mologous plasma for the animal's blood before the hemorrhage has in some unknown manner both reduced the bleeding volume and prevented the usual movement of fluid and protein into the circulation accompanying a hemorrhage to 35 mm Hg arterial pressure. There is no evidence that the same results might not have been obtained if autologous plasma had been used for the exchange. Unfortunately, neither the present experiments nor those of Lawson *et al*(4) permit even tentative conclusions concerning the mechanism producing this effect.

After hemorrhage, in spite of plasma leaving the circulation, the venous cell percentage decreased while the circulatory hematocrit did not change. Thus, it may be assumed that plasma from the small vessels or low hematocrit compartment entered the large vessel system or high hematocrit compartment(11). Consequently, the BVR_{cells} increased from 0.80 after the plasma-blood exchange to 0.91 after the hemorrhage.

Summary. The effect on plasma and red cell volume and protein concentration of exchanging homologous plasma for the dogs' own blood before a hemorrhage to 35 mm Hg mean arterial pressure was tested in anesthetized dogs. The measured cell and plasma volumes and protein concentration were in good agreement with those expected after the exchange. Therefore, homologous plasma successfully replaced the dogs' plasma removed during the exchange, and no reaction was seen attributable to the plasma infusion. When these animals were subjected to hemorrhage, the mean bleeding volume was 30% to reach a level of 35 mm Hg arterial pressure in contrast to 46% for dogs not receiving an exchange. Also 5.9 ml/kg of plasma and 0.46 g/kg of protein has escaped from the circulation while previously there was mobilization of fluid and protein after a hemorrhage.

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Some Characteristics of a Proteolytic System from *Phymatotrichum omnivorum.**† (28824)

ALEXIS A. BURGUM, J. M. PRESCOTT AND RALPH J. HERVEY‡
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The importance of *Phymatotrichum omnivorum* (Shear) Duggar as a plant pathogen has prompted extensive investigations of this fungus from morphological, physiological, and agronomic standpoints. Little consideration, however, has been given to the study of the enzymes produced by this organism. As part of a fundamental investigation of the metabolism of *P. omnivorum,* we have determined some characteristics of proteolytic systems found in culture filtrates from 2 pathogenic strains. The existence of proteinase ac-

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TABLE I. Medium for Growing *Phymatotrichum omnivorum*.

Component	Amt/l	Component	Amt/l
	g		mg
Sucrose	30.0	KCl	150
NH_4NO_3	1.2	$FeSO_4 \cdot 7H_2O$	50
K_2HPO_4	1.4	$MnSO_4 \cdot 2H_2O$	34
$MgSO_4 \cdot 7H_2O$	1.63	$ZnSO_4 \cdot 7H_2O$	9

tivity has also been established in 2 non-pathogenic strains.

Materials and methods. Five strains of *P. omnivorum* were isolated over a period of several years from infected cotton plants in central Texas, and were maintained in the laboratory on sterile sorghum seeds in soil. These strains are designated as 2, 5-2, 6, 17 and 26. Upon being cultured for 4 to 5 years in the laboratory, strains 2 and 5-2 lost their pathogenicity toward cotton seedlings, and became somewhat physiologically deteriorated, producing no sclerotia and exhibiting very slow growth rates. Their colonial appearances were atypical, being whiter and fluffier than normal, and on the soil-sorghum seed medium they produced a sheet of thin, soil-penetrating mycelia rather than the typical coarse, rope-like sclerotial strands, which characteristically penetrate the soil prior to formation of sclerotia. Strain 6 was deteriorated to a lesser degree, both pathogenically and physiologically. Strains 17 and 26, much more recently isolated, still showed high pathogenicity and only slight physiologic degeneration. In soil they produced coarse, thread-like sclerotial strands and turgid, typically shaped, viable sclerotia. The colonies on agar or liquid media were typical open-matrix colonies, which gradually changed from white to tan-colored mycelia. These 2 strains produced an abundant yellow-brown extracellular pigment.

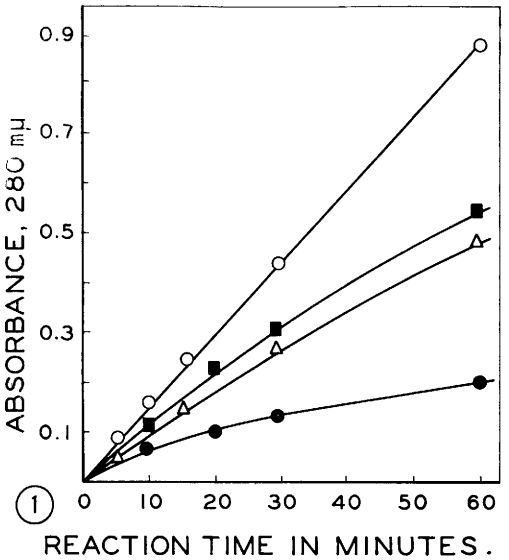
Inocula of *P. omnivorum* were prepared by infecting autoclaved sorghum seed in flasks containing sterile soil. A single seed was removed with sterile forceps and placed in a 2.5 liter low-form culture flask containing 300 ml of sterilized basal medium (Table I), which was similar to that of Rogers(1). Cultures were incubated at 22°C until good growth had occurred, usually 25 days. The mycelial pads were removed, any imbibed fluid was expressed by gentle pressure, and the metabolized medium was filtered through a layer of cotton. The culture filtrate thus obtained was frozen and stored at –10°C until used in enzyme experiments.

Tests for proteolytic activity were performed by the trypsin procedure of Anson(2), using a slightly modified urea-denatured hemoglobin substrate.§ Incubation of the reaction mixture was 37.5°C; extent of proteolysis was determined by reading the trichloracetic acid filtrate at 280 mμ in a Beckman DU spectrophotometer. Other protein substrates were assayed in the same general manner. Tests on milk clotting were performed by the method of Berridge(3).

Estimations of specific activities were based on protein concentrations determined by the method of Lowry *et al*(4), using crystalline bovine plasma albumin as the standard. Samples of enzyme to be assayed for protein were dialyzed, reacted with the Lowry reagent, and protein concentrations were read from the standard curve.

Results. Culture filtrates from all strains of *P. omnivorum* except strain 6 possessed proteolytic activity. Because of the light growth of strains 2 and 5-2, however, it was necessary to concentrate their culture filtrates approximately 5-fold by perevaporation in order to obtain appreciable activity. Proteolysis followed a typical time-response course for each active strain, as shown in Fig. 1. Other experiments showed enzymatic activity to be proportional to enzyme concentration. There was considerable variation in the protein concentrations of culture filtrates from the different strains, however, because of differences in amount of growth, and Table II shows proteolysis by the various strains expressed in terms of specific activity. From these data it is evident that there is no clear-cut relationship between proteolysis and the pathogenicity of the

§ Prepared by suspending 5 g salt-free lyophilized hemoglobin (Worthington Biochemical Corp.) with 80 g urea in 80 ml of water and incubating at 37.5°C for 1 hour. The mixture was then diluted to 250 ml with a 10% solution of urea made in 0.066 M phosphate buffer of the desired pH.



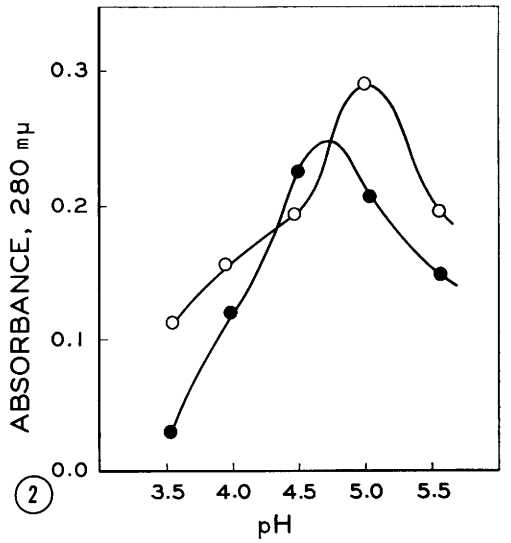


FIG. 1. Proteolysis by culture filtrates of 4 strains of *Phymatotrichum omnivorum*. ○ = strain 17 (0.22 mg protein per ml); ■ = strain 5.2 (0.45 mg protein per ml); △ = strain 26 (0.8 mg protein per ml); ● = strain 2 (0.26 mg protein per ml). Tests employed 1 ml of culture filtrate and 5 ml of hemoglobin substrate, pH 5.0. Incubation temperature, 37.5°C. Culture filtrates from strains 2 and 5-2 were concentrated approximately 5-fold prior to assay.

FIG. 2. Effects of pH on proteolysis by enzymes from *Phymatotrichum omnivorum*. ○ = strain 17; ● = strain 26. Conditions of reaction: 20 min at 37.5°C.

strain. This is also borne out by the absence of proteolytic activity in strain 6, since it was more active pathologically and grew more heavily than strains 2 and 5-2. The proteolytic enzymes appeared to be restricted to the extracellular fluid, since disruption and extraction of washed mycelial pads of strains 17 and 26 yielded no detectable proteolytic activity.

Although strains 2 and 5-2 possessed higher specific activity than strain 26, the physiologic deterioration of these 2 strains upon continued culture in the laboratory resulted in poor growth and low total proteinase production. Consequently, further studies on the properties of the proteolytic enzymes were confined to strains 17 and 26 because of their more abundant growth and total enzyme production.

TABLE II. Specific Activities of Proteolytic Enzymes from 4 Strains of *Phymatotrichum omnivorum*.

Strain No.	Plant pathogenicity	Specific activity (units*/mg protein)
17	+	14.1
26	+	2.5
5–2	—	4.9
2	—	3.5

* One unit is that amount of enzyme which produces a change in absorbance of 0.1 at 280 mμ in 20 min of incubation at 37.5°C.

pH dependence of proteolysis. Hemoglobin substrates were prepared in acetate buffers covering the range of pH 3 to 8. As shown in Fig. 2, strain 26 was maximally active between pH 4.5 and 5.0, and strain 17 was most active at pH 5.0. Similar results were obtained when a combination of phosphate and citrate buffers were used to cover the range of pH values.

Effects of temperature. A range of reaction temperatures from 6°C to 70°C was used to determine the effects of temperature on proteolysis, using a 20-minute reaction time. Under these conditions, maximum activity of strain 17 culture filtrates occurred at 60°C, while proteolysis by enzymes from strain 26 was maximal at 55°C. Rapid inactivation occurred at higher temperatures. When culture filtrates were exposed to a temperature of 70°C for varying time intervals, thermal inactivation of the proteinases occurred between 5 and 7.5 minutes in strain 17 and in less than 2.5 minutes in strain 26.

Effects of activators and inhibitors. To test for ionic activation or inhibition, culture filtrates of *P. omnivorum* were dialyzed at 5°C for 24 hours against 0.001 M phosphate buffer, pH 4.5. The dialyzed culture filtrates were then incubated with equal volumes of 0.01 M solutions of various salts for 1 hour before addition of the hemoglobin substrate. A number of divalent ions increased enzymatic activity, some by as much as 57% over that of the dialyzed culture filtrate. Stimulation was produced by Co^{++}, Ni^{++}, Zn^{++}, Ca^{++}, Mg^{++} and Mn^{++} ions, but Fe^{+++} reduced activity substantially. Addition of 1,10-phenanthroline to a final concentration of 0.001 M completely inhibited proteolysis, thus indicating the essentiality of metal ions. Addition of Co^{++} ions to a concentration of 0.0025 M, however, restored enzymatic activity to 50% of its original level in strain 26, and to 70% of the original level in strain 17. Similarly, a sample of enzyme from strain 17, which was dialyzed against 0.001 M 1,10-phenanthroline, was reactivated to varying extents by addition of Co^{++}, Ni^{++}, Zn^{++}, Mg^{++}, Mn^{++} or Ca^{++} ions at a concentration of 0.01 M. Of these, Co^{++} and Ni^{++} were the most effective.

Dialyzed culture filtrates were also tested for the effects of reducing agents by addition of KCN, thioglycollate, or cysteine at a level of 0.005 M. All 3 of these reagents reduced proteolysis slightly. The presence of *p*-chloromercuribenzoate failed to inhibit proteolytic activity of either strain of *P. omnivorum.*

When culture filtrate from strain 17 was reacted for one hour with 0.001 M diisopropylphosphofluoridate (DFP), a 20% reduction in proteinase activity occurred.

Activity toward other substrates. The enzymes from *P. omnivorum* attacked casein and egg albumin, but the rate of hydrolysis was less than that observed with hemoglobin under similar conditions. The enzymes also clotted milk, but at a slow rate.

Discussion. The data indicate similarities between the proteolytic enzymes of *P. omnivorum,* strains 17 and 26, but they do not permit a decision as to whether the enzymes of the two strains are identical. Hagihara (5) has reviewed the properties of a number of mold proteases, but the properties of the enzymes from *P. omnivorum* appear to be different from any of those described with respect to pH optimum and ionic activators. That the enzymes from *P. omnivorum* require a metal ion activator is evident from the ability of 1,10-phenanthroline to inhibit proteolysis. The restoration of activity by several divalent ions suggests that the specificity of the ionic requirement is rather low. It is possible that the culture filtrates from *P. omnivorum* contain mixtures of proteolytic enzymes, as do those of some other molds (5). The fact that only partial inactivation was produced by DFP suggests the presence of DFP-susceptible and non-susceptible enzymes. Alternatively, this observation may stem from an unusually slow reaction between DFP and the proteinase.

The proteolytic activity of *P. omnivorum* strains appears to have no clear-cut relationship either to pathogenicity or to physiologic vigor. Two of the 3 strains which had lost their pathogenicity toward cotton seedlings and much of their physiologic vigor showed higher specific proteolytic activities than one of the 2 rapidly growing, pathogenic strains. Strain 6, on the other hand, was less deteriorated both pathogenically and physiologically, yet produced no detectable amounts of proteolytic enzymes.

Work in progress is being directed toward obtaining homogeneous fractions with enzymatic activity, so that bond specificities and ionic requirements can be determined precisely.

Summary. The existence of proteolytic enzymes has been shown in 4 strains of *Phymatotrichum omnivorum* of varying stages of pathologic and physiologic vigor. One other strain failed to exhibit proteinase production in laboratory culture. Various properties of the enzymes from 2 strains have been studied. The enzymes were optimally active at pH 4.5 to 5.0, and required a divalent metal ion. This requirement could be met by Co^{++}, Zn^{++}, Ni^{++}, Mg^{++}, Mn^{++} or Ca^{++} ions; Fe^{+++} ions were inhibitory. Slight inhibition resulted from the presence

of reducing agents, and 0.001 M diisopropylphosphofluoridate reduced proteolysis partially. The enzymes from both strains were inactivated fairly quickly at 70°C.

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Anti-herpetic Activity of 5-Iodo-2'-deoxyuridine in Presence of Its Degradation Products. (28825)

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Kaufman and Maloney(1) noted that, after storage, 5-iodo-2'-deoxyuridine (IUDR) solutions appeared to lose antiviral activity in tissue culture. More recently these investigators(2) reported that an 'aged' solution of IUDR not only lost its activity against herpes simplex virus in tissue culture, but also interfered with the anti-herpetic activity of freshly prepared IUDR. They attributed this biological instability to the presence of the degradation products of IUDR, namely, 5-iodouracil (IU) and possibly deoxyuridine (DU). They concluded that the presence of minute amounts of IU inhibited the ability of IUDR to eliminate virus from herpes simplex-infected rabbit kidney tissue culture. The antagonism of IUDR by its degradation products, which was apparently observed in tissue culture, was not found *in vivo* in experimental keratitis in the rabbit (2). Thymidine has been previously reported (3) to antagonize the anti-herpetic activity of IUDR in tissue culture. This observation also has been confirmed in our laboratory. The purpose of this investigation is 2-fold: (a) to study further the effect of the degradation products, IU and DU, on anti-herpetic activity of IUDR and (b) to show the effect of prolonged storage of solutions of IUDR on anti-herpetic activity as determined in tissue culture.

Material and methods. Plaque inhibition tests using chick embryo cell cultures were carried out as described previously(4) using the HF strain of herpes simplex virus. Filter paper discs, impregnated with 0.1 ml of solutions of either 'fresh' IUDR, 'aged' IUDR, IU, DU, thymidine, and IUDR-IU combinations, were air-dried prior to use. The zone of inhibition of virus growth, evidenced by the absence of plaques surrounding each active disc, was measured in millimeters.

The chemotherapeutic activity of IUDR preparations also was determined in tube cultures of rabbit kidney. Tubes containing approximately 10^5 cells were infected with 100 $TCID_{50}$ of the Virtue strain of herpes simplex virus and treated simultaneously with varying concentrations of test material. Treatment was administered again 4 days after infection. Microscopic examination of the tubes and scoring of cytopathogenic effect (CPE) were done on the 4th and 6th days after infection; the experiment was terminated on the 6th day. The cytopathogenic changes were scored from 0 to 4+, where 0 represents a complete absence of viral CPE and 4+ represents total destruction of the cells. The minimal effective dose represents that concentration of test material that protects at least 50% of the cell layer from viral destruction.

Experimental results. Freshly prepared IUDR at a concentration of 50 μg/disc consistently produced considerable anti-herpetic activity, with zones of inhibition averaging