normal equine sera. These sera were not pooled. They were separately identified as equine serum number 1 and equine serum number 2.

(b) <u>Rabbit serum</u>. Pooled sera obtained from three apparently normal rabbits was employed as the normal rabbit serum.

g. <u>Serum protein fractions</u>. According to Dukes (14) the antibodies of the blood appear to be definitely associated with the globulins. They can be separated from the blood in the globulin fraction of the proteins. Bodansky (4, p. 23) states that the separation of serum protein into two fractions may be accomplished by half-saturation with ammonium sulfate. The globulin is salted out while the albumin remains in solution.

Each of the normal equine sera was half-saturated with ammonium sulfate, by adding an equal volume of a saturated solution of ammonium sulfate to a given volume of serum. The resultant mixtures were centrifuged for 15 minutes at 3,000 rpm. The supernate, containing the albumin fraction of the serum protein, of each sera was recovered. It was dialyzed for 16 hours aginst cold flowing tap water. The salted out globulin fraction of the serum protein of each sera was resuspended in standard diluent to original volume. This material was also dialyzed for 16 hours against cold flowing tap water.

After dialysis, the albumin and globulin fractions of each sera were tubed, and properly identified.

h. Apparatus

(1) 13 x 100 mm. test tubes were used in all serological test procedures.

(2) 16 x 150 mm. test tubes were used for making serum, antigen, and complement dilutions.

2. Method of procedure

The schedules employed in the following procedures for hemolysin, complement, antigen titration, and complement-fixation tests were the same as those described by Casals *et al* (8) in their specific complement-fixation test for infection with poliomyelitis virus.

a. Neutralization tests. Each of the anti-rabies sera and normal sera, described under materials and apparatus, were checked for the presence of rabies neutralizing antibodies. These sera were employed as immune and normal sera in a series of serum-virus neutralization tests in which the CVS strain of rabies virus was used as the standard virus. The tests were performed according to the method of Koprowski and Johnson (34).

b. Initial standardization of materials

(1) Titration of hemolysin. The titration of hemolysin was performed according to Schedule 1.

Schedule 1. Titration of hemolysin

| Amount of each component expressed in ml. | Hemolysin dilutions | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 |
| Amt. of ea. hemolysin dilution | 1.01 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Complement 1:25 dilution | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Standard diluent | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Sheep RBC 3% suspension | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

(Read* after 30 minutes in a 37 C. water bath.)

*4 = complete complement fixation (no hemolysis)
3, 2, 1 = partial complement fixation (partial hemolysis)
0 = no complement fixation (complete hemolysis).

One unit of hemolysin was defined as the smallest amount of hemolysin capable of producing complete hemolysis of 0.1 ml.

of a 3% suspension of sheep red blood cells in the presence of an excess of complement. A 1:3,000 dilution of hemolysin contained 1 unit per 0.1 ml. Therefore, 0.1 ml. of a 1:1,000 dilution would contain 3 units.

(2) Titration of complement. The preliminary titration of complement was performed according to Schedule 2.

Schedule 2. Titration of complement

| Amount of each component expressed in ml. | Serial dilutions of complement* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.20 | 0.18 | 0.16 | 0.14 | 0.12 | 0.12 | 0.08 | 0.07 | 0.06 | 0.04 |
| Amt. of ea. complement dilution | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Standard diluent | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Sensitized cells 0.1 of 3% sheep RBC 0.1 of 3 units of hemolysin | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| | (Read after 30 minutes in a 37 C. water bath.) | | | | | | | | | |

*A 1:25 dilution of complement was prepared and from it a graduated series of dilutions containing 0.20 through 0.02 ml. of the original dilution of complement in each 0.3 ml.

One unit of complement was defined as the smallest amount of complement giving complete hemolysis in the presence of 3 units of hemolysin. The results of this titration indicated the dilution of complement required to contain 2 units in 0.2 ml. after 30 minutes at 37 C.

(3) Titration of experimental antigens for hemolytic activity. Serial dilutions of "Rhozyme P-11" antigens number 1 and 2 and "Protease 15" antigens number 1 and 2 were titrated for evidence of hemolytic activity according to Schedule 3.

Schedule 3. Titration of antigens for hemolytic activity

| Amount of each component expressed in ml. | Antigen dilutions | | | | |
|---|---|---|---|---|---|
| | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 |
| Amount of each antigen dilution | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Complement, 2 units in | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Standard diluent | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Sheep RBC, 1.5% suspension | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

(Read after 30 minutes in a 37 C. water bath.)

(4) Titration of antigen components for anti-complementary activity. The anti-complementary activity of a 1:2 dilution of each of the four antigen components was measured as follows.

Five series of complement titrations were performed according to Schedule 2. One series was identical to that shown in Schedule 2. It served as the normal control. The other four series were similar to that shown in the schedule with the exception that one series was designated for each antigen component. In each of these series of complement titrations, 0.2 ml. of a 1:2 dilution of an antigen component was substituted for the 0.2 ml. of standard diluent shown in the schedule.

The smallest amount of complement in the presence of each antigen component required to produce complete hemolysis was determined. When compared to the normal control, this value served as an indication of the anti-complementary activity of each antigen component.

(5) Titration of complement in the presence of serial dilutions of antigens. A titration of complement was performed in the presence of each dilution of serial two-fold dilutions, 1:2 through 1:16, of each of the following antigens: "Rhozyme P-11" antigens number 1 and 2; "Protease 15" antigens number 1 and 2; and "N.Y." and CVS live virus antigens. Twenty-four titrations were performed simultaneously

according to Schedule 4.

As controls, two titrations of complement were performed according to Schedule 1. One of these standard diluent controls was incubated and read prior to the beginning of the test. The second control was handled and read in the same manner as the 24 titrations of complement in the presence of antigens.

Schedule 4. Titration of complement in the presence of serial dilutions of antigens

| Amount of each component expressed in ml. | Serial dilutions of 1:25 complement in 0.3 ml. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.20 | 0.18 | 0.16 | 0.14 | 0.12 | 0.10 | 0.08 | 0.07 | 0.06 | 0.04 |
| Amt. of ea. complement dilution | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| Antigen dilution | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| (Hold for 18 hrs. at 4 C. followed by 30 minutes at room temp.) | | | | | | | | | | |
| Sensitized cells 0.1 3% sheep RBC 0.1 3 units of hemolysin | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| (Read after 30 minutes in a 37 C. water bath) | | | | | | | | | | |

The smallest amount of complement in the presence of each antigen dilution required to produce complete hemolysis when compared with the normal control served as an indication of the anti-complementary activity, if any, of each antigen. It also indicated the amount of complement required, in the presence of the live virus antigens, to insure the availability of 2 units of complement at the end of 18 hours at 4 C. These results also indicated that 0.1 ml. of a 1:1,000 dilution of hemolysin provided adequate amounts of hemolysin for the test in the presence of the live virus antigens.

c. Standardization of materials for actual complement-fixation tests

(1) Re-titration of hemolysin and complement. All dilutions of the "Rhozyme P-11" antigens and some of the dilutions of the "Protease 15" antigens exhibited marked anti-complementary activity in the previous titration of complement in the presence of serial dilutions of antigens. Since no end-point was reached with these antigens in the previous titrations, it was necessary to re-titrate the hemolysin and complement. This was done in order to determine the dilution factors required for hemolysin and complement to provide the proper concentration of these materials in subsequent serological procedures employing any of these antigens.

(a) Titration of hemolysin in presence of

antigens. Eight sets of hemolysin titrations were performed to determine the dilution of hemolysin which would be required, in the presence of each of the "Rhozyme P-11" and "Protease 15", antigens, to contain 3 units of hemolysin in 0.1 ml. Schedule 1, previously described for the titration of hemolysin, was used with the following modifications.

A 1:2 dilution was prepared of each of the following antigens: "Rhozyme P-11" antigens number 1 and 2; and "Protease 15" antigens number 1 and 2. Two-tenths ml. volumes of these antigen dilutions were substituted for the same volume of standard diluent described in Schedule 1.

Due to the anti-complementary activity previously exhibited by the antigens being tested, two dilutions of complement, 1:15 and 1:20, were used. This was done in an attempt to be certain that an excess of complement was provided. Two hemolysin titrations were performed simultaneously with each antigen dilution, one with each dilution of complement.

No end-point was reached with the "Rhozyme P-11" antigens due to their extreme anti-complementary activity. End-points were obtained with both "Protease 15" antigens. Three units of hemolysin were determined to be contained in 0.1 ml. of a 1:500 dilution.

(a) Titration of complement in the presence of "Protease 15" antigen number 2. Having established that a

1:500 dilution of hemolysin contained 3 units per 0.1 ml. in the presence of both "Protease 15" antigens, it was necessary to perform a 30 minute and an 18 hour titration of complement in the presence of one of the antigens. This was done to determine the amount of complement required to provide 2 units per 0.2 ml. in the presence of the antigen.

The procedure employed was the same as described in Schedule 4, with the exception that a 1:30 dilution of complement was employed. On the basis of the results attained, one unit of complement was defined as the smallest amount of complement giving complete homolysis after 18 hours at 4 C., in the presence of the antigen and 3 units of hemolysin as determined in the presence of the antigen.

(2) <u>"Box" titration of antigens.</u>* A "box" titration of two-fold serial dilutions, 1:2 through 1:16, of "Protease 15" antigen number 2; and "N.Y." and CVS live virus antigen was performed. These titrations were performed against equine and rabbit anti-rabies sera. The smallest quantity of antigen which would fix complement in the presence of the highest dilution of serum was determined for each of the sera.

(a) <u>Actual test</u>. Schedule 5 as outlined below was identical for each serum and all antigen dilutions. The sera to be tested were diluted 1:2 and the native complement

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*"Box" titration of antigens is the term used by Casals <u>et al</u>. (8) to indicate a titration of serial dilutions of antigens in the presence of serial dilutions of immune serum.

Schedule 5. "Box" titration of antigens

| Amount of each component expressed in ml. | Serum dilutions | | | |
|---|---|---|---|---|
| | 1:5 | 1:10 | 1:20 | 1:40 |
| Amount of each serum dilution | 0.1 | 0.1 | 0.1 | 0.1 |
| Complement, 2 units in | 0.2 | 0.2 | 0.2 | 0.2 |
| Antigen dilution | 0.2 | 0.2 | 0.2 | 0.2 |
| (Hold for 18 hrs. at 4 C. followed by 30 minutes at room temp.) | | | | |
| Sensitized cells<br>0.1 3% sheep RBC<br>0.1 3 units of hemolysin | 0.2 | 0.2 | 0.2 | 0.2 |
| (Read after 30 minutes in a 37 C. water bath) | | | | |

inactivated at 55 C. in a water bath for 30 minutes prior to the preparation of the serial dilutions.

In order that 2 units of complement would be contained in 0.2 ml., two dilutions of complement were employed. With the "Protease 15" antigen number 2, a 1:19 complement dilution was used. With the live virus antigens, a 1:24 dilution of complement was required. The actual number of units of complement were identical for all antigens.

As determined by previous titrations of hemolysin as described in the text, a 1:500 hemolysin dilution was used

with the "Protease 15" antigen number 2 and a 1:1,000 hemolysin dilution with the live virus antigens. In each instance there were 3 units of hemolysin per 0.1 ml. in the presence of each antigen.

To measure the degree of anti-complementary activity of each serum four sets of controls were run. Two sets of controls consisted of substituting standard diluent in place of the antigen in the above schedule. This was done with each serum with both complement dilutions employed. The other two sets of controls consisted of essentially the same procedure except that the antibiotic solution, previously described under antigen components, was substituted for the antigen rather than standard diluent.

(b) Complement controls. Titrations of graduated dilutions of a 1:20 dilution of complement in the presence of serial dilutions of "Protease 15" antigen number 2; and a 1:25 dilution of complement in the presence of serial dilutions of "N.Y." and CVS live virus antigens were performed. These titrations were performed concurrently with the respective "box" titrations of antigens and served as complement controls. The method of procedure was described in Schedule 4.

One unit of complement was determined for each dilution of each antigen after 18 hours incubation at 4 C. These results served as an indication of the amount of complement available after 18 hours at 4 C. in each "box" titration in

the absence of serum.

d. Complement-fixation tests. The complement-fixing capabilities of "Protease 15" antigen number 2; "N.Y." and CVS live virus antigens; and normal rabbit brain antigen were investigated in two series of complement-fixation tests. In one series, equine anti-rabies serum was employed as the immune serum, and normal equine sera number 1 and 2 and their respective albumin and globulin fractions were employed as the normal sera. In the second series of tests, pooled rabbit anti-rabies serum was employed as the immune serum, and pooled normal rabbit serum as the normal serum. The titre of each serum was defined as the highest dilution of serum giving a reading of 3 or 4 fixation of complement. Evidence of complement fixation in the controls indicated any anti-complementary activity of the serum tested.

(1) Actual tests. Each immune and normal serum to be tested was diluted 1:2 and the native complement inactivated at 55 C. in a water bath for 30 minutes. Schedule 6 was employed for both series of complement-fixation tests.

Based upon the results of the "box" titrations of antigens, a 1:2 dilution of each antigen was employed with the equine sera. A 1:4 dilution of each antigen was used with the rabbit sera. Normal rabbit brain antigen was not employed with the rabbit sera.

Schedule 6. Complement-fixation test

| Amount of each component expressed in ml. | Serum dilutions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1:5 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1:320 | 1:640 |
| Amt. of ea. serum dilution | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Complement, 2 units in | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Antigen dilution | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| (Hold for 18 hrs. at 4 C. followed by 30 minutes at room temp.) | | | | | | | | |
| Sensitized cells<br>0.1 3% sheep RBC<br>0.1 3 units of hemolysin | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| (Read after 30 minutes in a 37 C. water bath) | | | | | | | | |

The complement and hemolysin dilutions were the same as for the "box" titrations of antigens described above. The standard diluent and antibiotic solution controls were performed in the same manner as described for the "box" titrations of antigens. Two sets of controls, as previously described, were run for each immune and normal serum tested.

(2) <u>Complement controls</u>. Titrations of graduated dilutions of complement in the presence of each antigen

employed in the complement-fixation tests were run. The procedure was identical to that employed in the complement controls for the "box" titrations of antigens.

# V. EXPERIMENTAL RESULTS

## A. Toxicity Produced in Mice by Intracerebral Inoculation of Various Enzymes

One hundred and ten groups of mice, each group containing four mice, were inoculated intracerebrally with 0.03 ml. of serial dilutions of various enzymes. Certain of these enzyme dilutions manifested toxic reactions resulting in the death of inoculated mice. The toxic activity of nine of the twelve enzymes tested appear in Table 1.

Table 1. Toxicity of serial dilutions of nine enzymes when inoculated intracerebrally into mice

| Enzymes | Grams of enzyme in 10 ml. of diluent | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1.0 | 0.5 | 0.25 | 0.12 | 0.10 | 0.06 | 0.04 | 0.03 | 0.015 |
| Trypsin | 4* | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bromelain | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Ficin | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 3 |
| Chymotrypsin | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Rhozyme P-11 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 |
| Protease 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Protease (trypsin) | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lysozyme | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Papain | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |

*Number of mice which died out of four which were inoculated.

From the results shown in Table 1, it is evident that "Protease 15" was the least toxic of the twelve enzymes tested. As much as 0.03 ml. of a 10% solution of the enzyme could be inoculated intracerebrally without producing any toxic effects in mice. Next in order of toxicity was lysozyme which was toxic in concentrations greater than 0.25 gm. per 10 ml. of diluent. Trypsin and "Protease (trypsin)" were of approximately equal toxicity. Concentrations greater than 0.125 gm. per 10 ml. of either enzyme were toxic to inoculated mice. "Rhozyme P-11" was twice as toxic as the latter two enzymes mentioned in that mice inoculated with concentrations greater than 0.06 gm. per 10 ml. exhibited toxic reactions.

Trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and lysozyme were markedly less toxic to mice than bromelain, ficin, chymotrypsin or papain. All four of these latter enzymes were toxic in concentrations as low as 0.015 gm. per 10 ml. of diluent. Since concentrations of less than 0.015 gm. per 10 ml. were not tested, with the exception of papain, it was not possible to ascertain the final end-point of the toxicity range of each of these enzymes. In the case of papain, three additional dilutions were prepared, which are not shown in Table 1, containing 0.0002, 0.0001, and 0.00005 gm. per 10 ml. of diluent. Each of these additional dilutions proved to be 100% fatal to inoculated mice.

Three enzymes, which are not shown in Table 1, were also

tested. These enzymes were prepared in serial dilutions of units of measurement which were based upon the manufacturers' assay. Two of these enzymes, pancreatic desoxyribonuclease and streptokinase-streptodornase exhibited no toxic activity when 100,000 units of each were suspended in 10 ml. quantities of diluent and inoculated into mice in 0.03 ml. dosages. The third enzyme was "Proteinase A". Three-tenths ml. quantities of serial dilutions in 10 ml. of diluent were inoculated into mice. The 25,000, 18,750 and 12,500 unit dilutions killed all of the mice inoculated. The 6,250 and 3,125 unit dilutions were fatal to half of each group of four inoculated mice. The 2,500 unit dilution killed one out of four inoculated mice. A 1,560 unit dilution was non-toxic to inoculated mice.

From the results obtained in this investigation, it was possible to calculate the minimal quantity of each enzyme required to produce fatal toxic reactions in inoculated mice. The minimal lethal mouse dose of each enzyme tested appears in Table 2.

As can be seen by a comparison of the minimal lethal mouse dosages shown in Table 2, bromelain, ficin, chymotrypsin, and papain were extremely toxic as compared to the other enzymes tested. The minimal lethal mouse dose of each of these enzymes was less than 0.00003 gm. For this reason these four enzymes were not employed in subsequent investigations.

Table 2. Minimal lethal mouse dose of twelve proteolytic enzymes

| Enzyme | Quantity of enzyme per 0.03 ml. dose | |
|---|---|---|
| | Grams | Units by Mfg. assay |
| Proteinase A | | >7.5 |
| Pancreatic desoxyribonuclease | | >300 |
| Streptokinase-streptodornase | | >300 |
| Trypsin | >0.0008 | |
| Bromelain | <0.00004 | |
| Ficin | <0.00003 | |
| Chymotrypsin | <0.00004 | |
| Rhozyme P-11 | <0.0002 | |
| Protease 15 | >0.001 | |
| Protease (trypsin) | >0.0005 | |
| Lysozyme | >0.003 | |
| Papain | <0.0000001 | |

The antibiotic solution employed as a bacteriostatic agent with each enzyme dilution did not appear to be associated with the toxic effects manifested by mice following inoculation of the enzyme preparations. Inoculation of an antibiotic solution containing the same quantity of antibiotics as the enzyme preparations failed to produce any manifestation of toxicity in mice. No toxic symptoms were manifested by mice which received two and three times as much antibiotics as were present in the enzyme preparations.

## B. Time Required to Degrade Brain Tissue by Digestion with Various Enzymes

Four distinct groups of turbidimetric determinations were made in an effort to determine the amount of time required to degrade brain tissue by digestion with various enzymes. The results of these determinations are shown in Table 3.

### 1. Determination of the amount of degradation occurring in suspensions of enzymes in the absence of a substrate

The percentage of decrease in total protein calculated from the colorimeter readings made on the five test solutions and the negative control are shown as Determination I in Table 3. The results of this determination indicate that trypsin, "Rhozyme P-11", "Protease 15" and "Proteinase A" evidenced no proteolytic activity when incubated at 37 C. for 24 hours in the absence of a substrate.

"Protease (trypsin)" did exhibit proteolytic activity when incubated at 37 C. for four hours or longer as evidenced by a 14% decrease in the protein value of the enzyme in the absence of a substrate. This indicates that "Protease (trypsin)" was an impure product probably containing some non-enzyme protein material. The manufacturer stated that it was not a purified enzyme preparation.

Table 3. Decrease in protein values occurring in five enzyme solutions incubated at 37 C. in the presence and absence of antibiotics, and the presence of brain tissue with and without antibiotics

| Test solutions | Percentage of decrease in protein values | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total protein | | | | | | | | Brain protein | | | |
| Substrate | No substrate | | | | Brain tissue | | | | Brain tissue | | | |
| Antibiotics | Present | | Absent | | Present | | Absent | | Present | | Absent | |
| Hours at 37 C. | 4 | 24 | 4 | 24 | 4 | 24 | 4 | 24 | 4 | 24 | 4 | 24 |
| Determination I | | | | | | | | | | | | |
| Trypsin | - | - | 0 | 0 | | | | | | | | |
| Rhozyme P-11 | - | - | 0 | 0 | | | | | | | | |
| Protease 15 | - | - | 0 | 0 | | | | | | | | |
| Protease (trypsin) | - | - | 14 | 14 | | | | | | | | |
| Proteinase A | - | - | 0 | 0 | | | | | | | | |
| Determination II | | | | | | | | | | | | |
| Trypsin | | | | | - | 17 | - | - | - | 17 | - | - |
| Rhozyme P-11 | | | | | - | 20 | - | - | - | 20 | - | - |
| Protease 15 | | | | | - | 14 | - | - | - | 14 | - | - |
| Protease (trypsin) | | | | | - | 26 | - | - | - | 12 | - | - |
| Proteinase A | | | | | - | 7 | - | - | - | 7 | - | - |
| Determination III | | | | | | | | | | | | |
| Trypsin | 0 | 0 | - | - | 15 | 15 | - | - | 15 | 15 | - | - |
| Rhozyme P-11 | 0 | 0 | - | - | 19 | 19 | - | - | 19 | 19 | - | - |
| Protease 15 | 0 | 0 | - | - | 11 | 11 | - | - | 11 | 11 | - | - |
| Protease (trypsin) | 14 | 14 | - | - | 26 | 26 | - | - | 12 | 12 | - | - |
| Proteinase A | 0 | 0 | - | - | 9 | 9 | - | - | 9 | 9 | - | - |
| Determination IV | | | | | | | | | | | | |
| Trypsin | 0 | - | 0 | - | 18 | - | 18 | - | 18 | - | 18 | - |
| Rhozyme P-11 | 0 | - | 0 | - | 20 | - | 20 | - | 20 | - | 20 | - |
| Protease 15 | 0 | - | 0 | - | 11 | - | 11 | - | 11 | - | 11 | - |
| Protease (trypsin) | 12 | - | 12 | - | 26 | - | 25 | - | 14 | - | 13 | - |
| Proteinase A | 0 | - | 0 | - | 9 | - | 9 | - | 9 | - | 9 | - |
| Determinations I, II, III, IV | | | | | | | | | | | | |
| 37 C. neg. control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 C. neg. control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

2. Determination of the amount of degradation occurring in suspensions of enzymes in the presence of a substrate and antibiotics

A suspension of rabbit brain emulsion containing a standard amount of antibiotics was employed as the substrate in Determination II shown in Table 3. The percentage of decrease in total protein and brain protein calculated from the colorimeter readings made on the five test solutions of enzymes and negative controls are shown in the table.

By comparing the percentage of decrease in the protein value of the substrate in the presence of each enzyme with the values obtained with the negative controls, it was indicated that antibiotics in the presence of the substrate did not possess proteolytic activity.

3. Determination of the rate of degradation occurring in suspensions of enzymes in the presence of antibiotics in conjunction with the presence or absence of a substrate

In this series of determinations, solutions of enzymes and negative controls identical to those employed in Determination II were used as one set of test solutions. These were compared with solutions of enzymes and negative controls containing identical quantities of antibiotics in the absence of a brain emulsion substrate. The percentage of decrease in

total protein and brain protein, when applicable, was calculated from the colorimeter readings made at 2, 4, 6, 12 and 24 hour intervals on each test solution. The protein values for the fourth and twenty-fourth hour are shown as Determination III in Table 3.

None of the five enzymes tested produced any measurable amount of proteolysis by the end of the second hour. Maximum proteolysis occurred between the second and fourth hour of incubation. No increase in the amount of proteolysis was detectable after the fourth hour of incubation.

A comparison of the results obtained with the five enzymes in the presence of brain tissue and antibiotics with the results obtained with antibiotics without brain tissue indicate that the antibiotics did not exert any detectable influence on the proteolytic activity of the enzymes. These results tend to confirm those of Determination II.

4. Determination of the amount of degradation occurring in suspensions of enzymes in, (a) the presence and absence of antibiotics without substrate, and (b) the presence of substrate with or without antibiotics

The fourth group of determinations shown in Table 3 constituted a re-check of the previous three groups of determinations. Four series of suspensions of identical quantities of enzymes used in the previous three groups of determinations

were employed as test solutions. The decrease in total protein and brain protein, when applicable, occurring in each series of suspensions and controls after 4 hours incubation at 37 C. was determined.

The results obtained with the five enzymes in the absence of a substrate and antibiotics in Determination IV confirm the results shown for Determination I. These collective results indicate more definitely that with the exception of "Protease (trypsin)" none of the enzymes evidenced proteolytic activity when incubated at 37 C. in the absence of a substrate. In Determination IV it was again indicated that the proteolytic activity of the five enzymes is not detectably influenced by the presence of antibiotics, either alone or in the presence of brain tissue.

In Determination IV, the results obtained with the five enzymes in the presence of brain tissue were identical to those obtained in Determinations II and III. In each of these groups of determinations it was apparent that with all five enzymes, maximum proteolysis occurred by the fourth hour of incubation at 37 C. No increase in the amount of proteolysis was detectable after the fourth hour of incubation.

The results shown in Table 3, also tend to indicate the degree of proteolytic efficiency of each enzyme preparation. Trypsin and "Rhozyme P-11" appear to be more efficient than "Protease 15", "Protease (trypsin)" and "Proteinase A".

"Protease 15" and "Protease (trypsin)" were equally efficient. "Proteinase A" was the least efficient of any of the five enzymes tested. The procedures employed in the turbidimetric determinations of the proteolytic activity of trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A" were repeated a sufficient number of times to ascertain that the results obtained were consistent within a range of 2%.

## C. Rabies Virus Elution in the Presence of Five Proteolytic Enzymes

In this investigation, suspensions of rabies virus rabbit brain emulsions were subjected to the proteolytic action of trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A".

### 1. Identification of stock viruses

Five strains of rabies street virus and one strain of fixed virus were employed in this investigation. The street viruses were: "N.Y.", FAAML 237, 309, 363 and 372. The fixed virus was the CVS strain of rabies virus.

The identity of each virus was established by a combination of procedures. As shown in Table 4, each rabbit inoculated with a stock virus developed typical symptoms of rabies.

The incubation period in the case of each inoculated rabbit was within the typical range described for rabies virus infection.

The microscopical examinations of impression smears prepared from the brain of each infected rabbit, as shown in Table 4, revealed Negri bodies in each case of street rabies. No Negri bodies were observed in the case of the fixed virus.

Table 4. Identification of the stock viruses used in the study

| Virus strain | Days past inoculation when symptoms were observed in rabbits | | Microscopical exam. of smears of rabbit brain for Negri bodies | Index of mouse $LD_{50}$ of virus neutralized in serum-virus neutralization tests |
|---|---|---|---|---|
| | Paralysis | Death | | |
| CVS | 7 | 13 | negative | >24,450 |
| "N.Y." | 6 | 8 | positive | >2,191 |
| FAAML 237 | 14 | 16 | positive | >326 |
| FAAML 309 | 12 | 13 | positive | >1,638 |
| FAAML 363 | 11 | 13 | positive | >128 |
| FAAML 372 | 15 | 18 | positive | >1,850 |

All six of the stock viruses gave indicies of mouse $LD_{50}$ of virus neutralized in excess of 100 $LD_{50}$ in the serum-virus neutralization tests. The index of mouse $LD_{50}$ of virus neutralized by each individual virus are shown in Table 4. The

results of these procedures, establish the identity of each virus employed as a stock virus as rabies.

2. Titration, in animals, of serial dilutions of stock virus brain emulsions subjected to proteolytic enzymatic digestion

Aliquots of pools of rabbit brain emulsions of each of the stock viruses were subjected to proteolytic enzymatic digestion with five different enzymes, as shown in Table 5. Separate suspensions of each enzyme with each virus strain were subjected to 4 hours incubation at 37 C. Two controls were employed for each strain of virus. One control was held for 4 hours at 4 C. while the other control was incubated at 37 C. along with the enzyme preparations.

Serial ten-fold dilutions, $10^{-1}$ through $10^{-5}$, of each enzyme and control preparation were titrated in mice. At the end of the 21 day observation period, the $LD_{50}$ of each serial dilution was calculated. From the $LD_{50}$ of each serial dilution, the number of residual $LD_{50}$ of live virus remaining in each 0.03 ml. of brain emulsion after 37 C. for 4 hours and proteolytic digestion was determined, as shown in Table 5.

The experimental results shown in Table 5 give some indication of the separate influences exerted by heat and proteolytic enzymes on rabies virus. By comparison of the residual number of $LD_{50}$ of live virus in each 0.03 ml. of brain

Table 5. Effects of five proteolytic enzymes on six strains of rabies virus

| Virus strains | Controls | | Test preparations: Proteolytic enzymatic digestion at 37 C. | | | | |
|---|---|---|---|---|---|---|---|
| | 4 C. | 37 C. | Trypsin | Rhozyme P-11 | Protease 15 | Protease (trypsin) | Proteinase A |
| CVS | 100,000* | 19,900 | 10 | 30 | 199 | 15 | 10 |
| "N.Y." | 3,160 | 1,000 | 100 | 19 | 30 | 10 | 19 |
| FAAML 237 | 30 | 10 | - | - | 10 | - | - |
| FAAML 309 | 10,000 | 1,990 | - | - | 10 | 30 | 10 |
| FAAML 363 | 316 | 199 | - | - | - | - | - |
| FAAML 372 | 50 | 19 | - | - | - | 15 | - |

*Number of residual $LD_{50}$ of live virus in 0.03 ml. of brain emulsion after 4 hours incubation.

emulsion after 37 C. for 4 hours to the 4 C. control, a number of inferences can be drawn as to the effect of heat on rabies virus.

Each virus was treated by exposure of a 10% suspension of rabbit brain emulsion to 37 C. for 4 hours. In every case, a marked decrease was noted in the number of $LD_{50}$ of live virus in each 0.03 ml. of brain emulsion, as shown in Table 6.

Table 6. Decrease in $LD_{50}$ of rabies virus occasioned by incubation at 37 C. for 4 hours

| Virus | Test preparation | $LD_{50}$ per 0.03 ml. of brain emulsion | $LD_{50}$ lost by incubation at 37 C./ 4 hrs. | Approx. per cent of decrease |
|---|---|---|---|---|
| CVS | 4 C. control | 100,000 | | |
| | 37 C. control | 19,900 | 80,100 | 80% |
| "N.Y." | 4 C. control | 3,160 | | |
| | 37 C. control | 1,000 | 2,160 | 66% |
| FAAML 237 | 4 C. control | 30 | | |
| | 37 C. control | 10 | 20 | 66% |
| FAAML 309 | 4 C. control | 10,000 | | |
| | 37 C. control | 1,990 | 8,010 | 80% |
| FAAML 363 | 4 C. control | 316 | | |
| | 37 C. control | 199 | 117 | 37% |
| FAAML 372 | 4 C. control | 50 | | |
| | 37 C. control | 19 | 31 | 60% |

Table 6 shows that the decrease in $LD_{50}$ of rabies virus exposed to 37 C. for 4 hours is not a constant characteristic. It varies between different strains of virus from 30% to 80%. Each strain of virus possessed an individual level of heat resistance. This ability to resist heat bears some relationship to the original titre of the virus. The CVS, "N.Y." and FAAML 309 strains of virus which possessed a relatively high titre before heat treatment appeared to be proportionally

less resistant to the effects of heat.

By comparison of the residual number of $LD_{50}$ of live virus in each 0.03 ml. of brain emulsion after treatment with the five proteolytic enzymes to the 37 C. controls, a number of inferences can be drawn as to the effect of each enzyme on rabies virus. All five of the proteolytic enzymes exhibited harmful effects on each of the six strains of virus, as shown in Table 7.

Table 7. Decrease in $LD_{50}$ of rabies virus occasioned by incubation at 37 C. for 4 hours in the presence of five enzymes

| Virus strain | Decrease in $LD_{50}$ per 0.03 ml. of brain emulsion | | Approx. per cent of decrease in $LD_{50}$ in enzyme preps. above the decrease caused by 37 C. alone |
|---|---|---|---|
| | 37 C. control | Range of 5 enzyme preparations | |
| CVS | 80,100 | 99,800 - 99,990 | 19% |
| "N.Y." | 2,160 | 3,060 - 3,150 | 33% |
| FAAML 237 | 20 | > 20 | < 33% |
| FAAML 309 | 8,010 | 9,960 - 9,990 | 19% |
| FAAML 363 | 117 | >306 | >60% |
| FAAML 372 | 31 | 40 - 44 | 26% |

As shown in Table 5, a decrease in the number of $LD_{50}$ of live virus per 0.03 ml. of brain emulsion was evidenced in each titration of virus treated by any one of the five enzymes. These decreases, although they varied in extent for each strain of virus, exceeded the loss in the number of $LD_{50}$ previously attributed to the effect of 37 C. for 4 hours, as shown in Table 7. The collective results shown in Tables 5, 6 and 7, indicate that the virulence of each strain of virus tested was markedly antenuated by subjecting the virus to proteolysis with either trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" or "Proteinase A" for 4 hours at 37 C.

### D. Challenge of Mice Which Survived in the Titration of Serial Dilutions of Stock Virus Brain Emulsions Subjected to Proteolytic Enzymatic Digestion

Those mice which survived for 21 days in the titrations of rabies virus subjected to proteolysis were challenged by intracerebral inoculation with 4 $LD_{50}$ of homologous virus in 0.03 ml. The concentration of the challenge virus suspensions was based on the $LD_{50}$ titre of the 4 C. controls of the titrations of homologous stock viruses. The challenged mice and controls were observed for a 21 day period following inoculation. The mortality ratios of challenged mice which

survived are shown in Table 8.

As shown in Table 8, a sufficient number of mice survived the challenge inoculation to indicate that some degree of immunity had been established in the mice. This immunity did not appear to be associated with the residual number of $LD_{50}$ of live virus present in each enzyme-brain emulsion preparation, as shown in Table 5. There was no visible correlation between the number of residual $LD_{50}$ of live virus demonstrated in the original titrations and the survival of mice following challenge.

By comparing the survival rates observed subsequent to challenge, as shown in Table 8, the dilution of residual live virus did not appear to have any visible correlation with any of the survival rates. In some of the control and enzyme-brain emulsion titrations, the challenge survival rates were higher in the groups of mice which had survived in the lower dilutions of the original titrations. Just as frequently, the reverse of these findings was true. These findings were so inconsistant that their influence on the immunity observed was discounted.

The immunity developed by the surviving mice in the various titrations appeared to be associated with the amount of dead virus in each control and enzyme-brain emulsion titration. This can be seen by comparing the results shown in Table 5 to those shown in Table 8. A correlation between the

Table 8. Results of the challenge of mice which survived in the titrations of rabies virus subjected to proteolytic enzymatic digestion

| Virus strain and LD $_{50}$ titre of 4 C. control | Challenge dilution of virus represented by 4 LD $_{50}$ in 0.03 ml. | Original test preparation of rabies virus subjected to proteolysis at 37 C. for 4 hrs. | Mortality ratio of mice which survived in each serial dilution of rabies virus subjected to proteolytic enzymatic digestion | | | | |
|---|---|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| CVS | 1:25,000 | Challenge control | 6/6 | - | - | - | - |
| $10^{-5}$ | | 4 C. control | - | - | - | 0 | 0/2 |
| | | 37 C. control | - | - | - | 0/1 | 0/4 |
| | | Trypsin | - | 0/4 | 0/4 | 1/4 | 1/4 |
| | | Rhozyme P-11 | - | 0/2 | 0/4 | 0/4 | 0/4 |
| | | Protease 15 | - | 0/1 | 0/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | 2/2 | 0/3 | 0/4 | 1/4 | 1/4 |
| | | Proteinase A | - | 1/4 | 0/4 | 2/4 | 2/4 |
| "N.Y." | 1:790 | Challenge control | 6/6 | - | - | - | - |
| $10^{-3.5}$ | | 4 C. control | - | - | - | 3/4 | 2/4 |
| | | 37 C. control | - | - | 2/2 | 2/4 | 2/4 |
| | | Trypsin | - | 1/2 | 3/4 | 3/4 | 2/4 |
| | | Rhozyme P-11 | 0/1 | 1/4 | 1/4 | 0/4 | 0/4 |
| | | Protease 15 | 1/1 | 2/3 | 1/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | - | 2/4 | 3/4 | 1/4 | 1/4 |
| | | Proteinase A | 0/1 | 2/4 | 3/4 | 3/4 | 2/4 |
| FAAML 237 | 1:8 | Challenge control | 6/6 | - | - | - | - |
| $10^{-1.5}$ | | 4 C. control | - | 1/4 | 4/4 | 4/4 | 4/4 |
| | | 37 C. control | 0/2 | 0/4 | 4/4 | 4/4 | 2/4 |
| | | Trypsin | 1/2 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Rhozyme P-11 | 4/4 | 3/4 | 3/4 | 3/4 | 2/4 |
| | | Protease 15 | - | 3/3 | 3/3 | 2/3 | 2/3 |
| | | Protease (trypsin) | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Proteinase A | 2/2 | 1/4 | 4/4 | 4/4 | 2/4 |

Table 8. (continued)

| Virus strain and $LD_{50}$ titre of 4 C. control | Challenge dilution of virus represented by 4 $LD_{50}$ in 0.03 ml. | Original test preparation of rabies virus subjected to proteolysis at 37 C. for 4 hrs. | Mortality ratio of mice which survived in each serial dilution of rabies virus subjected to proteolytic enzymatic digestion | | | | |
|---|---|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| FAAML 309 | 1:2,500 | Challenge control | 6/6 | - | - | - | - |
| $10^{-4}$ | | 4 C. control | - | - | - | 2/2 | 4/4 |
| | | 37 C. control | - | - | 1/1 | 4/4 | 4/4 |
| | | Trypsin | 0/4 | 3/4 | 2/4 | 4/4 | 2/4 |
| | | Rhozyme P-11 | 1/4 | 1/4 | 0/4 | 0/4 | 0/4 |
| | | Protease 15 | 2/2 | 1/4 | 2/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | - | 1/4 | 1/4 | 1/4 | 0/4 |
| | | Proteinase A | 2/2 | 0/4 | 0/4 | 0/4 | 0/4 |
| FAAML 363 | 1:79 | Challenge control | 6/6 | - | - | - | - |
| $10^{-2.5}$ | | 4 C. control | - | 0/4 | 3/3 | 4/4 | 4/4 |
| | | 37 C. control | - | 0/1 | 4/4 | 4/4 | 4/4 |
| | | Trypsin | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Rhozyme P-11 | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Protease 15 | 4/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| | | Protease (trypsin) | 4/4 | 4/4 | 4/4 | 4/4 | 3/4 |
| | | Proteinase A | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| FAAML 372 | | Challenge control | 6/6 | - | - | - | - |
| $10^{-1.7}$ | | 4 C. control | 0/1 | 0/2 | 0/4 | 2/4 | 2/4 |
| | | 37 C. control | 0/1 | 0/4 | 1/4 | 2/4 | 2/4 |
| | | Trypsin | 1/4 | 1/4 | 3/4 | 3/4 | 1/4 |
| | | Rhozyme P-11 | 0/4 | 0/4 | 3/4 | 2/4 | 3/4 |
| | | Protease 15 | 2/4 | 2/4 | 3/4 | 0/4 | 1/4 |
| | | Protease (trypsin) | 1/2 | 2/3 | 2/4 | 2/4 | 1/4 |
| | | Proteinase A | 2/4 | 2/4 | 0/4 | 3/4 | 2/4 |

decrease in the number of $LD_{50}$ per 0.03 ml. of brain emulsion in the control and enzyme-brain emulsion preparations and the survival rate of the mice following challenge appeared to exist. Although there were some exceptions the survival rate for all control and enzyme-brain emulsion preparations following challenge was highest in those cases where the original titrations were determined to have a loss of 3,000 or more $LD_{50}$ of virus, as shown in Table 7. The CVS, "N.Y." and FAAML 309 strains of virus each exhibited a loss of 3,000 or more $LD_{50}$ of virus in the original titrations. In the case of these viruses, the strength of the immunity exhibited subsequent to challenge, as shown in Table 8, appeared to be proportional to the amount of dead virus determined to be present in the original titrations, as shown in Table 7. Such a correlation did not appear to exist for those viruses in which the loss of $LD_{50}$ was less than 3,000 in the original titrations. In those titrations in which the loss of $LD_{50}$ was less than 3,000 the survival rates subsequent to challenge were extremely low.

"Rhozyme P-11" and "Protease 15" enzyme-brain emulsion preparations appeared to establish an immunity against 4 $LD_{50}$ most consistently. With both enzyme preparations, an immunity of some significance appeared to have been established against the CVS, "N.Y.", FAAML 309 and FAAML 372 strains of the total of six virus strains tested.

E. Attempts to Immunize Mice against Rabies by a Single Inoculation of a Supernate of CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

1. <u>Titration in mice of serial dilutions of the second rabbit passage of the CVS strain of rabies virus</u>

In the titration of a 10% suspension of brain emulsion of the second rabbit passage of the CVS strain of rabies virus, the $LD_{50}$ titre was determined to be $10^{-4.6}$. This indicated that there were 39,820 $LD_{50}$ of virus in 0.03 ml. of pooled brain emulsion. The number of $LD_{50}$ per 0.03 ml. of the pooled brain emulsion from the second rabbit passage represented a decrease in titre of 60,180 $LD_{50}$, when compared to the titre of the first rabbit passage. The $LD_{50}$ titre of a 10% suspension of rabbit brain emulsion prepared from a single brain of the first rabbit passage of the virus was $10^{-5}$. This titre indicated that there were 100,000 $LD_{50}$ of virus in 0.03 ml. of brain emulsion of the first rabbit passage.

This variance in titre indicated that the pooling of the rabbit brains of the second rabbit passage may have had an adverse effect on the $LD_{50}$ titre of the virus. The titre of the virus material employed in the preparation of the experimental vacines in this and subsequent investigations was less than the titre of the same strain of virus which was employed in the investigation in rabies virus elution in the presence

of enzymes.

2. Test of the viability of the experimental vaccines

Serial ten-fold dilutions, $10^{-1}$ through $10^{-3}$, of "Rhozyme P-11" vaccine number 1, and "Protease 15" vaccine number 1, were titrated by intracerebral inoculation of mice. No deaths occurred among any of the groups of inoculated mice during the ensuing 14 day observation period. Since no detectable residual live virus was present in either vaccine, the $LD_{50}$ titre of the vaccines was demonstrated to be less than $10^{-1}$.

3. Attempted immunization of mice with experimental and control vaccines

No deaths occurred among any of the groups of mice which were inoculated intraperitoneally with "Rhozyme P-11" vaccine number 1, "Protease 15" vaccine number 1 or the phenolized control vaccine. The mice were observed for 14 days without any symptoms of any type of toxicity being demonstrated.

4. Challenge of mice vaccinated with experimental and control vaccines

On the fourteenth day of the post-inoculation period, each group of 30 mice inoculated with experimental and control

vaccines and a group of 30 normal mice were divided into six lots of five mice each. One lot of mice of each group was inoculated intracerebrally with 0.03 ml. quantities of serial dilutions of challenge virus, 124 $LD_{50}$ through 12,443 $LD_{50}$ per dose. These challenged mice were observed for an additional 14 day period. The mortality ratio experienced per serial dilution of challenge virus in the groups of vaccinated and unvaccinated mice is shown in Table 9.

Table 9. Results of the challenge of mice vaccinated with a single dose of experimental vaccine

| Groups of mice inoculated with a single 0.5 ml. dose of vaccine | Number of $LD_{50}$ in 0.03 ml. of challenge virus | | | | | |
|---|---|---|---|---|---|---|
| | 124 | 1,244 | 3,110 | 6,221 | 9,331 | 12,443 |
| Unvaccinated controls | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Vaccinated controls Phenolized vaccine | 4/5* | 4/5 | 4/5 | 4/5 | 5/5 | 5/5 |
| Rhozyme P-11 vaccine no. 1 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Protease 15 vaccine no. 1 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 | 5/5 |

*Mortality ratios of mice inoculated with 0.03 ml. of serial dilutions of challenge virus.

All of the mice which had been vaccinated with the two experimental vaccines died subsequent to challenge, as did the mice in the unvaccinated controls. In the group of mice which received a 10% suspension of phenolized vaccine, the survival rates were extremely poor. Of these vaccinated controls, only one mouse out of five survived in the groups inoculated with serial dilutions of challenge virus, 124 $LD_{50}$ through 6,221 $LD_{50}$ per dose.

"Rhozyme P-11" and "Protease 15" vaccines number 1 were not capable of inducing an immune response in mice inoculated intraperitoneally with a single 0.5 ml. dose of either vaccine when challenged with 124 $LD_{50}$ of homologous virus. A 10% suspension of standard phenolized rabies vaccine of caprine origin was not capable of inducing a significant immune response under identical conditions.

## F. Attempts to Immunize Mice against Rabies by Multiple Inoculations of Vaccines Produced from CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

### 1. Titration in mice of serial dilutions of the third rabbit passage of the CVS strain of rabies virus

A pooled rabbit brain emulsion of the third rabbit passage of the CVS strain of rabies virus was employed as the sub-

strate for the experimental vaccines used in this investigation. The $LD_{50}$ titre of this substrate was determined to be $10^{-4.6}$ by intracerebral titration in mice. From this titre it was determined that 0.03 ml. of the substrate contained 39,820 $LD_{50}$ of live virus.

2. Test of the viability of the experimental vaccines

The following vaccines: "Rhozyme P-11" vaccine suspension and vaccine supernate number 2; "Protease 15" vaccine suspension and vaccine supernate number 2; and the heat inactivated control vaccine suspension and vaccine supernate were titrated in mice. Deaths occurred among the $10^{-1}$ group of mice of each vaccine titration. The $LD_{50}$ of the vaccine titrations was calculated in those cases where a sufficient number of deaths occurred that a 50% end point was reached. From the $LD_{50}$ titre of a vaccine titration, the number $LD_{50}$ of residual live virus in each 0.03 ml. of vaccine was calculated. These results are shown in Table 10.

In order to accurately evaluate these results, it is necessary to compare the method employed in the preparation of the vaccines used in this investigation to the method used in the preparation of the vaccines employed in the preceding investigation. In the preparation of "Rhozyme P-11" and "Protease 15" vaccines number 1, the enzyme-brain emulsion preparations were incubated at 50 C. for 4 hours. No detectable

Table 10. Intracerebral titration in mice of six experimental vaccines

| Vaccine titrations | Mortality ratios of mice inoculated with 0.03 ml. of serial dilutions of vaccine | | | Residual $LD_{50}$ of live rabies virus in each 0.03 ml. of vaccine |
|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | |
| Rhozyme P-11 vaccine suspension | 3/5 | 0/5 | 0/5 | 15 |
| Rhozyme P-11 vaccine supernate | 2/5 | 0/5 | 0/5 | <10 |
| Protease 15 vaccine suspension | 1/5 | 0/5 | 0/5 | <10 |
| Protease 15 vaccine supernate | 1/5 | 0/5 | 0/5 | <10 |
| Heat inactivated vaccine suspension | 4/5 | 0/5 | 0/5 | 25 |
| Heat inactivated vaccine supernate | 4/5 | 0/5 | 0/5 | 25 |

residual live virus was found in either of these two vaccines when they were titrated by intracerebral inoculation in mice. The method of proteolysis employed in the preparation of the vaccines listed in Table 10 differed from the method employed in the preceding investigation, in that the enzyme-brain emulsion preparations were incubated at 50 C. for only 1 hour followed by incubation at 37 C. for 3 hours. A 10% suspension of untreated brain emulsion was subjected to the same heat treatment and is shown in Table 10 as heat inactivated vaccine.

The period of time for which the enzyme-brain emulsion preparations were held at 50 C. was decreased to one hour because available information in the literature indicated that 1 hour at 50 C. should be sufficient to inactivate rabies virus, and also because it was felt that the unsatisfactory results obtained in the previous investigation might have been due to the effect of 50 C. for 4 hours on the specificity of the enzyme reaction. The assumption that 50 C. for 1 hour would inactivate the virus was disproved by the results obtained in the titrations of the vaccines as depicted in Table 10. In the case of each vaccine suspension and supernate there was sufficient evidence to indicate that exposure to 50 C. for 1 hour was insufficient to completely inactivate the virus.

As previously determined in the titration in mice of serial dilutions of the third rabbit passage of the CVS strain of rabies virus, the brain emulsion from which the experimental vaccines were prepared had a titre of 39,820 $LD_{50}$ of live virus per 0.03 ml. By comparing the residual $LD_{50}$ of virus in 0.03 ml. of vaccine, as shown in Table 10, to the $LD_{50}$ titre of the untreated brain emulsion, it is evident that "Rhozyme P-11" and "Protease 15", plus heat, inactivated a minimum of 39,800 $LD_{50}$ of virus per 0.03 ml. of brain emulsion. By making a similar comparison with the titres shown for the heat inactivated vaccines, it is apparent that the

action of the heat treatment, alone, inactivated a minimum of 39,790 $LD_{50}$ of virus per 0.03 ml. of brain emulsion.

On the basis of the results described above, it was concluded that the effect of the heat treatment employed in the preparation of the vaccines accounted for over 99% of the total amount of virus inactivated. The small amount of virus shown to be inactivated by the action of the enzymes, over and above that accounted for by the effects of heat, was insignificant.

The brains of the mice which died after the fifth day of each vaccine titration were harvested and pooled. Each pool of this brain material was subjected to the modified serum-virus neutralization test. The results of these tests are shown in Table 11.

That the deaths of the mice observed in the vaccine viability titrations were caused by rabies virus was demonstrated by the results obtained in the serum-virus neutralization tests. These results indicate that rabies virus was present in each pool of brain material harvested from the mice which died in each vaccine titration. On the basis of the results observed in the vaccine viability titrations, as shown in Table 10, and the confirmation afforded by the serum-virus neutralization tests that the vaccines did contain live virus, it was concluded that exposure of the CVS strain of rabies virus to 50 C. for 1 hour followed by 37 C. for 3 hours was

Table 11. Identification of virus material harvested from brain tissue of mice which died in the experimental vaccine viability titrations

| Vaccine viability titration from which the virus was recovered | Modified serum-virus neutralization tests: Mortality ratios of mice inoculated with 0.03 ml. of a mixture of equal volumes of serum dilution and 1:360 dilution of virus | |
|---|---|---|
| | 1:10 Normal serum | 1:100 Immune serum |
| Rhozyme P-11 | | |
| vaccine suspension | 6/6 | 0/6 |
| vaccine supernate | 6/6 | 0/6 |
| Protease 15 | | |
| vaccine suspension | 6/6 | 0/6 |
| vaccine supernate | 5/6 | 0/6 |
| Heat inactivated | | |
| vaccine suspension | 6/6 | 0/6 |
| vaccine supernate | 6/6 | 0/6 |

insufficient to completely inactivate the virus.

3. Attempted immunization of mice with experimental and control vaccines

No deaths occurred among the four groups of mice which were inoculated intraperitoneally with six 0.5 ml. doses of the four control vaccines: heat inactivated control vaccine suspension and vaccine supernate; and phenolized control vaccine suspension and vaccine supernate. Several deaths oc-

curred in each of the four groups of mice which were inoculated in a similar manner with the four enzyme vaccines: "Rhozyme P-11" vaccine suspension number 2 and vaccine supernate number 2; and "Protease 15" vaccine suspension number 2 and vaccine supernate number 2. These deaths occurred within 10 to 15 minutes following inoculation. The mice exhibited symptoms similar to those seen in the enzyme toxicity investigation. The deaths were attributed to the toxicity of the enzyme vaccine preparations.

4. Challenge of mice vaccinated with multiple inoculations of experimental and control vaccines

On the fourteenth day of the immunization period, 20 mice from each group of mice inoculated with experimental and control vaccines were challenged intracerebrally with a dilution of homologous virus containing 500 $LD_{50}$ per 0.03 ml. dose. The percentage of survivors in each group of vaccinated mice, subsequent to challenge with 500 $LD_{50}$, was calculated at the end of the 14 day observation period. These results and the mortality ratios of the challenged mice are shown in Table 12.

Table 12 shows that eighty per cent of the mice which were vaccinated with a 10% suspension of commercially prepared phenolized vaccine of caprine origin survived when challenged. In the case of those mice which were vaccinated with

Table 12. Percentage of survivors in groups of mice vaccinated with multiple doses of experimental and control vaccines and challenged with rabies virus

| Vaccines | Mortality ratios of mice challenged with 500 $LD_{50}$ of rabies virus | Percentage of survivors 14 days after challenge |
|---|---|---|
| Prenolized control | | |
| vaccine suspension | 4/20 | 80 |
| vaccine supernate | 15/20 | 25 |
| Heat inactivated control | | |
| vaccine suspension | 15/20 | 25 |
| vaccine supernate | 6/20 | 70 |
| Rhozyme P-11 | | |
| vaccine suspension | 20/20 | 0 |
| vaccine supernate | 20/20 | 0 |
| Protease 15 | | |
| vaccine suspension | 20/20 | 0 |
| vaccine supernate | 20/20 | 0 |

the supernate of a 10% suspension of heat inactivated vaccine, only 70% of the mice survived when challenged. According to Habel (20), to be a valid test, the challenge given to the vaccinated mice should be between 100 and 1,000 $LD_{50}$, and 50% of the vaccinated mice should survive. The results obtained with these two vaccines were more than adequate to pass the screening potency test.

Only 25% of the mice vaccinated with a supernate of a 10% suspension of phenolized vaccine survived a 500 $LD_{50}$ dose

of challenge virus. Identical results were obtained with the mice which were vaccinated with a 10% suspension of heat inactivated vaccine. Neither of these vaccines passed the screening potency test. However, the results indicated that some degree of immunity was induced by both preparations.

None of the mice which were vaccinated with a 10% suspension or with supernate prepared from pooled CVS brain emulsion subjected to proteolysis with either "Rhozyme P-11" or "Protease 15" survived a 500 $LD_{50}$ dose of challenge virus. All four of the vaccines failed to pass the screening potency test.

From the results obtained in the vaccine potency tests, it appeared that the failure of the enzyme-brain emulsion preparations to induce an immunity in mice was not necessarily related to the low $LD_{50}$ titre of the brain emulsion from which the vaccines were prepared. The satisfactory immunity established in those mice which were vaccinated with a supernate of a 10% suspension of heat inactivated vaccine tend to indicate that the $LD_{50}$ titre of the brain emulsion was adequate for the production of an acceptable vaccine.

5. Titration in mice of the challenge virus

From the titration of challenge virus performed concurrently with the challenge of the vaccinated mice, it was determined that the $LD_{50}$ titre of the virus was $10^{-4.6}$.

Therefore, 0.03 ml. of the brain emulsion of challenge virus contained 39,820 LD $_{50}$. The 1:79 dilution of brain emulsion used for the challenges contained the 500 LD $_{50}$ per 0.03 ml. dose as indicated in Table 12.

## G. Evaluation of Complement-fixing Antigens Produced from CVS Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion

### 1. Neutralization tests

The results of the serum-virus neutralization tests performed with a known strain of rabies virus and the anti-rabies and normal sera employed in this investigation are shown in Table 13.

Table 13. Serum-virus neutralization tests performed to detect presence of rabies neutralizing antibodies

| Serum | Mortality ratios of mice inoculated with serum and dilutions of rabies virus | | | | | Index of mouse LD $_{50}$ of virus neutralized |
|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | |
| Normal equine no. 1 | 6/6 | 6/6 | 6/6 | 5/6 | 0/6 | - |
| Normal equine no. 2 | 6/6 | 6/6 | 6/6 | 6/6 | 0/6 | - |
| Anti-rabies equine | 2/6 | 0/6 | 0/6 | 0/6 | 0/6 | >20,000 |
| Normal rabbit | 6/6 | 6/6 | 6/6 | 6/6 | 0/6 | - |
| Anti-rabies rabbit | 6/6 | 3/6 | 0/6 | 0/6 | 0/6 | >300 |

The equine and rabbit anti-rabies sera possessed rabies neutralizing antibodies. The normal sera did not possess such antibodies. The results of these serum-virus neutralization tests served to definitely establish the identity of each serum used in this investigation as either an anti-rabies serum or a normal serum.

2. Initial standardization of materials

a. Titration of hemolysin. One-tenth ml. of a 1:3,200 dilution was found to contain 1 unit of hemolysin. This was the smallest amount of hemolysin capable of producing complete hemolysis of an equal volume of a 3% suspension of sheep red blood cells in the presence of an excess of complement.

b. Titration of complement. One unit of complement was found to be contained in 0.06 ml. of a 1:25 dilution of complement. This was the smallest amount of complement giving complete hemolysis of 0.1 ml. of a 3% suspension of sheep red blood cells in the presence of 3 units of hemolysin.

c. Titration of experimental antigens for hemolytic activity. No hemolytic activity was exhibited by any of the experimental antigens at the end of 30 minutes at 37 C. in the presence of an equal volume of a 1.5% suspension of sheep red blood cells and 2 units of complement. These results indicated that any hemolysis that might occur in subsequent

serological procedures in which these antigens were used was due to the action of hemolysin or some material other than the antigens.

d. <u>Titration of antigen components for anti-complementary activity</u>. When a 1:2 dilution of each antigen component was titrated for 30 minutes at 37 C. in the presence of serial dilutions of 1:25 complement with 3 units of hemolysin and an equal volume of a 3% suspension of sheep red blood cells, varying degrees of anti-complementary activity were exhibited by each component. The degree of anti-complementary activity can be expressed as the number of units of complement inactivated by each component as compared with the amount of complement available in the control. The number of units of complement found to be inactivated by each 1:2 dilution of antigen components were: "Rhozyme P-11", more than 2.5 units; "Protease 15", 1 unit; Krebs-Ringer-phosphate solution, 0.4 units; and antibiotic solution, 0.4 units.

These results indicated that the anti-complementary activity exhibited by the experimental enzyme antigens was due, for the most part, to the action of the enzymes themselves. The elimination of the antibiotics or the Krebs-Ringer-phosphate solution from the composition of the enzyme antigens was not likely to greatly reduce the degree of anti-complementary activity exhibited by such antigens.

e. Titration of complement in the presence of serial dilutions of antigens. Titrations of serial dilutions of a 1:25 dilution of complement were performed in the presence of physiological salt solution and serial dilutions of each experimental and control antigen. The minimum and maximum number of units of complement available in the physiological salt solution controls at the beginning and end of the test were determined. By comparing these results to those of the titrations containing antigens, it was possible to determine the number of units of complement that were inactivated in the presence of each antigen, as shown in Table 14.

The results obtained in this series of titrations, as shown in Table 14, indicated that dilutions of 1:2 through 1:16 of both "Rhozyme P-11" antigens, "Protease 15" antigen number 1, and dilutions 1:2 and 1:4 of "Protease 15" antigen number 2 were markedly anti-complementary in the presence of a 1:25 dilution of complement. No end-point was reached with any of these antigen dilutions. In order to determine the amount of complement required in the presence of the antigens to provide a minimum of 2 units at the end of 18 hours, it was necessary to repeat the titrations using a more concentrated complement dilution.

The results obtained with the 1:8 and 1:16 dilutions of "Protease 15" antigen number 2, and all dilutions of the "N.Y." and CVS live virus antigens and the normal rabbit

Table 14. Titration of serial dilutions of 1:25 complement in the presence of antigens

| Antigen dilution | Units of complement available | | Units of complement inactivated |
|---|---|---|---|
| | Minimum | Maximum | |
| | (After 30 minutes at 37 C.) | | |
| Physiological salt solution beginning of test | 1 | 4.5 | 0 |
| | (After 18 hours at 4 C.) | | |
| end of test | 1 | 1.6 | 2.9 |
| Rhozyme P-11 number 1 1:2 through 1:16 | 0 | 0 | 4.5 |
| Rhozyme P-11 number 2 1:2 through 1:16 | 0 | 0 | 4.5 |
| Protease 15 number 1 1:2 through 1:16 | 0 | 0 | 4.5 |
| Protease 15 number 2 | | | |
| 1:2 and 1:4 | 0 | 0 | 4.5 |
| 1:8 and 1:16 | 1 | 1.6 | 2.9 |
| N.Y. Live Virus | | | |
| 1:2 through 1:8 | 1 | 1 | 3.5 |
| 1:16 | 1 | 1.4 | 3.1 |
| CVS Live Virus | | | |
| 1:2 through 1:8 | 1 | 1 | 3.5 |
| 1:16 | 1 | 1.2 | 3.3 |
| Normal Rabbit Brain | | | |
| 1:2 through 1:8 | 1 | 1 | 3.5 |
| 1:16 | 1 | 1.4 | 3.1 |

brain antigen indicated that these antigens exhibited only a minor amount of anti-complementary activity. It was determined that 0.06 ml. of a 1:24 dilution of complement should provide a minimum of 2 units of complement at the end of 18 hours at 4 C. in the presence of each of these antigens.

3. Standardization of materials for the actual complement-fixation tests

a. Titration of hemolysin in the presence of antigens. Due to the anti-complementary activity evidenced by "Rhozyme P-11" antigens number 1 and 2 and "Protease-15" antigens number 1 and 2, titrations of serial dilutions of hemolysin were performed in the presence of these antigens. The results obtained in the hemolysin titrations indicated that "Rhozyme P-11" antigens number 1 and 2 were extremely anti-complementary in their activity. Both of these antigens inactivated all available complement in a 1:15 dilution of complement. No end-point was reached in any of the hemolysin titrations performed due to this anti-complementary activity. For this reason, the "Rhozyme P-11" antigens were eliminated from further consideration in this problem.

In the hemolysin titrations performed with the "Protease 15" antigens and a 1:20 dilution of complement, definite end-points were obtained. One-tenth ml. of a 1:1,600 dilution of hemolysin was found to contain 1 unit of hemolysin in the

presence of a 1:2 dilution of "Protease 15" antigens number 1 and 2. This was the smallest amount of hemolysin in the presence of the antigen capable of producing complete hemolysis of an equal volume of a 3% suspension of sheep red blood cells in the presence of 0.3 ml. of a 1:20 dilution of complement.

b. <u>Titration of complement in the presence of "Protease 15" antigen number 2</u>. Serial dilutions of a 1:20 dilution of complement were titrated in the presence of a 1:2 dilution of "Protease 15" antigen number 2. The number of units of complement available at the beginning of the titration was compared to the number of units available at the end of the test after 18 hours at 4 C. At the beginning of the titration, 3 units of complement were available. At the end of the titration, only 1 unit was available. The results of this titration indicated that 0.12 ml. of a 1:19 dilution of complement should provide a minimum of 2 units of complement at the end of 18 hours at 4 C. in the presence of the antigen and 3 units of hemolysin as determined in the presence of the antigen.

c. <u>"Box" titration of antigens</u>. "Box" titrations of two-fold serial dilutions, 1:2 through 1:16, of "Protease 15" antigen number 2, and "N.Y." and CVS live virus antigens were performed in the presence of two-fold serial dilutions, 1:5 through 1:40, of equine and rabbit anti-rabies sera. Upon

completion of these 18 hour titrations the results were read visually. The degree of hemolysis exhibited in each tube of the titrations was graded 0, 1, 2, 3 or 4, as shown in Table 15. Four represented no hemolysis and zero represented complete hemolysis. Any doubtful reactions in the 0 to 1 gradation were recorded as 0.

Table 15. "Box" titration of experimental and control antigens

| Dilutions of anti-rabies serum | 1 to | Antigen dilutions: Protease 15 | | | | NY Live virus | | | | CVS Live virus | | | | Controls: Phy. salt | Controls: Anti-bio. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| serum | 1 to | 2 | 4 | 8 | 16 | 2 | 4 | 8 | 16 | 2 | 4 | 8 | 16 | 0 | 0 |
| Equine | 1:5 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1:10 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1:20 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1:40 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Rabbit | 1:5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 3 |
| | 1:10 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 |
| | 1:20 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 |
| | 1:40 | 4 | 4 | 0 | 0 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 0 |

From the results shown in Table 15, it was possible to determine the greatest dilution of each antigen capable of fixing complement in the greatest dilution of each serum. With the equine serum it was determined that a 1:2 dilution of "Protease 15" antigen number 2 was the greatest dilution which would fix complement in the presence of a 1:40 dilution

of serum. In the case of the live virus antigens, no end-point was obtained and dilutions of each of these antigens in proportions of 1:2 or higher were incapable of fixing complement in the presence of the serum.

From the results obtained with the titrations performed with rabbit anti-rabies serum, it was ascertained that a 1:4 dilution of the "Protease 15" antigen number 2 was the greatest dilution which would fix complement in the presence of serial dilutions of serum. The results shown for the "N.Y." and CVS live virus antigens indicate that any dilution from 1:2 through 1:16 would fix complement in the presence of the serum. Since no end-point was obtained with the 1:16 dilutions of these latter antigens, it is presumed that even greater dilutions would have fixed complement in the presence of the rabbit serum.

The complement controls which were performed concurrently with the antigen titrations indicated that in the absence of serum a minimum of 1 unit of complement would have been available at the end of 18 hours at 4 C. in each antigen titration. These results confirmed that any complement-fixation which took place in the presence of the anti-rabies sera, as shown in Table 15, was due to the fixation of complement by the experimental and control antigens concerned.

4. Complement-fixation tests

The complement-fixing capabilities of "Protease 15" antigen number 2; "N.Y." and CVS live virus antigens; and normal rabbit brain antigen were investigated in two series of complement-fixation tests. The results of these tests were read visually. The degree of hemolysis exhibited in each tube of each test was graded 0, 1, 2, 3, and 4. Four represented no hemolysis and 0 represented complete hemolysis. Any doubtful reactions in the 0 to 1 gradation were read as 0. The titre of each serum was expressed as the greatest dilution of serum giving a 3 or 4 reading as shown in Table 16.

As shown in Table 16, the complement-fixation tests performed with the equine immune serum, the normal equine sera number 1 and 2 and their respective albumin and globulin fractions gave conflicting results. Neither of the normal sera nor their albumin and globulin fractions were found to be markedly anti-complementary, as shown by the readings obtained with the respective physiological salt solution and antibiotic solution controls.

Normal equine serum number 1 and its globulin fraction demonstrated a titre of complement fixation greater than 1:640 in the presence of all four antigens. A titre greater than 1:640 was obtained with the immune serum only in the presence of the "Protease 15" antigen number 2. With the immune serum no titre was demonstrated with the other three

Table 16. Results of complement-fixation tests employing experimental and control antigens

| Serum | Titre of serum | | | | | |
|---|---|---|---|---|---|---|
| | Antigen dilutions | | | | Controls | |
| | Protease 15 | "N.Y." | CVS | Rabbit | Phy. salt | Anti-biotic. |
| | (1:2) | (1:2) | (1:2) | (1:2) | (1) | (1) |
| Equine immune | >640 | 0 | 0 | 0 | 0 | 0 |
| Normal equine #1 | >640 | >640 | >640 | >640 | 0 | 0 |
| albumin fraction | 0 | 0 | 0 | 0 | 0 | 0 |
| globulin fraction | >640 | >640 | >640 | >640 | 0 | 0 |
| Normal equine #2 | 5 | 40 | 40 | 20 | 20 | 20 |
| albumin fraction | 0 | 0 | 0 | 0 | 0 | 0 |
| globulin fraction | 5 | 40 | 40 | 40 | 20 | 20 |
| | (1:4) | (1:4) | (1:4) | ( - ) | (1) | (1) |
| Rabbit immune | >640 | 160 | 640 | - | 0 | 0 |
| Rabbit normal | 5 | 80 | 80 | - | 20 | 20 |

antigens shown in Table 16. In comparing the titre of the immune serum to that of the normal serum number 1 and its globulin fraction, it is impossible to state whether or not greater dilutions of each serum would have demonstrated any significant differences between their respective titres. It was demonstrated that in a dilution of 1:640, the end-point of complement fixation had not been reached in either serum. The fact that the normal equine serum number 1 and its globulin fraction demonstrated titres greater than 1:640 in the

presence of normal rabbit brain antigen, and that its albumin fraction did not fix complement, suggests that non-specific complement fixation may account for the results obtained. This possibility was further indicated by data in the clinical record of the horse from which the serum was obtained. The clinical record stated that the animal had been found to be affected with a sub-acute suppurative metritis several weeks before the serum sample was collected. At the time the serum sample was received the author was not aware of the previous clinical history of the donor.

A second sample of normal equine serum, designated as number 2, was obtained from a horse with a verified normal clinical history. The results obtained with this serum sample and its serum fractions, as seen in Table 16, were decidedly different from those obtained with equine serum number 1. A titre of 1:5 was demonstrated in the presence of "Protease 15" antigen as compared to a titre greater than 1:640 in the immune serum. These results indicated a greater than sevenfold difference in titre between the normal and immune serum was obtained with the "Protease 15" antigen number 2.

Complement-fixation tests employing the same antigens as used with the equine sera, with the exception of the normal rabbit antigen, were performed with pooled immune and normal rabbit sera. As shown in Table 16, a titre of greater than 1:640 was obtained with the immune serum as compared to a

titre of 1:5 in the normal serum with "protease 15" antigen. This represented a greater than seven-fold difference in titre between the immune and normal rabbit serum. With the same sera, only a one-fold difference in titre was obtained with the "N.Y." and a three-fold difference in titre with the CVS live virus antigen.

The complement controls which were performed concurrently with the complement-fixation tests indicated that in the absence of serum a minimum of 1 unit of complement would have been available at the end of 18 hours at 4 C. in each complement-fixation test. These results confirmed that any complement-fixation which took place in the presence of the sera, shown in Table 16, was due to the fixation of complement by the experimental and control antigens concerned.

# VI. DISCUSSION

The twelve enzymes which were originally selected for use in this study of some effects of proteolytic enzymes on rabies virus were considered as proteolytic enzymes. Although there are several classification systems referred to in the literature by which they could have been classified, the method of classification described by Sumner (51) places all twelve of these enzymes within the group referred to as proteolytic enzymes.

In contemplating the use of proteolytic enzymes for the elution of virus from animal tissue, several problems were evident. The action of most proteolytic enzymes, and enzymes in general, is specific. This specificity exists, according to Bodansky (4, p. 138), not only as to the relation between a given enzyme preparation and the general chemical structure of the substances which react in its presence but also as to specific atomic groups or linkages in the molecule of the substrate. This factor would have a tendency to restrict the use of certain enzymes due to their limited range of protolytic activity. Lysozyme, pancreatic desoxyribonuclease, and streptokinase-streptodornase, although non-toxic <u>in vivo</u> in high concentrations, were eliminated from further consideration because of their limited range of proteolytic activity.

Many of the proteolytic enzymes which might be considered

for use in the attempted elution of virus by proteolysis produce toxic reactions when inoculated into mice. One means of avoiding such toxic reactions might be the inactivation of the enzyme after completion of the proteolytic reaction. Enzymes can be inactivated by various methods, such as: alteration of the pH of the reaction; elevation of the temperature of the reaction; and exposure to light. Any procedure which might be used to inactivate an enzyme or alter an enzyme reaction could very likely have a detrimental effect on most viruses. The problem is further complicated by the fact that enzymes are proteins. The inactivation of an enzyme's proteolytic activity would not necessarily remove the cause of its toxic properties for mice. Jobling, et al. (24), found that active and inactive trypsin were equally capable of producing toxic reactions in dogs when injected intravenously. For these reasons, it appeared that the problem of enzyme toxicity could best be solved by searching for suitable enzymes that were non-toxic or relatively so. No attempt was made to find means of inactivating enzymes which would not harm the rabies virus.

Very few references appear in the literature concerning the effects manifested in laboratory animals following the injection of proteolytic enzymes. The work done by various investigators on the effect of various enzyme preparations on rabies virus, as cited in the review of pertinent literature,

offered little insight into the problem since the enzyme preparations used were all crude products, containing extraneous non-enzyme material. To answer the question of enzyme toxicity in mice, it was necessary to inoculate groups of mice with uniform dosages of serial dilutions of the twelve enzyme preparations under consideration. The intracerebral route of inoculation was employed in these toxicity tests since this was the normal route used in the isolation of rabies virus. By following this procedure it was possible to determine which enzymes were toxic for mice and the concentrations which were toxic.

All mice which evidenced toxic reactions following inoculation with serial dilutions of proteolytic enzymes presented symptoms which although they resembled severe anaphylactic shock, could more correctly be designated as proteolytic enzyme shock. Jobling, et al. (24), described similar symptoms which resembled anaphylaxis in cases of peptone shock in dogs. In his discussion of syndromes similar to the anaphylactic reaction, Godlowski (16), likens peptone and trypsin shock to anaphylactic shock. He draws the distinction that there is no sensitization period in peptone or trypsin shock. It is believed that the same distinction should apply to the results obtained with the various enzymes employed in this investigation. In no instance was there any known sensitization period in the case of any of the enzymes tested. It is

difficult to conceive how there could have been any natural prior sensitization of an animal such as a mouse to any of the enzymes employed in this study, particularily with such enzymes as ficin, bromelain, and papain.

From the results obtained in the enzyme toxicity tests it appears that there is some relationship between the specificity of a proteolytic enzyme as to the substrate on which it acts and its toxicity in mice. In general, enzymes with a limited range of proteolytic activity were comparatively non-toxic when inoculated intracerebrally in mice in comparatively concentrated dosages. In the same respect, enzymes with wider ranges of proteolytic activity were toxic for mice. The degree of toxicity being progressively greater with those enzymes which had the widest ranges of proteolytic activity.

Out of the group of twelve enzymes which were originally considered for use in this problem, three were eliminated because of their limited range of proteolytic activity as reported by the manufacturers. Four of the nine remaining enzymes under consideration were eliminated from further consideration although they each possessed wide ranges of proteolytic activity. They were found to produce proteolytic enzyme shock in mice when inoculated in dilutions greater than 0.00004 gm. of enzyme per minimal mouse dose.

Five enzymes were finally selected for further investiga-

tion in this problem. All five of these enzymes were non-toxic to mice in concentrations which were adequate for degradation of suitable protein substrates, according to the various manufacturers' data sheets. Two of these enzymes were trypsin preparations, one being a purified crystalline preparation and the other an unpurified preparation. The other three enzymes consisted of a fungal protease, a bacterial protease and a filtrate of a culture of an aerobic soil saprophyte.

The use of these five enzymes were particularily suitable for this problem. The action on rabies virus of several enzymes of animal and plant origin have been reported in the literature by various investigators. The two trypsin preparations served as standards since considerable data was available in the literature as to the action of trypsin preparations on rabies virus. No reference could be found in available literature as to the effect on rabies virus of the more recently isolated fungal and bacterial enzyme preparations. Therefore the use of the three enzymes of fungal or bacterial origin in this problem would serve to help complete the picture concerning the general effects of proteolytic enzymes on rabies virus.

Several methods exist for the measurement of the proteolytic activity of pure and crude proteolytic enzyme preparations. In general, methods of assaying such enzymes depend

upon correlating the proteolytic activity manifested by the enzyme on a standard substrate, Christensen (10), and Northrop (38); or the determination of the amount of a specific enzyme inhibitor required to inhibit the proteolytic activity of the enzyme, Kunitz (35). None of the methods of assay reported in the literature appeared to offer a solution to the problems associated with this study.

A method was needed to determine the amount of time required by a given quantity of a given proteolytic enzyme, at a given temperature, to produce maximum proteolysis of a specific substrate. The maximum limitations as to the duration and temperature of the reaction were predetermined on the basis of the known effects of these physical forces on the viability of rabies virus. The maximum quantity which could be used of any given enzyme was pre-determined by known non-toxic dose levels established for intracerebral inoculation of mice. Within these arbitrary limits, it was necessary to determine the minimal amount of enzyme which would produce maximum proteolysis of a brain tissue substrate.

The method evolved was a modification of the Klett-Summerson method for the colorimeter determination of protein in cerebrospinal fluid. This method is a standard laboratory diagnostic procedure considered to be sufficiently accurate for routine purposes. Essentially, the modified procedure consisted of turbidimetric determinations of the percentage

of decrease in the protein value of a brain tissue substrate occasioned by proteolysis.

In the series of turbidimetric determinations conducted in this study, the pH of each proteolytic enzyme reaction was ascertained to be in the range of 7.4 to 7.6. Although this pH range was not the optimal pH for each enzyme used, it was within the effective pH range of each of the five enzymes employed. According to Bodansky (4, p. 145), the activity of an enzyme depends upon the pH at which the reaction takes place. At a certain pH, the enzyme may undergo destruction while it acts on the substrate. On the other hand, even though there is no destruction of the enzyme, the rate at which it acts depends on the pH of the solution. There is a certain pH for every enzyme at which the reaction velocity is optimal. It is apparent that the pH, had it been at an optimal concentration for each enzyme, could have resulted in either a greater degree of proteolysis or a decrease in the reaction time.

The incubation temperature of each proteolytic enzyme reaction conducted for turbidimetric determinations was held at 37 C. since temperature also has a definite relationship to the velocity of an enzyme reaction. For each enzyme there is an optimal temperature range at which the reaction velocity is greatest. Grob (18), in his discussion of conditions which affect the activity of proteolytic enzymes states that most

of these enzymes are very active at 37 C., slightly increased in activity by short exposure to a temperature between 37 C. and 60 C., slightly impaired by a temperature of 65 C., and destroyed, if in solution, above 70 C. The necessary temperature varies inversely with the length of time of the reaction. The temperature of 37 C. was within the effective temperature range of each of the five proteolytic enzymes tested. This temperature was selected since the enzymes under investigation were intended for later use in the study of their effects on rabies virus. It was essential to assay the activity of the enzymes in relation to their subsequent utilization. The temperature of 37 C. was decided upon after reviewing the literature as to the effect of heat on rabies virus. It appeared that this temperature was the highest temperature which could be employed without adversely affecting the virus, while at the same time providing an effective temperature for the enzyme reactions.

The results obtained in the turbidimetric determinations correlated well with the factors, as described by Bodansky (4, p. 141), and Grob (18, p. 220), which control the velocity of enzyme reactions, such as: concentration of substrate; concentration of enzyme; pH of the reaction; temperature of the reaction; and inactivation of the enzyme. The belief that the decrease in the protein value of the substrate was a direct indication of the proteolytic activity of the enzymes

tested is based on the following factors: significant decreases in the protein value of the substrate were recorded when the substrate was incubated at 37 C. for 4 to 24 hours in the presence of each enzyme; no decrease in the protein value of the substrate was recorded when the substrate was handled in a similar manner in the absence of enzyme.

By means of this turbidimetric procedure it was possible to determine that each of the five proteolytic enzymes used in this study were capable of degrading brain protein when employed in concentrations that would not produce proteolytic enzyme shock in mice. The procedure also made it possible to ascertain the amount of time required at 37 C. for each enzyme to accomplish maximum proteolysis of the brain tissue. In this regard it was particularly interesting to find that with each enzyme-brain emulsion preparation, the end-point of the reaction was reached by the fourth hour of incubation at 37 C. This finding made it possible to establish a standard incubation schedule of 4 hours at 37 C. for the subsequent investigations of the effect of these enzymes on rabies virus.

The fact that the enzymes were capable of producing a 9 to 20% decrease in brain protein, depending upon the enzyme employed, in addition to the amount of degradation of protein accomplished by emulsification of the brain tissue was promising. If an equivalent per cent of live rabies virus was

released with this proteolysis, it could result in a significant increase in the titre of the virus in an infected brain tissue suspension. This possibility prompted the subsequent investigation of rabies virus elution in the presence of the five proteolytic enzymes.

In view of the results obtained with the modified Klett-Summerson method for the colorimeter determination of protein, it is felt that this method might be suitable for assaying the activity of proteolytic enzymes on any suitable substrate. The method could lend itself to the determination of the influence on the rate of proteolysis of various factors which might influence the velocity of a given proteolytic enzyme reaction.

Throughout the investigation of rabies virus elution in the presence of five proteolytic enzymes and in subsequent investigations in which mice were used for animal inoculation studies, 20 gm., adult mice were employed. According to Casals (7), the age difference in the susceptibility of mice to street virus are not especially noteworthy, but are significant with fixed virus which has been passed artifically from animal to animal. He found that 7 to 9 day old mice were more susceptible than older mice to the injection of fixed or street virus by any route. Twenty day old mice were reported to be no more susceptible to intracerebral injection of street or fixed virus than 60 day old mice. Koprowski

(32, p. 56), states, "mice of all ages are susceptible to intracerebrally introduced rabies virus. However, it is easiest to inoculate, maintain, and observe mice which are 21-35 days old, (8-12 gm. weight) at the time of inoculation."

Since the use of young mice, commonly recommended in the literature, appeared to be based for the most part on the ease of handling, the same criterion influenced the use of 20 gm. mice in these investigations. A large number of the mice used in this problem were received from a producer located several hundred miles from our laboratory. Due to regional transportation difficulties, each shipment of mice was in transit several days. Twenty gm. mice sustained shipment better than younger mice. It is felt that the age and weight of the mice employed bears no discernable influence on the experimental results obtained. The factor of age and weight of the mice used for animal inoculation was maintained constant throughout the course of this study.

In the investigation of rabies virus elution in the presence of five proteolytic enzymes and in subsequent investigations employing stock emulsions of rabies infected brain material, every effort was made to insure that each suspension of a particular brain emulsion was of uniform titre. That these efforts were successful is demonstrated by the fact that identical $LD_{50}$ titres were obtained in four titrations of untreated suspensions of each brain emulsion although

picked at random.

The LD $_{50}$ titre of the 4 C. controls in the titrations of rabies virus subjected to proteolysis was $10^{-4.6}$ as compared to a titre of $10^{-5}$ obtained with the homologous virus in the serum-virus neutralization tests. The cause of this divergence was not definitely ascertained. The virus suspensions employed in the neutralization tests were 20% suspensions, whereas those employed in the titrations of rabies virus subjected to proteolysis were 10% suspensions. Different diluents with slightly different pH were employed in these two series of tests. These two factors could account, in part, for the different LD $_{50}$ titres obtained in the two series of tests.

The titrations in mice of brain emulsions of six different strains of rabies virus which had been subjected to proteolytic enzymatic digestion indicated that each of the five enzymes employed exerted detrimental effects as evidenced by reduction in the titre of each virus. The results obtained with each individual enzyme so closely approximated each other that a separate consideration of each enzyme is unnecessary. Therefore, they can be described as a group. The effect of the enzymes on the viruses was not uniform in so far as each individual strain of virus was concerned since the percentage of decrease in LD $_{50}$ of live virus, following proteolysis at 37 C. for 4 hours, varied between strains from

88% to 99%. This would appear to indicate that each strain possessed a slightly different degree of resistence to the antenuating effects of the enzymes. These results correlate well with the results reported by several other investigators to the effect that trypsin can have a detrimental effect on rabies virus. It was found that proteolytic enzymatic digestion performed with trypsin or "Protease (trypsin)" occasioned a greater than 99% decrease in the titre of each strain of virus tested.

It was demonstrated with each virus, that infected rabbit brain emulsions when subjected to 37 C. for 4 hours in the absence of any enzyme produced decreases in the titre of the viruses ranging from 37% to 80%. An inverse relationship was found to exist between the percentage of decrease in titre of a strain attributable solely to treatment at 37 C. for 4 hours to the percentage of decrease in titre attributed solely to the effect of the enzymes. When the decrease in titre of a strain of virus occasioned by heat was great, the decrease by proteolysis was small. Conversely, when decrease in titre by heat was small, the decrease by proteolysis was greater.

Since proteolytic enzymatic digestion of brain emulsions containing rabies virus produced severe, almost total, decreases in the titre of live virus due to the combined action of heat and enzymes, it is obvious that elution of live virus

was not successfully accomplished by the enzymes. However, it appeared that an increase in the elution of dead virus was accomplished by the enzymes and that the quantity of dead virus released by this method exceeds that which can be released solely by emulsion of infected brain tissue followed by inactivation at 37 C. for 4 hours. If such were true, an immune response might possibly be induced by inoculation of mice with suspensions of rabies virus brain emulsion subjected to proteolytic enzymatic digestion. This possibility prompted the challenge of those mice which survived in the titrations of rabies virus brain emulsions subjected to proteolysis.

In the challenge of the surviving mice, 4 $LD_{50}$ of homologous virus was used as the challenge virus since it was felt that this concentration would provide a minimal lethal dose. It was feared that the use of a standard challenge dose of virus as employed in routine vaccine potency tests might possibly obscure any immunity that might conceivably have been induced by a single intracerebral inoculation of 0.03 ml. of attenuated virus.

A sufficient number of mice survived the challenge inoculation to indicate that in four out of six of the viruses used, some degree of immunity had been established in those mice which had been inoculated with rabies infected brain emulsions subjected to proteolytic enzymatic digestion. This immunity was sufficient to protect against 4 $LD_{50}$ of

homologous virus and appeared to be associated with the amount of dead virus known to be present in each of the original control and enzyme-brain emulsion suspensions.

The results obtained in the challenge tests described above indicated that it might have been possible to induce a higher level of immune response if larger quantities or more concentrated suspensions of enzyme-brain emulsions had been used to inoculate the mice. To explore the possible use of enzymes in the preparation of rabies vaccines, two experimental vaccines were prepared by proteolytic enzymatic digestion of brain tissue pools of CVS rabies virus. The first rabbit passage of the CVS virus was used as the seed virus and one fungal and one bacterial protease as the enzymes.

Although the seed virus conformed with the requirements for a rabies seed virus, as described by Kaplan (28), the $LD_{50}$ titre of the brain tissue pool from the resultant second rabbit passage of the virus was not high enough to properly conform to the standard requirements for vaccine production. This brain tissue pool was employed for the preparation of the experimental vaccines disregarding its failure to conform to standard requirements for commercial vaccine production. The purpose of this investigation was to study the effect of the enzymes on the antigenicity of the virus and not to produce a commercial vaccine. Since the brain tissue pool from the second rabbit passage of the CVS

virus possessed the highest titre of any of the readily available viruses it was deemed to be the most suitable for the production of experimental vaccines.

In the production of the experimental vaccines the method of proteolysis of the brain emulsion substrate differed from that employed in the investigation of the elution of rabies virus in the presence of five proteolytic enzymes. The incubation temperature employed for the proteolytic reaction in the production of the vaccines was 50 C. for 4 hours as compared to 37 C. for 4 hours in the elution of rabies virus by proteolysis. The incubation temperature was increased in order to inactivate the residual live virus which was present in the enzyme-brain emulsion preparations. Due to the specificity of enzyme reactions it was assumed that the increased temperature increased the velocity of the reaction. It was also assumed on the basis of previous results that it did not increase the degree of proteolysis obtained as compared to a temperature of 37 C. for 4 hours.

When groups of mice were inoculated with a single 0.5 ml. dose of either "Rhozyme P-11" or "Protease 15" vaccine number 1 and challenged 14 days later with serial $LD_{50}$ dilutions of homologous virus, no immune response was evidenced. This would seem to indicate that the experimental vaccines possessed no antigenic properties capable of inducing the formation of neutralizing anti-bodies. However, no such conclusion

was warranted since a single dose of the commercial phenolized vaccine also produced unsatisfactory results. No conclusions were drawn from this experiment other than the fact that it should be repeated using multiple doses as specified in standard methods for assaying the potency of rabies vaccines.

In the attempts to immunize mice by multiple inoculations of the experimental vaccines, the results confirm the belief that the "Rhozyme P-11" and "Protease 15" exerted an adverse effect on the antigenicity of the virus. When the mice which has been vaccinated with "Rhozyme P-11" and "Protease 15" vaccine suspensions number 2 and vaccine supernates number 2 were challenged with a standard challenge dose of 500 $LD_{50}$ of homologous virus, none of the mice survived. This would seem to indicate that the vaccines did not stimulate the production of neutralizing anti-bodies, or that the titre of the neutralizing anti-bodies that were produced was too low to withstand 500 $LD_{50}$ of virus. The latter deduction is probably more accurate since it was previously demonstrated that a single intracerebral inoculation of similar enzyme-brain emulsion preparations could induce an immune response sufficient to protect against 4 $LD_{50}$. There is some indication in the literature that the enzymes might interfere, in some manner, with the antigenic properties of the virus associated with the stimulation of the production of neutralizing anti-bodies. Johnson (25, p. 287), states

that tissue enzymes liberated from brain material used in the preparation of rabies vaccine undoubtedly have a deleterious effect on the antigenicity of the virus. In the proteolysis of brain tissue it would not be illogical to assume that foreign enzymes added to those liberated from the brain tissue could also result in a deleterious effect on the antigenicity of the virus.

Eighty per cent of the mice which were vaccinated with a 10% suspension of commercially prepared phenolized vaccine survived when challenged with 500 $LD_{50}$ of CVS virus, whereas only 25% of the mice vaccinated with a supernate of this suspension survived the challenge. The difference in the level of immunity induced by the vaccination of mice with suspensions and supernates of phenolized vaccines could possibly be explained on the effects of centrifugation. D'Silva, _et. al_. (13), draw attention to the fact that upon centrifugation of brain emulsions containing rabies virus, the brain tissue is deposited taking with it over 90% of the virus. This explanation could account for the variance in the antigenicity of the suspensions when compared to the supernates of the phenolized vaccine. It would not explain the results obtained with the heat inactivated vaccines.

In the case of the heat inactivated vaccines, the supernates were decidedly more antigenic than the suspensions. These results were the reversal of those obtained with the

phenolized vaccine suspensions and supernates. The amount of residual live virus present in the suspensions and the supernates of the heat inactivated vaccines was found to be approximately the same. Although this phenomenon cannot be fully explained a possible explanation might be based on the assumption that the virus which was freed by the emulsion of the brain tissue and subsequently inactivated by heat was of such particle size as to prevent its sedimentation by centrifugation at low speeds. If such a condition did exist, it could result in the greater portion of freed dead virus remaining in the supernate. The procedure of decanting the supernate and pooling it would serve to concentrate the freed dead virus in the supernate vaccine. To come to any definite conclusions, this portion of the investigation should be repeated a sufficient number of times to determine if the phenomenon was a constant characteristic of such preparations.

In the preparation of the enzyme vaccines and the heat inactivated vaccines, the enzyme-brain emulsion and non-enzyme-brain emulsion preparations were incubated at 50 C. for 1 hour followed by 37 C. for 3 hours. The procedure differed from that used in the preparation of the "Rhozyme P-11" and "Protease 15" vaccines number 1 which were incubated at 50 C. for 4 hours. Information available in the literature indicate that one hour at 50 C. should have been sufficient to inactivate the virus. However, on the basis of the results

obtained when serial dilutions of the experimental and heat inactivated vaccines were titrated in mice it is evident that exposure of the CVS strain of rabies virus to 50 C. for 1 hour followed by 37 C. for 3 hours was insufficient to completely inactivate the virus. Deaths occurred in the $10^{-1}$ dilution of each vaccine which was titrated and these deaths were proved to be due to rabies virus. These results are markedly different from those described by other investigators.

Although it was found that the effect of the heat employed in the incubation of the enzyme and heat inactivated vaccines accounted for over 99% of the total virus inactivated, this finding does not mean that the enzyme vaccines could not have contained a greater concentration of dead virus. The method of calculating the amount of virus killed by the action of the heat ranges used for the incubation of the vaccine preparations consisted of comparing the live virus titre of the vaccines to that of the original untreated brain emulsion. Numerous references in the literature concede that in the emulsion of infected brain tissue, only a portion of the live virus is recoverable by methods currently available for the harvest of the virus. If any additional amounts of virus were freed and killed by the proteolytic action of the enzymes it would not be possible to estimate the amount of dead virus in the vaccines prepared by proteolysis since the original amount of live virus freed and subsequently inacti-

vated would not be detectable except by the demonstration of the increased antigenicity of such a preparation. As has already been shown, the enzymes also inhibit the antigenicity of the virus at least in so far as its ability to stimulate the formation of neutralizing anti-bodies is concerned. It might be possible that the enzymes completely degraded the dead virus. Due to the specificity of enzymes in their action on a substrate this possibility does not seem too likely. At any rate, further investigation was warranted in order to more fully define the effect that the enzymes exerted on the antigenicity of the virus. For this reason, the investigation of the complement-fixing capabilities of the enzyme-brain emulsion preparations was considered.

Supernates of "Rhozyme P-11" and "Protease 15" vaccines number 1 and 2 were originally intended to be used as antigens in the evaluation of the complement-fixing antigens produced from CVS rabbit brain emulsions subjected to proteolytic enzymatic digestion. The "Rhozyme P-11" antigens were determined to possess marked anti-complementary activity and were therefore unsuitable for use as antigens. Both "Protease 15" antigens exhibited some anti-complementary activity which could be readily compensated for by balancing the complement and hemolysin systems. Although both of these antigens were suitable for use in so far as their anti-complementary activity was concerned only "Protease 15" antigen number

2 was selected for use in the actual complement-fixation tests. The sole reason for eliminating "Protease 15" antigen number 1 from further consideration was that insufficient quantities of this antigen were available and the expense involved in the preparation of additional quantities was not warranted. The dilutions of each "Protease 15" antigen which were determined to be suitable for use in the "box" titrations of antigens had exhibited similar characteristics in all the procedures which were performed for the standardization of the materials to be used in the complement-fixation tests.

The selection of a suitable complement-fixation test schedule presented some problems since the quantity of materials required in most schedules would have exceeded the quantities available for this purpose. Although specific complement-fixation test schedules have been developed for rabies, Casals and Palacios (9), and Bernkopf and Nachitigal (3), neither of the two reported in the literature were adaptable to this problem. The schedule which was selected was the one described by Casals, <u>et al</u>. (8), in their specific complement-fixation test for infection with poliomyelitis virus. This schedule was well adapted to the effective utilization of small quantities of materials and gave satisfactory results with the antigens employed in this problem.

In the complement-fixation tests performed with the

equine anti-rabies serum and the normal equine serum number 1 and its serum fractions, there was some evidence that non-specific complement fixation may have accounted for the unsatisfactory results obtained. In the tests performed with the equine anti-rabies serum and the normal equine serum number 2 and its serum fractions, there was a greater than seven-fold difference in titre between the immune and normal serum obtained with the "Protease 15" antigen. The end-point of complement fixation was not demonstrated with this antigen since complete complement fixation occurred in the 1:640 dilution of the immune serum which was the greatest dilution employed in the test. No evidence of complement fixation was demonstrated in the immune serum by either of the control antigens.

In the complement-fixation tests performed with the pools of rabies immune and normal rabbit serum there was a greater than seven-fold difference in titre between the immune and normal serum obtained with the "Protease 15" antigen. Again, the end-point of complement fixation was not demonstrated with this antigen since complete complement fixation occurred in the 1:640 dilution of the immune serum. With the same sera a one-fold difference in titre was obtained with the "N.Y." and a three-fold difference in titre with the CVS live virus antigen.

On the basis of the results obtained in the two series

of complement-fixation tests, it appears that the control antigens were capable of fixing complement in rabies immune serum as shown in the tests with the rabbit immune serum. If the titres of complement fixation attributed to the "Protease 15" antigen are assumed to be due to the complement-fixing capabilities of this antigen, it would indicate that complement-fixing antibodies were definitely available in both of the immune sera. Although it was not definitely proved it appears that the failure of the control antigens to fix complement in the equine anti-rabies serum could have been due to a low titre of complement-fixing antibodies in this serum. The rabbit immune serum was collected from animals which had just recently been vaccinated against rabies and therefore could be expected to contain a high titre of complement-fixing antibodies. On the other hand, the equine anti-rabies serum was a commercially prepared concentrated serum developed for its neutralizing antibody titre by repeated inoculations over a period of serveral months. It is conceivable that the complement-fixing antibody titre of such a serum could be considerably lower than would have been expected if the serum had been secured from freshly vaccinated animals.

The approximate concentration of live rabies virus in the "Protease-15" antigen and the live virus control antigens had been previously determined. The "N.Y." live virus

antigen contained approximately 390 times more live virus than the "Protease 15" antigen per given quantity of antigen. The CVS live virus antigen contained approximately 3,900 times more live virus than the "Protease 15" antigen. It would appear that the live virus content of the antigens was not the determining factor in the comparative abilities of the antigens to fix complement. If the live virus was the determining factor in the ability of an antigen to fix complement it would seem logical to expect that the results obtained in the complement-fixation tests would have been the opposite of those which were observed.

The actual concentration of dead virus in the "Protease 15" antigen and the control antigens was not definitely known. It had been previously determined that proteolytic enzymatic digestion of a brain emulsion of CVS or "N.Y." virus by "Protease 15" resulted in a greater than 99% reduction in the number of $LD_{50}$ in a given quantity of emulsion as compared to the titre of an identical quantity of untreated brain emulsion of the same virus. On the basis of this information it is assumed that the "Protease 15" antigen contained a much greater concentration of dead virus than either control antigen. If this assumption is correct it would indicate that the concentration of dead virus in an antigen was closely related to the ability of an antigen to fix complement.

These theories would satisfactorily explain the results obtained with each antigen employed in the complement-fixation tests. They would also tend to support the belief that proteolytic enzymatic digestion of rabies infected brain tissue results in the elution of greater quantities of virus from the tissue than would be possible by mechanical emulsification alone, and that the virus which is released by proteolysis is subsequently inactivated by the combined action of heat plus enzyme. It does not appear that the enzyme adversely effects the antigenicity of the virus in so far as the complement-fixing capabilities of the virus are concerned. It definitely does adversely effect the ability of the virus to induce the formation of neutralizing antibodies.

Further investigation of the potentials of the "Protease 15" antigen should encompass a more significant number of homologous and heterologous immune sera, and higher dilutions of sera should be employed. The ability of suspensions of rabies brain emulsion which have been subjected to proteolytic enzymatic digestion to induce the formation of complement-fixing antibodies in the serum of inoculated animals should also be investigated.

# VII. CONCLUSIONS

The following conclusions were made concerning the effects of five proteolytic enzymes on rabies virus.

1. Trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A" exert a marked attenuating effect on the virulence of each strain of rabies virus tested.

2. The employment of trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A" as a means of elution of dead rabies virus from brain material appeared to be successful.

3. The employment of the 5 proteolytic enzymes, studied in this problem, as a means of elution of live rabies virus was not successful.

4. The inoculation of mice, intracerebrally, with a 10% suspension of rabies infected rabbit brain emulsion subjected to proteolysis with either "Rhozyme P-11" or "Protease 15" may induce an immune response in the host. This immune response, as observed with four out of six strains of virus tested, is sufficient to protect against an intracerebral challenge of 4 $LD_{50}$ of homologous virus.

5. Suspensions of brain material from rabbits infected with rabies virus when subjected to proteolysis with either "Rhozyme P-11" or "Protease 15" are antigenically unsuitable for employment as vaccines. The titre of neutralizing anti-

bodies induced in mice inoculated with either preparation is too low to protect against standard doses of challenge virus. Both enzymes exert a deleterious effect on the antigenicity of the CVS strain of rabies virus in so far as the ability of the virus to stimulate the production of neutralizing antibodies is concerned.

6. Concentrations of 0.1 gm. of "Rhozyme P-11" in either physiological salt solution or supernates of 10% suspensions of rabbit brain emulsion exhibit marked anticomplementary activity.

7. An antigen prepared by the proteolysis of a 10% suspension of rabies infected rabbit brain emulsion with "Protease 15" gave indications of being a suitable complement-fixing antigen warranting further investigation. This enzyme does not appear to exert any deleterious effect on the antigenicity of the CVS strain of virus in so far as the ability of the virus to fix complement is concerned.

The following conclusions were made concerning the effect of heat on rabies virus.

1. Exposure of a 10% suspension of rabies infected rabbit brain emulsion to 37 C. for a period of 4 hours produces a marked decrease in the infectivity of brain tissue. The percentage of decrease of infectivity varies from 30% to 80% depending on the original titre of the virus.

2. Exposure of a 10% suspension of rabies infected rabbit brain emulsion to 50 C. for 4 hours inactivates the virus.

3. Exposure of a 10% suspension of rabbit brain emulsion of the CVS strain of fixed virus to 50 C. for 1 hour, followed by 37 C. for 3 hours, produces a 99% decrease in the number of $LD_{50}$ of live virus in the brain tissue. The virus is not completely inactivated.

# VIII. SUMMARY

The toxicity induced in mice by 12 proteolytic enzymes was determined by the intracerebral inoculation of serial dilutions of solutions of each enzyme. Five enzymes: trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A" were found to be relatively non-toxic in low dilutions. Three enzymes: lysozyme, pancreatic desoxyribonuclease and streptokinase-streptodornase were characterized by a narrow range of proteolytic activity. Although non-toxic to mice, they were eliminated from further onsideration. Four enzymes: bromelain, ficin, chymotrypsin and papain were found to be toxic to mice in high dilutions and were eliminated from further consideration for this reason.

A turbidimetric method for the assay of the velocity of proteolytic enzyme reactions, under given conditions which effect this rate of velocity, was described. By means of this method, trypsin, "Rhozyme P-11", "Protease 15", "Protease (trypsin)" and "Proteinase A" were assayed for their proteolytic effect on a brain tissue substrate. Significant decreases in the protein content of the substrate were produced by proteolysis with each of the five enzymes. In the case of all five enzymes, no detectable proteolysis occurred until after the second hour at 37 C. No additional increase in the amount of proteolysis occurred after the fourth hour.

Rabbit brain emulsions of six strains of rabies virus were individually subjected to proteolysis with each of five enzymes. After completion of the proteolytic reaction, serial dilutions of each enzyme-brain emulsion preparation of each strain of virus were prepared. Groups of mice were inoculated intracerebrally per dilution. A significant number of mice survived in each titration of enzyme-brain emulsion preparations indicating that all five of the enzymes possessed the ability to markedly decrease the viability of each strain of virus tested.

The survivors of each titration of an enzyme-brain emulsion preparation were challenged 21 days subsequent to inoculation. The challenge was administered intracerebrally and consisted of 4 $LD_{50}$ of homologous virus. An immunity to 4 $LD_{50}$ was established, with four of the six strains of virus employed, in mice which had received an intracerebral inoculation of enzyme-brain emulsion preparations prepared with either "Rhozyme P-11" or "Protease 15".

Attempts were made to immunize mice against rabies by a single intraperitoneal inoculation of a supernate of a 10% suspension of rabies infected rabbit brain emulsion subjected to proteolysis with either "Rhozyme P-11" or "Protease 15". Challenge of inoculated mice, 14 days subsequent to inoculation, with serial $LD_{50}$ dilutions of homologous virus failed to confirm the existence of any immunity attributable to

either enzyme-brain emulsion preparation.

Attempts to immunize mice with multiple inoculations of the enzyme-brain emulsion preparations, described above, following the procedure for the modified Habel potency test, were equally fruitless. No immunity was evidenced by either enzyme-brain emulsion preparations when inoculated mice were challenged with 500 $LD_{50}$ of homologous virus. Results obtained with the control vaccines indicated that a supernate of a heat inactivated rabbit brain emulsion containing rabies virus induced a satisfactory immune response. This immunity compared favorably to the immunity established with the commercial phenolized rabies vaccine employed as a control.

The complement-fixing antigenic potentials of supernates of "Rhozyme P-11" and "Protease 15" enzyme-brain emulsion preparations were investigated. Preparations containing "Rhozyme P-11" were found to be markedly anti-complementary in their activity. With preparations containing "Protease 15" a balanced complement-hemolysin system was developed. A "Protease 15" brain antigen for rabies was employed in a series of complment-fixation tests with equine and pooled rabbit immune and normal sera. Albumin and globulin fractions of each of the normal equine sera were also tested.

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