

# Herbicide Transformation

## II. Studies with an Acylamidase of *Fusarium solani*

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Replacement cultures liberated 3,4-dichloroaniline (DCA) from 3,4-dichloropropionanilide (propanil). The kinetics of the conversion suggest a requirement for de novo enzyme synthesis, but the system was not influenced by chloramphenicol or puromycin. Enzyme activity was detected when acetanilide ( $K_m = 0.195$  mM) was used to replace propanil as substrate. Fungal acylamidase (E.C. 3.5.1., an aryl acylamine amidohydrolase) was concentrated by salt precipitation and characterized. The *Fusarium solani* acylamidase exhibited an optimum at pH 7.5 to 9.0 and was inactivated in 10 min at 50 C. The enzyme was not sensitive to methyl-carbamate or organophosphate insecticides, but the herbicide, Ramrod (*N*-isopropyl-2-chloroacetanilide), acted as a competitive inhibitor of acetanilide hydrolysis ( $K_i = 0.167$  mM). Hydrolysis rates were decreased by various *para* substitutions of acetanilide. Chloro substitution in the acyl moiety of acetanilide also reduced the rate of hydrolysis. 3,4-Dichloroacetanilide was less susceptible to enzyme action than acetanilide, but 3,4-dichloropropionanilide was hydrolyzed much more rapidly than propionanilide. The fungal acylamidase was highly specific for *N*-acetylarylamines. It did not catalyze hydrolysis of formanilide, butyranilide, dicryl, Karsil, fenuron, monuron, or isopropyl-*N*-phenylcarbamate. It appears to differ from acylamidases that have been isolated from rice, rat liver, chick kidney, and *Neurospora*.

Chemical configuration is perhaps the most important factor that determines the persistence of a compound in nature. Virtually all of the biocidal compounds employed in pest control are the creations of organic chemists. As such, they are often structurally different from compounds of biological origin and therein lies the explanation, in major part, of their non-biodegradability or molecular recalcitrance (1). Apparently, a synthetic compound is susceptible to enzymatic attack because it bears a structural resemblance to some natural product that is sufficient to be mistaken for this substance and used as substrate by the enzyme involved.

This paper describes the isolation of an enzyme of *Fusarium solani* (10) responsible for propanil hydrolysis and examines its substrate range and affinity.

### MATERIALS AND METHODS

**Analytical procedures.** Protein concentrations were determined by the method of Lowry et al. (12) by using crystalline bovine serum albumin as standard.

The procedure used for analysis of the various primary arylamines was modified from that of Goldberg

et al. (6). In all cases, 1.0 ml of a 40% trichloroacetic acid solution was added to 3 ml of the aqueous sample to be analyzed. To determine the anilines released during replacement culture hydrolysis of acylanilides, 1.0-ml portions of the culture medium were transferred to 2.0 ml of distilled water.

After addition of the trichloroacetic acid, 1.0 ml of freshly prepared sodium nitrite solution (4 mg/ml) was added. When diazonium ion formation was complete (3 min), excess nitrate was destroyed by 1.0 ml of ammonium sulfamate solution (20 mg/ml), and, after a second 3-min interval, 2.0 ml of *N*-(1-naphthyl) ethylene diamine dihydrochloride solution (1.5 mg/ml of 95% ethyl alcohol) was added. Azo dye formation was complete after 15 min at room temperature in most cases. The exception was *p*-anisidine which required 45 min at 75 C for full color development. The intensity of the color was measured with a Klett-Summerson photoelectric colorimeter equipped with a green filter (no. 54). The concentrations were estimated by comparison to standard curves prepared with authentic samples of the appropriate arylamines.

**Preparation of replacement culture.** Czapek-Dox broth was distributed in 100-ml portions in 250-ml Erlenmeyer flasks and sterilized by autoclaving at 121 C for 15 min. The flasks were inoculated with spores of *F. solani* harvested from slants of Czapek-Dox agar and incubated for 72 hr at 28 C on a rotary shaker. Growth was harvested on paper (Whatman no. 41) by filtration and washed with distilled water. The

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mycelium collected from each of two flasks was combined and homogenized in 15 ml of distilled water for 15 sec at 14,000 rev/min in a mixer run in an ice bath. The resulting hyphal fragments were concentrated by centrifugation, suspended in 200 ml of 0.05 M phosphate buffer (pH 7.4) which contained either 30 mg of propanil or 54 mg of acetanilide, and divided into two 250-ml Erlenmeyer flasks. These replacement cultures were incubated on a rotary shaker at 28 C, and their ability to hydrolyze acylanilide was determined periodically. Arylamine analyses were made on 1.0-ml samples of the culture solutions that were freed of cells by filtration through paper (Whatman no. 542).

**Preparation of crude enzyme extract.** Replacement cultures used as a source of enzyme were obtained as described above, with acetanilide at a concentration of 270 mg/liter of buffer. Preliminary tests indicated that maximal enzyme concentration was obtained after exposure of replacement cultures to acetanilide for 6 to 7 hr. Exposed cells were collected by centrifugation, washed free of residual aniline and acetanilide, suspended in 15 ml of cold distilled water in a 50-ml tube, and sonicated for 90 sec at 0 to 5 C by a MSE-Mullard Ultrasonic Disintegrator (Instrumentation Associates, Inc., New York, N.Y.). Unbroken cells and cellular debris were sedimented by centrifugation at  $78,480 \times g$  for 30 min (Spinco model L ultracentrifuge, no. 30 head); the supernatant fluid, designated crude enzyme extract, was collected.

**Ammonium sulfate fractionation.** Acylamidase was partially purified and concentrated from crude extracts of *F. solani* by the addition of finely ground ammonium sulfate with constant stirring at room temperature. The major portion of the active protein was precipitated at salt concentrations ranging from 28 to 42% (w/v). The active material was collected by centrifugation, suspended in distilled water, and stored at -20 C, at which temperature it remained without significant loss of activity for at least 2 months.

**Enzyme assays.** Tests were performed in tubes containing 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of enzyme, and 1.0 ml of substrate solution. Reactions were terminated after 20 or 25 min at 28 C by addition of 40% trichloroacetic acid, and the amount of arylamine produced was determined. The acid served to terminate enzyme action and to provide protons for the diazotization reaction.

During enzyme purification and determination of optimal assay conditions and specific activity, acetanilide at a concentration of 2.0 mM was used as substrate. Specific activity of the *Fusarium* acylamidase was measured as nanomoles of aniline produced per milligram of protein per 20 min under assay conditions, and a unit of enzyme was defined as the amount required for the formation of 10 nmoles (0.93  $\mu\text{g}$ ) of aniline under the same conditions.

The relatively low solubility of some compounds used in enzyme specificity studies necessitated the use of a lower (0.5 mM) substrate concentration. All tests were repeated at least twice, and results were reported as the mean of corresponding analyses.

**Inhibitors.** To study the effects of carbaryl (1-naphthyl-*N*-methyl-carbamate) and parathion (O,O-diethyl-O-*p*-nitrophenyl thiophosphate) on the rate of

acetanilide hydrolysis by the fungal acylamidase, the insecticides were dissolved ( $10^{-5}$  and  $10^{-6}$  M) in 0.1 M phosphate buffer at pH 7.5. One ml of enzyme preparation was treated with 1.0 ml of insecticide solution and incubated at 28 C for 15 min prior to the addition of 1.0 ml of substrate (2.0 mM acetanilide). Analyses of aniline released were performed after 20 min.

**Substrates.** Propanil was a gift from Rohm and Haas Co. (Philadelphia, Pa.); dicryl (3',4'-dichloromethacrylanilide), Karsil (3',4'-dichloro-2-methylvalerianilide) and 3,4-dichloroacetanilide were supplied by Niagara Chemical Div., FMC Corp. (Middleport, N.Y.); monuron [3-(4-chlorophenyl)-1,1-dimethylurea] and fenuron (3-phenyl-1,1-dimethylurea) were provided by E. I. duPont de Nemours and Co. (Wilmington, Del.); and IPC (isopropyl-*N*-phenylcarbamate) was a gift from Pittsburgh Plate Glass Co. (Pittsburgh, Pa.). Acetanilide, 4-nitroacetanilide,  $\alpha$ -chloroacetanilide, and formilanilide were purchased (Aldrich Chemical Co., Inc., Milwaukee, Wis.), and propionanilide, butyranilide, *p*-acetaniside, and 4-chloroacetanilide were synthesized by the acylation of their corresponding arylamines by the appropriate acid chloride as described by Huffman and Allen (7). The products were recrystallized from suitable solvents until all detectable arylamine had been removed.

## RESULTS AND DISCUSSION

**Replacement cultures.** Previous studies of the growth of *F. solani* in mineral medium with propanil as sole source of carbon demonstrated that only minimal development could be obtained be-

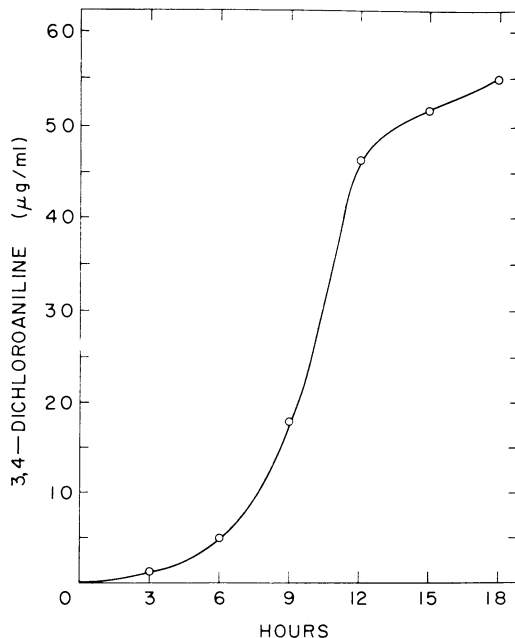


FIG. 1. Production of 3,4-dichloroaniline from propanil by replacement cultures of *Fusarium solani*.

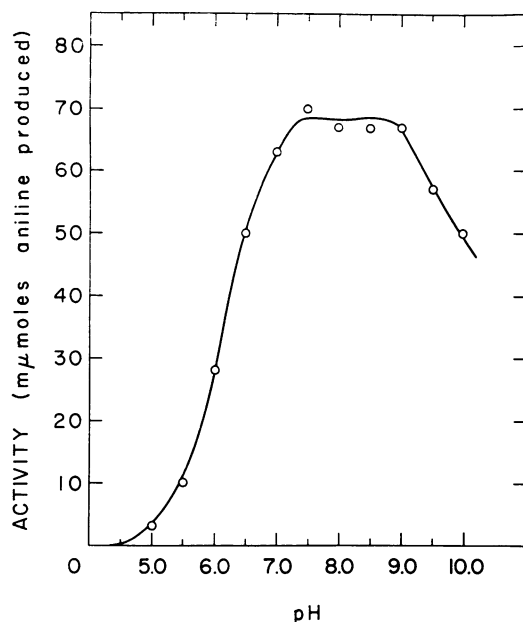


FIG. 2. Influence of pH on enzyme activity was tested by a combination of acetate (pH 5.0 to 5.5), phosphate (6.0 to 7.5), Veronal (pH 8.0 to 9.0) and carbonate (pH 9.5 to 10.0) buffers. The reaction mixtures contained 1.0 ml of 0.1 M buffer, 1.0 ml of enzyme, and 1.0 ml of a 2.0 mM acetanilide solution, and activities were measured after 20 min at 28 C.

fore a toxic level of 3,4-dichloroaniline (DCA) accumulated in the medium, preventing further growth (10). Subsequent tests showed that growth was limited by the eventual accumulation of DCA in the medium when a propanil-mineral salts medium was supplemented with other carbon sources. Moreover, cell-free extracts of *F. solani* grown in the absence of propanil had very low or no propanil-hydrolyzing activity. For these reasons, enzyme induction in replacement cultures was attempted.

The course of release of DCA from propanil by replacement cultures is illustrated in Fig. 1. The kinetics of the conversion shows that none or very low enzyme levels are initially present in fungal cells grown in the absence of propanil. However, after approximately 6 hr of exposure to propanil, there was a rapid increase in the rate of propanil hydrolysis suggesting de novo enzyme synthesis. To determine whether the lag period that preceded rapid DCA release was required for enzyme synthesis, chloramphenicol and puromycin were used as specific inhibitors. At a level of 100 μg/ml, neither of these antibiotics significantly influenced the rate or extent of propanil degradation by whole cells but showed increased levels of enzyme protein in cell-free extracts.

Of acylanilides, acetanilide was selected as an inducer of the enzyme. Replacement cultures hydrolyzed acetanilide, and the kinetics of the reaction were similar to those obtained with propanil used as substrate. Consequently, acetanilide was used as substrate during the purification and characterization of an acylamidase of *F. solani*.

**Factors influencing enzyme activity.** The influence of pH on the activity of the partially purified enzyme is illustrated in Fig. 2. The enzyme was active over a wide pH range with a rather broad optimum at pH 7.5 to 9.0. This was similar to the enzyme produced by *Pseudomonas striata* which hydrolyzed phenylcarbamate, propanil, and its acetic acid homologue (9). Moreover, alkaline, optima have been reported also for the propanil-hydrolyzing acylamidase isolated from rice plants (G. G. Still and D. S. Frear, 1968, Abstr. Amer. Chem. Soc. Sec. A, no. 48) and that found in rat livers (18).

The effect of temperature on activity was measured at 3, 15, 28, 38, and 50 C. (Fig. 3). Activity increased with increasing temperature to a maxi-

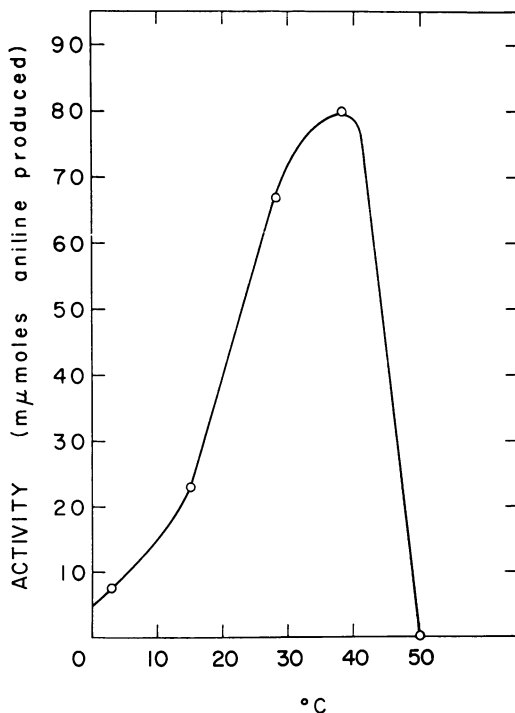


FIG. 3. Influence of temperature on enzyme activity. A 1.0-ml amount of 0.1 M phosphate buffer (pH 7.5) and 1.0 of enzyme were mixed and permitted to equilibrate for 10 min at each temperature tested. A 1.0-ml amount of a 2.0 mM acetanilide solution, held at the test temperature, was then added to initiate the reaction, and the amount of aniline produced in 20 min was determined.

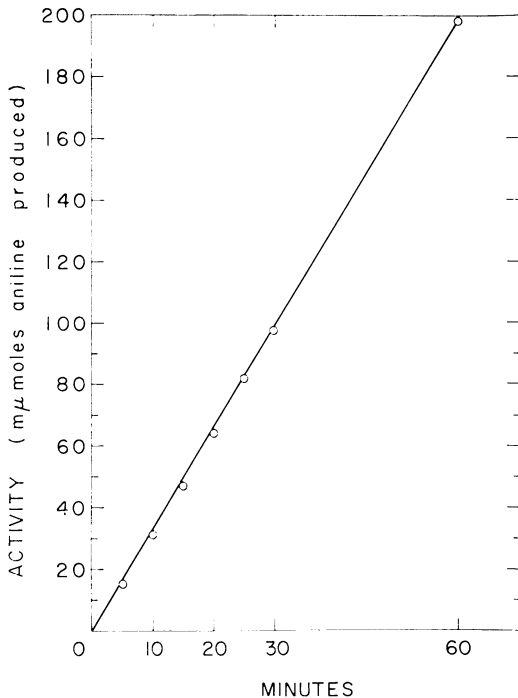


FIG. 4. Influence of time on enzyme activity. The reaction mixture contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of enzyme, and 1.0 of a 2.0 mM acetanilide solution, and activities were measured at 28 C.

mum at 38 C, but the enzyme was relatively heat labile and was completely inactivated after 10 min at 50 C. The effect of time on enzyme activity is illustrated in Fig. 4. Activity was linear for 60 min, after which 9.9% of the substrate was hydrolyzed.

The effect of enzyme concentration on initial activity was tested, and the data (Fig. 5) show that within the limits of the test, activity was a linear function of enzyme concentration.

Since substrate concentration is a major factor in determining the velocity of enzyme reactions, the effect of different levels of acetanilide on its hydrolysis was determined. The results (Fig. 6) suggest a zero-order reaction that depends on a simple dissociation and is characteristic of hydrolases in general. The concentration of acetanilide required for the reaction to proceed at half-maximal velocity ( $K_m$ ) was determined graphically by two methods. The method of Lineweaver and Burk (11) as illustrated by Fig. 7 gave a  $K_m$  value of 0.17 mM. A plot of  $[S]/v$  against  $[S]$  as illustrated in Fig. 8 gave a  $K_m$  value of 0.24 mM. The average of these values, a  $K_m$  value of 0.195 mM, is within an order of magnitude of several other acylamidases acting on similar substrates (8, 15).

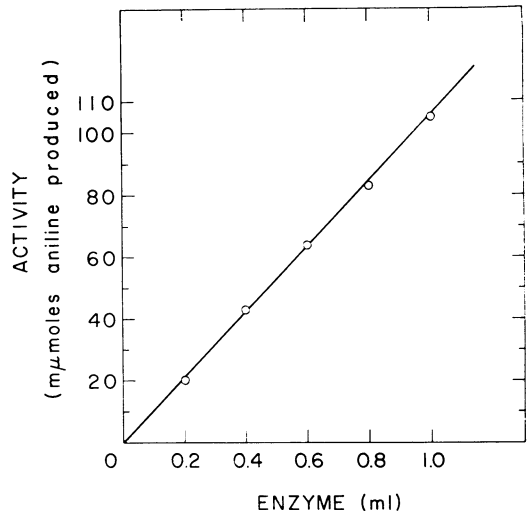


FIG. 5. Influence of enzyme concentration on activity. The reaction mixtures contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of 2.0 mM acetanilide solution, and 0.2 to 1.0 ml of an enzyme preparation with a concentration of 0.22 mg of protein per ml. All the test solutions were made to a final volume of 3 ml by the addition of distilled water. The reactions were terminated after 20 min at 28 C.

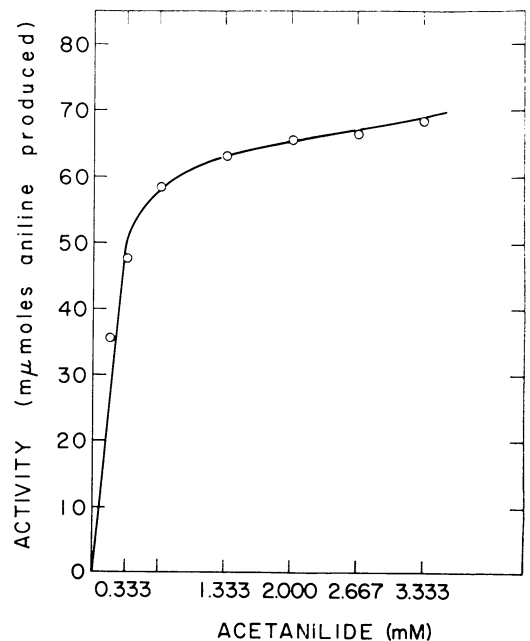


FIG. 6. Influence of substrate concentration on enzyme activity. The reaction mixtures contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of enzyme, and 1.0 ml of acetanilide solution which varied in concentration from 0.5 to 10.0 mM. The final concentration of substrate was, therefore, one-third the molar concentration of the added substrate solutions. The reaction was terminated after 20 min at 28 C.

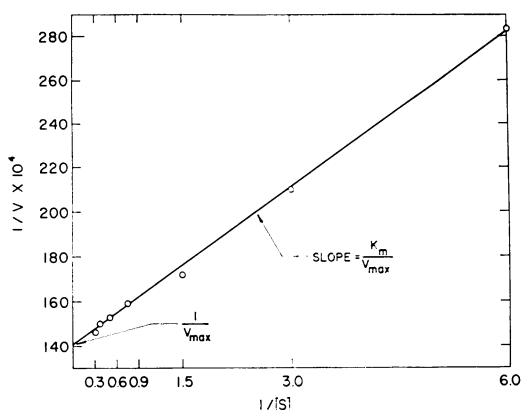


FIG. 7. Lineweaver-Burk plot of data in Fig. 6 for estimation of  $K_m$ .

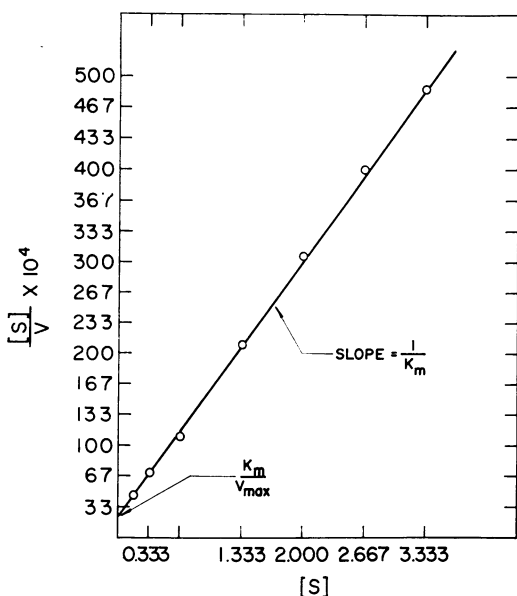


FIG. 8. Graphic estimation of  $K_m$  from data in Fig. 6.

**Inhibitors.** Propanil is a highly selective herbicide which discriminates between rice and certain weed plants, especially barnyard grass (*Echinochola crusgalli*). McRae et al. (Abstr. Weed Soc. Amer., 1964, p. 37) and Still and Kuzirian (17) suggested that the basis of herbicide selectivity and the tolerance of rice plants to propanil rests on the ability of the plant to detoxify the herbicide by enzymatic hydrolysis. This possibility gained support from the purification and properties of a rice enzyme which can hydrolyze propanil (Still and Frear, 1968, Abstr. Amer. Chem. Soc. Sec. A, no. 48).

Combined treatment of paddy fields with cer-

tain insecticides and with propanil caused injury to rice plants (4, 5). Organophosphorus insecticides and carbamate insecticides both enhance greatly the herbicidal activity of propanil on rice. This effect was explained by an ability of these insecticides to interfere with the metabolism of propanil by the rice plants. In fact, Still and Frear (Abstr. Amer. Chem. Soc. Sec. A, no. 48, 1968) demonstrated that as little as  $1.0 \times 10^{-6}$  M carbaryl and other carbamate insecticides inhibited propanil hydrolysis by an enzyme partially purified from rice. Matsunaka (13) showed the rice system was also sensitive to the organophosphorus insecticides parathion and paraoxon. Williams and Jacobson (18) also demonstrated inhibition of an acylamidase from rat livers by very low concentrations of carbaryl and parathion. There was, however, no effect of carbaryl and parathion on the activity of the fungal enzyme.

In a study of the biochemical transformation in soil of anilide herbicides, Bartha (2) reported that Ramrod (*N*-isopropyl-2-chloroacetanilide) withstood extensive degradation, but propanil, dicryl, and Karsil did not. Since Bartha suggested that the alkyl substitution of the amide-nitrogen of Ramrod was responsible of the stability of the compound in soil, it was of interest to ascertain whether the herbicide interacted in any way with the acylamidase of *F. solani*. The capacity of Ramrod (0.067 mM) to interfere with hydrolysis of acetanilide (0.167 to 1.377 mM) was examined to determine whether both compounds competed for a common active site associated with the fungal acylamidase. As the concentration of acetanilide was increased, it was found that the per cent inhibition decreased. The suggestion that Ramrod was a competitive inhibitor of acetanilide hydrolysis was supported when the results were plotted by the double reciprocal procedure of

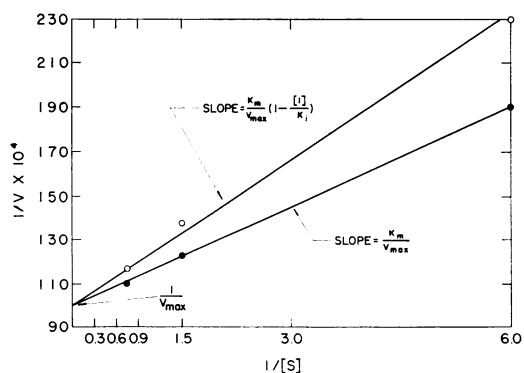


FIG. 9. Graphic illustration of the competitive inhibition of acetanilide hydrolysis by Ramrod.

Lineweaver and Burk (Fig. 9). Moreover, the affinity of the enzyme ( $K_i = 0.167$ ) was similar for Ramrod and acetanilide ( $K_m = 0.195$  mM).

**Substrate specificity.** A study was conducted to determine influence of chemical configuration on the rate of hydrolysis of various structurally related acylanilides. Modification of ring substituents, acyl groups, and certain phenylurea and phenylcarbamate herbicides were used.

The results of this study are listed in Table 1. The data indicated that the effect of *para* substitution was generally to decrease the rate of hydrolysis relative to acetanilide. The extent of inhibition was not constant. It was least in the case of chloro substitution and greatest for methoxy substitution. The inhibitory effects varied directly with increased molecular size and may be attributable to steric factors. The order of the present results is nearly the reverse of those obtained by Nimmo-Smith (15). He reported that the rates of hydrolysis of *p*-nitro, *p*-methoxy, and *p*-chloro acetanilide were 1.7, 2.2, and 3.8 times that of the unsubstituted compound, respectively, in a system catalyzed by chick kidney acylamidase.

The effects of *meta*-, *para*-dichloro substitution on hydrolysis of acetanilide and propionanilide

are summarized in Table 2. The rate of hydrolysis of 3,4-dichloroacetanilide was approximately 70% that of acetanilide. Apparently chlorine in the *meta* position contributed little to this effect, since *p*-chloro acetanilide had a resistance to hydrolysis (78.5%, Table 1) similar in magnitude to that of the dichloro compound. In contrast, 3,4-dichloropropionanilide (propanil) showed a twofold increase in the rate of hydrolysis over that of the unsubstituted compound. Of particular interest was the great difference between susceptibility of acetanilide and propionanilide to enzymatic hydrolysis. The former compound was hydrolyzed at a rate 42 times that of the latter. Since the acylamidase of *F. solani* had a much greater affinity for acetanilide than for propanil, initial difficulties in detecting enzyme activity in crude extracts when the herbicide was used as substrate were understandable. The order of preference for substrate displayed by the *F. solani* acylamidase was the reverse of that reported for rat liver (18) and rice (*C. C. Still, personal communication*) enzymes, both of which hydrolyzed propanil more rapidly than acetanilide.

The effect of the acyl chain length on anilide hydrolysis was determined in an experiment with formanilide, acetanilide, propionanilide, and butyranilide as substrate. Neither formanilide nor butyranilide was hydrolyzed by the fungal enzyme, and the rate of hydrolysis of acetanilide was approximately 42-fold that of propionanilide (Table 2). Thus, the enzyme displayed very high specificity for *N*-acetylarylamines. Acyl chain length was a major factor influencing hydrolysis rates, and, in this regard, the *F. solani* enzyme was similar to chick kidney (15), rat liver (14, 15), and *Neurospora* (8) acylamidases. However, both the rat liver and *Neurospora* enzymes (kynurenine formamidases) were most active with *N*-formylarylamines substrates, and increasing the acyl chain length drastically reduced activity. The enzyme from chick kidney was most active when the acyl chain was *N*-acetyl or *N*-propionyl, and activity depended also on the substitution of the aromatic ring. Hydrolysis was most rapid with the *p*-propionamido derivatives of benzene, anisole, and phenetole, and with the *p*-acetamido derivatives of benzoic acid, phenylacetic acid, and hippuric acid.

The introduction of chlorine atom into the acyl chain of acetanilide reduced the rate of hydrolysis by the *F. solani* enzyme by approximately 67%. In contrast to this result, a more rapid hydrolysis of chloroacetamido than acetamido derivatives was observed with both chick kidney and rat liver acylamidases (15). Similarly, an acylase from hog kidney consistently hydrolyzed *N*-chloroacylated

TABLE 1. Influence of *para* substitution on hydrolysis of acetanilide<sup>a</sup>

Substituent	Rate of hydrolysis relative to acetanilide <sup>b</sup>
H	100.0
Cl	78.5
NO <sub>2</sub>	54.0
OCH <sub>3</sub>	30.6

<sup>a</sup> Reaction mixtures contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of enzyme, and 1.0 ml of 0.5 mM substrate solution, and activities were measured after 25 min at 28 C.

<sup>b</sup> Average of two or more determinations.

TABLE 2. Influence of *meta*-, *para*-dichloro substitution on hydrolysis of acetanilide and propionanilide<sup>a</sup>

Compound	Rate of hydrolysis relative to acetanilide <sup>b</sup>
Acetanilide	100.0
3,4-Dichloroacetanilide	70.2
Propionanilide	2.4
3',4'-Dichloropropionanilide (propanil)	4.2

<sup>a</sup> Reaction mixtures contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 1.0 ml of enzyme, and 1.0 ml of 0.5 mM substrate solution, and activities were measured after 25 min at 28 C.

<sup>b</sup> Average of two or more determinations.

amino acids more rapidly than their *N*-acylated homologue (3).

**Phenylamide herbicides as substrates.** The acylamidase of *F. solani* was unable to hydrolyze the phenylurea herbicides, monuron and fenuron, or the phenylcarbamate herbicide, IPC. The acylanilide herbicides, dicryl and Karsil, were also unaffected by the fungal system, presumably due primarily to the specificity of the enzyme for an acyl chain of limited length. The hydrolysis of these herbicides in soil, as reported by Bartha (2), may be mediated by organisms other than *F. solani* (16).

#### ACKNOWLEDGMENTS

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