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Biochemical Decomposition of the Herbicide *N*-(3,4-Dichlorophenyl)-2-Methylpentanamide and Related Compounds

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Organisms capable of decomposing *N*-(3,4-dichlorophenyl)-2-methylpentanamide (Karsil) were isolated, identified, and tested for their ability to hydrolyze this herbicide. Primary products of Karsil decomposition by cells and cell-free extracts of a *Penicillium* sp. were identified as 2-methyl-valeric acid and 3,4-dichloroaniline. The Karsil acylamidase (EC 3.5.1.a aryl acylamine amidohydrolase) was an induced enzyme. It was partially purified and tested for its ability to hydrolyze 25 related compounds. Some relations between the structures of these compounds and their susceptibility to enzymatic hydrolysis were discerned.

The compound *N*-(3,4-dichlorophenyl)-2-methylpentanamide (Karsil) is one of several important acylanilide herbicides. It has exceptional activity against emerged annual grasses, and broadleaved weeds in celery (J. R. Orsenigo, 1960. Weed Soc. Amer. Abstr., p. 12). Of all acylanilides tested, Karsil proved to be the strongest inhibitor of photosynthesis in turnip chloroplasts (D. E. Moreland and K. L. Hill, 1960. Weed Soc. Amer. Abstr., p. 41). Its susceptibility to microbial attack was demonstrated by respirometric studies (3) and by the detection of the degradation products 3,4-dichloroaniline (DCA) and 3,3′,4,4′-tetrachloroazobenzene (TCAB) in soil treated with Karsil (1).

Another acylanilide herbicide, *N*-(3,4-dichlorophenyl)-propionamide (propanil) was also transformed in soil. A strain of *Fusarium solani* isolated from soil, was able to hydrolyze propanil. Crude cell-free extracts and a partly purified enzyme preparation catalyzed the hydrolysis of propanil and related compounds to their corresponding anilines (R. P. Lanzilotta, Ph.D. Thesis, Rutgers University, 1968). The purpose of this study was to isolate the organisms and the appropriate enzymes which catalyze the transformation of Karsil and compare their substrate specificities with that of *F. solani*.

MATERIALS AND METHODS

Isolation of Karsil-decomposing microorganisms. Karsil-degrading microorganisms were isolated after enrichment in a mineral medium ($NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; distilled water, 1 liter) containing 41.6 mg (0.1 mmole) Karsil/liter medium as the only organic carbon source. Isolation and purification of these organisms were carried out on Karsil-mineral salts agar. Isolated colonies were transferred to flasks containing a Karsil-mineral salts solution. Flasks were incubated on a rotary shaker at 28 C. After 7 to 10 days of incubation, a test for DCA was performed on 1-ml samples of the culture solution. This test was based on the Bratton-Marshall (5) reaction and procedure modified from that of Pease (10) as follows: each sample received 1 ml of glacial acetic acid and 6 ml of N HCl; 0.5 ml of sodium nitrite solution was then added, and the diazotization reaction was allowed to proceed for 5 min; to destroy excess nitrite, 0.5 ml of ammonium sulfamate solution was added; after 10 min, each sample received 1 ml of *N*-(1-naphtyl) ethylenediamine dihydrochloride solution (1% in 0.12 N HCl), with another 10 min allowed for complete development of color.

Efficiency of hydrolysis. The ability of three isolated fungi to decompose Karsil in pure culture was studied as a function of time using the herbicide either as the sole carbon source, or in the presence of sucrose. Each of the 250-ml flasks received 1 ml of ethyl alcohol containing 0.01 mmole of Karsil. The solution was then evaporated and 100 ml of either Czapek's mineral salts solution or Czapek's medium was added to each flask. The flasks were then sterilized, inoculated in four replicates with washed cells of the three fungi, and incubated on a rotary shaker at 28 C. The amount of DCA accumulated in the media was estimated at various intervals using 1-ml samples of culture solution. Samples from uninoculated Karsil flasks, and of organisms grown without Karsil, served as controls. For the DCA analysis

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and standard curve preparation, the procedure described above was employed, and the intensity of the color was measured using a Klett-Summerson colorimeter equipped with a green filter no. 54.

The test for TCAB formation was performed on cultures with Karsil as the sole carbon source. Samples from 50-ml cultures were extracted with 50 ml of ether. The ether was evaporated; the residues were redissolved in acetone and tested for TCAB employing gas chromatographic procedures, as described by Bartha and Pramer (2).

Further experiments involving other Karsil metabolites and the isolation of the hydrolytic enzyme were performed with *Penicillium* no. 1.

Detection of 2-methylvaleric and other volatile acids. Since the side chain of Karsil is 2-methylvaleric acid, initial hydrolysis should yield this compound. To test this hypothesis, *Penicillium* no. 1 was grown in Czapek's medium containing 15.5 mg of Karsil per 100 ml of medium. After 7 days of incubation, the mycelium was separated from the culture solution by filtration. Culture filtrate (400 ml) was adjusted to *p*H 13.0 with NH_4OH, then extracted twice with 100-ml portions of ether in order to remove nonhydrolyzed Karsil. The culture filtrate was neutralized, and 8 ml of concentrated H_2SO_4 was added. The culture filtrate was steam-distilled and 4,000 ml of distillate was collected, brought to *p*H 13.0 with NH_4OH, and concentrated to 5 ml in a flash evaporator at 55 C. Culture filtrate of *Penicillium* no. 1 grown without the herbicide was treated in the same manner. Paper chromatography, as described by Kennedy and Barker (6), was used to separate and identify the volatile acids in the concentrated extracts. Ammoniacal silver nitrate (5% $AgNO_3$ in concentrated NH_4OH) was used as a specific spray for formic acid.

Growth on 2-methylvaleric acid as sole carbon source. To each of four culture flasks containing 100 ml of Czapek's salt solution, 0.2 ml of 2-methylvaleric acid was added and the *p*H was adjusted to 7.5 with NH_4OH. These flasks, in addition to four control flasks, were sterilized, inoculated, and incubated for 7 days. The mycelium was separated and washed; cell dry weights were then determined.

Enzyme induction. *Penicillium* no. 1 was grown in Czapek's medium with and without Karsil (0.01 mmole/100 ml). After 82 hr of growth, the mycelium was harvested and washed with phosphate buffer (0.05 M, *p*H 7.0) until no DCA was detected in the filtrate. The following equal fractions of mycelium were transferred to duplicate flasks containing 50 ml of buffer and 0.01 mmole of Karsil: (i) Karsil-grown mycelium; (ii) mycelium grown in the absence of Karsil; (iii) mycelium grown in the absence of Karsil. Chloramphenicol (5 mg) was added in the last series. Suspensions were incubated on a rotary shaker at 28 C. At various intervals, duplicate 1-ml samples were taken from each flask and mycelium-free filtrates were analyzed for their DCA content.

Enzyme isolation and purification. *Penicillium* no. 1 was grown on Karsil-supplemented Czapek's medium, harvested, washed, suspended in phosphate buffer (0.05 M, *p*H 7.0), and macerated for 2 min in an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) at 0 to 2 C. Cell breakage was accomplished by sonic treatment for 2 min at 0 to 2 C in an MSE Mullard Ultrasonic Disintegrator (Instrumentation Associates Inc., New York, N.Y.). Unbroken cells and cellular debris were sedimented by centrifugation at 1,000 × *g* for 30 min at 2 C. To 1 ml of the cell-free extracts, 1 μmole of Karsil was added and the mixture was incubated for 3 hr at room temperature. The concentration of released DCA was measured as described previously. In this analysis, addition of acetic and hydrochloric acids to the reaction mixture served to terminate enzyme action and to provide protons for the diazotization reaction.

Protein was determined according to Lowry et al. (8), crystalline, bovine-serum albumin was used as standard. Specific activity was determined as nanomoles of DCA produced per mg of protein in 3 hr.

Cell-free extracts were first treated with streptomycin sulfate (0.2 volume of a 5% solution), and precipitation was allowed to proceed for 1 hr in an ice bath. The preparation was then centrifuged for 30 min to remove precipitated nucleic acids. Proteins in the supernatant fluid were fractionated with ammonium sulfate at 0 C with continuous stirring. Fractions precipitated at different ammonium sulfate concentrations were collected and suspended in buffer; their activity and protein concentration were then measured. The active fraction was stored at −20 C.

Substrates. The activity of the partially purified enzyme was tested on 23 acylanilides and two related compounds, which varied in their ring substitution, and the length and substitution of the *N*-acyl group (Table 2 and Fig. 4). A 1-μmole amount of each compound in 0.1 ml of ethyl alcohol was added to a test tube, ethyl alcohol was evaporated, and 1 ml of buffer was added to each tube. Enzyme solution (0.5 ml) was added and the reaction allowed to proceed for 3 hr at room temperature. Analysis for aniline released was performed as described previously on duplicates of enzyme substrate mixture, substrate alone, and enzyme alone. In this comparison, appropriate substituted anilines served as standards, and the activity on each substrate was expressed as nanomoles of arylamine released. All substrates employed were in recrystallized form and were generously supplied by FMC Corp., Niagara Chemical Division, Middleport, N.Y., and by Rohm and Haas Co., Philadelphia, Pa.

RESULTS

Of more than 15 different bacteria and fungi isolated on Karsil agar plates, only three fungi gave a positive aniline test. Two of these are *Penicillium* species no. 1 and no. 2. One of them (*Penicillium* no. 1) has the characteristics of *P. piscarium* (11). The third is a yeastlike organism, and was tentatively identified as a *Pullularia* sp. On malt agar and Czapek's agar its colonies are white-colored when young, and turn greenish and finally dark brown when older. The colony edge is strongly rhizoidal. It is able to grow on

sucrose or glucose as a carbon source, but not on maltose, galactose, or lactose. As a source of nitrogen, the organism can utilize NO_3, NH_4, peptone, urea, or asparagine, but not NO_2. Morphological and physiological characteristics indicate that this organism is a variant of *Pullularia pullulans*.

The amounts of DCA per 1 ml of culture liberated from Karsil by the three fungi when the herbicide was added as the sole carbon source are shown in Fig. 1. *Penicillium* no. 2 hydrolyzed Karsil slowly during the first two days; after this period, the amount of DCA increased rapidly. By the end of the experiment (11 days) about 64% of added Karsil was converted to DCA. During growth on Karsil the 3-mg (dry weight) inoculum increased to 7.6 mg. *Penicillium* no. 1 hydrolyzed Karsil less effectively, but at the termination of the experiment, 28% of added Karsil was converted to DCA with a net increase in mycelium of 4.0 mg (dry weight). *Pullularia* was the least active in decomposing the herbicide; at the 11th day, only 5% of added Karsil was converted to DCA. The net increase in dry weight due to growth on Karsil was 1.7 mg. Tests for TCAB showed that none of the three fungi formed this compound.

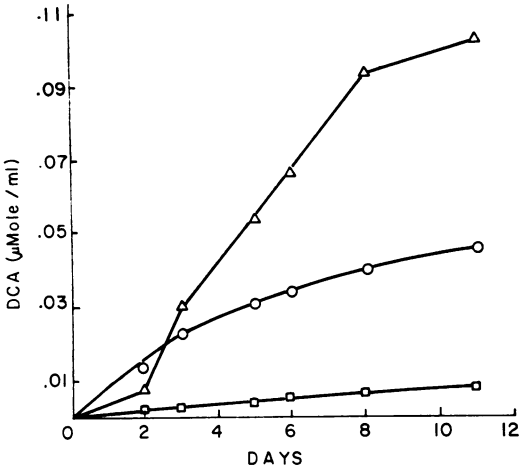

FIG. 1. *Comparison of the effect of time on Karsil decomposition by three fungi, with the herbicide as sole carbon source in the medium, measured by accumulation of the product DCA. Symbols:* ○, *Penicillium no. 1;* △, *Penicillium no. 2;* □, *Pullularia sp.*

TABLE 1. *DCA production from Karsil by microorganisms grown in the presence of sucrose*

Time (hr)	DCA concn[a]		
	Penicillium no. 1	*Penicillium* no. 2	*Pullularia*
24	0.4	0.4	1.5
48	19.0	2.3	4.5
72	38.3	5.2	26.5
82	46.0	7.0	32.5

[a] Expressed as nanomoles per milliliter of culture.

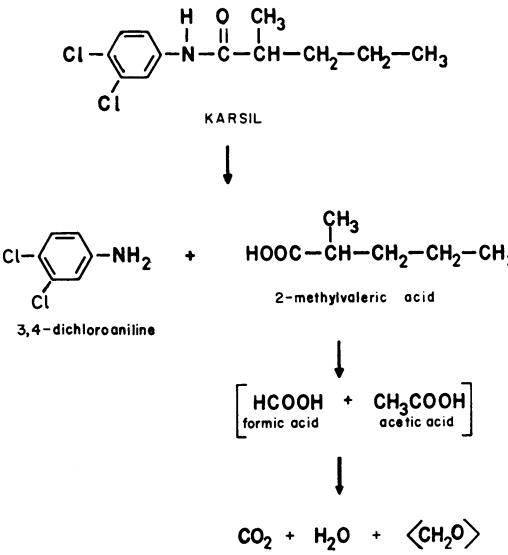

FIG. 2. *Suggested scheme of the transformation of N-(3,4-dichlorophenyl)-2-methylpentanamide by Penicillium no. 1.*

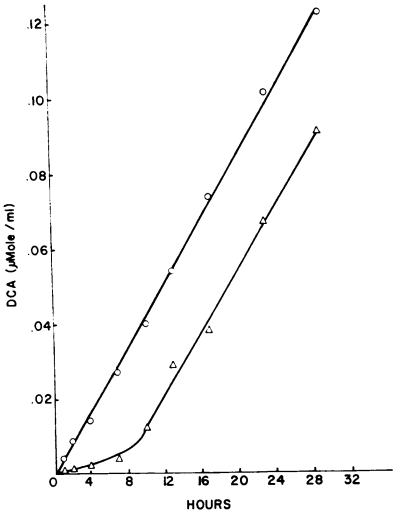

FIG. 3. *Influence of time on DCA production from Karsil by replacement cultures of Penicillium no. 1. Symbols:* ○, *cells previously grown in the presence of Karsil;* △, *cells previously grown without Karsil.*

Herbicide hydrolysis by the three organisms grown in presence of sucrose (Table 1) was completely different. *Penicillium* no. 1 was the most active, followed by *Pullularia*. *Penicillium* no. 2, which was the most active when grown in the absence of sucrose, had very limited activity when additional carbon source was present.

With 2-methylvaleric acid as the only carbon source, *Penicillium* no. 1 was able to grow rapidly as indicated by an increase in dry weight of mycelium from 2.8 mg (inoculum size) to 78.1 mg in 7 days per 100 ml of medium.

When 25 μliter samples of the concentrated steam distillates were chromatographed on paper (Whatman no. 1), a spot with the same R_F value (0.68) as that of authentic 2-methylvaleric acid was observed only in the extract of the Karsil-grown culture. In addition to the 2-methylvaleric acid, two spots, not completely separated, with R_F values of 0.38 and 0.36 were observed and corresponded to acetic and formic acid, respectively. Though the employed procedure could not completely resolve the mixture of acetic and formic acids, silver nitrate ammoniacal spray showed an increase of formic acid in Karsil culture as indicated by the size and intensity of the dark spot compared to that of the control. A suggested scheme of Karsil transformation by *Penicillium* no. 1 is shown in Fig. 2.

The production of DCA from Karsil by *Penicillium* no. 1 replacement cultures is illustrated in Fig. 3. Cells previously exposed to the herbicide rapidly hydrolyzed Karsil, and DCA concentration in the medium continued to increase as a linear function of time. Karsil hydrolysis by cells grown in medium without the herbicide was very slow during the first 7 hr, but increased rapidly thereafter. By the 9th hr, the rate of DCA release was equal to that of cells grown in the presence of Karsil. Chloramphenicol did not

TABLE 2. *Name, structure, and susceptibility to enzymatic hydrolysis of various acylanilides and related compounds*

No.	Name	Side chain R[a]	Ring substituents				Activity[b]
			2	3	4	5	
1	*N*-(3,4-Dichlorophenyl)-2-methylpentanamide (Karsil)	(m)	H	Cl	Cl	H	70
2	*N*-(3,4-Dichlorophenyl)-4-methylpentanamide	(n)	H	Cl	Cl	H	144
3	*N*-(3,4-Dichlorophenyl)-2-hydroxypentanamide	(l)	H	Cl	Cl	H	835
4	*N*-(3,4-Dichlorophenyl)-3,3-dimethylbutyramide	(k)	H	Cl	Cl	H	7
5	*N*-(3,4-Dichlorophenyl)-2-ethylbutyramide	(j)	H	Cl	Cl	H	0
6	*N*-(3,4-Dichlorophenyl)-2,2-dimethylpropionamide	(i)	H	Cl	Cl	H	0
7	*N*-(3,4-Dichlorophenyl)-2-methylbutyramide	(h)	H	Cl	Cl	H	48
8	*N*-(3,4-Dichlorophenyl) methacrylamide (Dicryl)	(f)	H	Cl	Cl	H	40
9	*N*-(3,4-Dichlorophenyl) propionamide (propanil)	(b)	H	Cl	Cl	H	520
10	*N*-(3,4-Dichlorophenyl)-2-chloropropionamide	(c)	H	Cl	Cl	H	340
11	*N*-(3,4-Dichlorophenyl) acetamide	(a)	H	Cl	Cl	H	218
12	*N*-(2,5-Dichlorophenyl)-2-methylpentanamide	(m)	Cl	H	H	Cl	0
13	*N*-(2,4,5-Trichlorophenyl)-2-methylpentanamide	(m)	Cl	H	Cl	Cl	11
14	*N*-(4-Chlorophenyl)-2-methylpentanamide	(m)	H	H	Cl	H	106
15	*N*-(3-Chlorophenyl)-2-methylpentanamide	(m)	H	Cl	H	H	117
16	*N*-(3-Chloro-4-methylpenyl)-2-methylpentanamide (Solan)	(m)	H	Cl	CH_3	H	55
17	*N*-(3,4-Dimethylphenyl)-2-methylpentanamide	(m)	H	CH_3	CH_3	H	0
18	*N*-(3-Chloro-4-methylphenyl) propionamide	(b)	H	Cl	CH_3	H	430
19	*N*-(3,4-Dimethylphenyl) propionamide	(b)	H	CH_3	CH_3	H	0
20	*N*-(4-Bromophenyl) propionamide	(b)	H	H	Br	H	188
21	*N*-(Phenyl) butryamide	(e)	H	H	H	H	1,000
22	*N*-(Phenyl) propionamide	(b)	H	H	H	H	262
23	*N*-(Phenyl) acetamide	(a)	H	H	H	H	4
24	*N'*-(3,4-Dichlorophenyl)-*NN*-dimethylurea (diuron)	(d)	H	Cl	Cl	H	0
25	Isopropyl *N*-(3-chlorophenyl) carbamate (CIPC)	(g)	H	Cl	H	H	0

[a] For formulas, *see* Fig. 4.

[b] Expressed as nanomoles of arylamine released per micromole of substrate per 3 hr.

influence the period of induction or the rate of hydrolysis. The results obtained from replacement culture in the presence of the antibiotic were not significantly different from results obtained without it.

Cell-free extracts of induced *Penicillium* no. 1 were able to hydrolyze Karsil. The active protein fraction was precipitated at 45% ammonium sulfate saturation. A three-fold increase in specific activity was obtained employing this procedure.

The names, structures, and susceptibilities to hydrolysis by the partially purified enzyme of 25 related compounds are listed in Table 2. There was marked variation in the enzyme activity on different substrates. The substituted *N'*-(3,4-dichlorophenyl)-*NN*-dimethylurea (diuron, no. 24, Table 2) and isopropyl *N*-(3-chlorophenyl) carbamate (CIPC, no. 25) were not hydrolyzed. The extent of acylanilide hydrolysis varied with structure in a manner which suggested the following relationships.

Effect of N-acyl group. In case of acylanilides with unsubstituted aniline rings having unbranched *N*-acyl groups, the enzyme was most active on *N*-(phenyl)butyramide (no. 21). Activity on the three-carbon side chain compound (no. 22) was one-fourth that of the four-carbon one. No activity was observed on the two-carbon side chain compound *N*-(phenyl)acetamide (no. 23). In case of 3,4-dichloro-substituted acylanilides, activity on a three-carbon side chain compound (no. 9) was higher than that on a two-carbon side chain compound (no. 11).

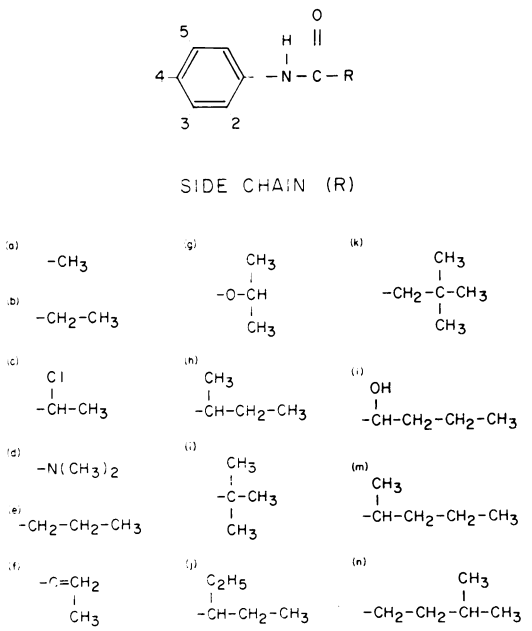

FIG. 4. *Structural formulas of various acylanilides and related compounds. Letters in parentheses refer to Table 2.*

Branching, or substitution of the acyl group affected the extent of hydrolysis. In case of five-carbon length acyl groups, activity was higher on the 4-methyl-(no. 2) than on the 2-methyl-(no. 1) substituted compounds, and activity was highest with the 2-hydroxy substituted compound (no. 3). Compounds with 2-ethyl (no. 5), 3,3-dimethyl (no. 4), and 2,2-dimethyl (no. 6) substitution on the acyl group were not hydrolyzed. The herbicide, *N*-(3,4-dichlorophenyl) methacrylamide (Dicryl, no. 8), which has a methacrylic acid side chain, was hydrolyzed only to a limited degree. Chlorine substitution on the α-carbon (no. 10) reduced activity by about 50% as compared to *N*-(3,4-dichlorophenyl)propionamide (propanil, no. 9).

Ring substitution. With Karsil analogues, the enzyme had a greater activity on the monochloro- (no. 14 and 15) than on the dichloro- (no. 1) substituted compounds. However, the influence of chlorine substitution in the case of shorter side chain compounds was different. The activity on propanil (no. 9) was twice as high as on *N*-(phenyl) propionamide (no. 22). Activity on *N*-(3,4-dichlorophenyl) acetamide (no. 11) was much higher than on *N*-(phenyl) acetamide (no. 23). Methyl substitution on the phenyl ring decreased enzymatic hydrolysis. *N*-(3-Chloro-4-methylphenyl)-2-methylpentanamide (Solan no. 16), which has 3-chloro-4-methyl substitutions, was hydrolyzed to a lesser degree than the 3,4-dichloro-substituted Karsil (no. 1). The 3,4-dimethyl-substituted analogue (no. 17) was not hydrolyzed. In case of propanil analogues, activity on the 3-chloro-4-methyl-substituted compound (no. 18) was lower than on the 3,4-dichloro-substituted compound (no. 9), and there was no activity on the 3,4-dimethyl-substituted compound (no. 19). Bromide substitution in the 4-position decreased hydrolysis; the compound *N*-(4-bromophenyl)propionamide (no. 20) was hydrolyzed to a lesser degree than *N*-(phenyl)-propionamide (no. 22). Compounds with 2,5-dichloro (no. 12) and 2,4,5-trichloro substitutions (no. 13) were not hydrolyzed.

DISCUSSION

The three isolated fungi were able to hydrolyze Karsil, but differed in the level or in the activity of their hydrolytic enzymes, or both. There was a major difference in the ability to hydrolyze herbicides between cells grown in the presence of sucrose and those grown with Karsil alone.

With *Penicillium* no. 2, organic matter appeared to have a sparing effect on herbicide decomposition. However, with *Fusisporium* and *Penicillium* no. 1, the rate of hydrolysis increased significantly in the cells grown in the presence of sucrose.

DCA was the major product of Karsil decomposition; it accumulated in the medium without further transformation to DCAB. As peroxidases were shown to catalyse the conversion of anilines to azobenzenes (4), apparently these organisms lacked such enzymes under the conditions described.

Another product of Karsil decomposition, 2-methylvaleric acid, was further metabolized by *Penicillium* no. 1. This was apparent from the lack of accumulation of this intermediate in the medium and from the fact that the organism was able to grow on 2-methylvaleric acid as sole carbon source. Whether the increase in the amount of herbicide and acetic acid in the medium was a direct result of 2-methylvaleric acid oxidation, or whether they were produced by the fungus under the influence of DCA or Karsil, remains to be studied.

The fungal acylamidase (EC 3.5.1.13, aryl acylamine amidohydrolase) of *Penicillium* no. 1 is an inducible enzyme with an induction period of 7 to 9 hr. Chloramphenicol, a known protein synthesis inhibitor in some fungi (7), did not affect the acylamidase production of *Penicillium* no. 1. The inductive synthesis of the same enzyme remained unaffected also in *A. solani* (Lanzilotta, 1968).

Cell-free extracts of *Penicillium* no. 1 hydrolyzed the herbicide slowly. A threefold increase in specific activity was obtained by ammonium sulfate fractionation.

The extent of enzymatic hydrolysis was related to the chemical structure of the substrate. Activity increased with increased side chain length up to four carbons. However, this was not the case with the *A. solani* enzyme, which hydrolyzed acetanilide at a rate 42-fold higher than propionanilide. A major difference between *Penicillium* no. 1 enzyme and that of *A. solani* is the inability of the latter to hydrolyze Karsil and Dicryl. Deacylation by *Penicillium* no. 1 acylamidase, like that of the rat liver one (12), was higher on propanil than on acetanilide. *Penicillium* no. 1 acetylamidase differed from the chick kidney enzyme (9) in that the former exhibited its highest activity on four-carbon side chain compounds, whereas the latter on three-carbon side chain compounds.

Substitution or branching of the *N*-acyl group affected enzyme activity. This effect was related to the nature and the position of the substituent, as could be observed with 2-methyl and 4-methyl (no. 1 and no. 2, Table 2), and with the 2-hydroxy (no. 3) Karsil analogues. The 2-methyl (no. 5) or dimethyl- (no. 4) substituted analogues were not hydrolyzed. A 2-chloro substitution on the side chain of propanil (no. 10) also reduced activity.

Deacylation was also influenced by the nature and position of substituents on the phenyl ring. The 3,4-dichloro substitution on compounds with two- to three-carbon side chains increased hydrolysis in a similar manner as did the 4-chloro substitution in case of the chick kidney enzyme (9). A similar effect was reported for the *D. solani* enzyme, which hydrolyzed propanil at a faster rate than propionanilide. However, when the side chain was longer than four carbons, chlorination of the aromatic ring had a reverse effect; Karsil analogues became increasingly susceptible to enzymatic hydrolysis with decreasing chlorination.

Effect of *N*-acyl group. A 2-chloro substitution of the aromatic ring seems to prevent hydrolysis as observed in case of compounds no. 12 and no. 13. Nemagon exhibited a similar effect with the chick kidney enzyme (9). This loss of activity could be a result of steric hindrance of the hydrolysis by the *ortho* chloro substituent. Ring substitution by methyl groups reduced hydrolysis. The 3-chloro-4-methyl-analogues of Karsil and propanil (no. 16 and no. 18) were deacylated to a lower degree than Karsil and propanil, respectively. The 3,4-dimethyl analogues of Karsil and propanil (no. 17 and no. 19) were not hydrolyzed at all. The enzyme had no activity on either diuron or CIPC.

The three isolated fungi are known as common soil inhabitants. They may account for a substantial part of acylanilide herbicide degradation (1–3).

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Microbial identification using effluents backflushed from exhausted urban and rural tank resins and cleaned resins containing the sulfonated copolymer of styrene and divinylbenzene (SDB) were completed, along with microbial assessment of the concentrated stock salt brine. Forty-four different bacterial and fungal genera were identified. Extensive biochemical and animal virulence tests completed on one of the six bacterial salt brine isolates indicated a pathogenic staphylococcal strain. The retention of *Staphylococcus aureus*, a *Flavobacterium* sp, and *Escherichia coli* B bacteriophage was demonstrated both by using the nonexhausted sodium-regenerated resin and by using the same resin exchanged with different mono-, di-, and trivalent cations. Effluent counts completed after bacterial seepage through the resins indicated the Pb^{++}-exchanged resin removed 55% of the bacteria; Na^{+}, Fe^{++}, and Al^{+++} removed 31 to 36% and Ca^{++} and Cu^{++} removed about 10 to 15%. Seventy per cent or more of the bacteriophage was removed by Fe^{++}, Cu^{++}, and Al^{+++}, whereas the Ca^{++} and Na^{+} cations removed 25 to 31%. Over a 77-day period, nonsterile tap water was passed through bacterial seeded and uninoculated SDB (Na) resin columns. Effluent and resin elution counts demonstrated the growth and survival of 2 different bacteria per column. Increased bacterial retention, survival, and multiplication occurred concomitantly with accumulation of organic and inorganic materials and the Ca^{++} and Mg^{++} cations from the tap water. Furthermore, microbial elution from resin particles taken from column depths of 1, 8, and 16 cm indicated a bacterial diminution with increasing depths.

Innumerable studies have described microbial survival and dissemination in public water supply systems. We have contributed to this body of knowledge by relating paralytic poliomyelitis cases to the water-borne virus (1), and by relating septicemia and deaths in prematures to a water-borne *Achromobacter* bacterium (13). Moreover, certain microorganisms indigenous to water, such as the *Flavobacterium* (6), *Pseudomonas* (26), *Alcaligenes* (10), and *Paracolobactrum* (23), have been associated with troublesome nursery infections caused by inhalation therapy equipment in hospitals or contaminated water from other sources (21). Although such infections have not been attributed to or associated with microorganisms cultured from cationic exchange resins used in water-softener units, they are directly involved in water microbiology. It seemed reasonable, therefore, to study these resins. Consider, for example, (i) their microbiological entrapment capabilities, (ii) their favorable environment for microbial maintenance and multiplication, (iii) their longevity (7 to 9 years), and (iv) their reuse capability. Moreover, their microbial dissemination potential cannot be completely discounted when we consider their ubiquitous distribution in water softener units.

This report describes field and laboratory studies completed on a synthetic cationic exchange resin. Our objectives were simply to provide: (i) a reasonable microbiological assessment of water-softener resins, a study which entailed the generic identification of types and total numbers of the bacteria and fungi recoverable from the first backflush contents of the exhausted water-softener resin tanks servicing mainly urban and rural homes, but also hospitals and dairies; (ii) a microbiological assessment of the 26% "stock salt brine" used to regenerate the cleaned resins; (iii) information regarding bacterial survival time and filtration capabilities using both the nonexhausted sodium-regenerated resin (SDB) and the spent resin, determining whether these phenomena are the same when the resin is exchanged with different mono-, di-, and trivalent cations at the same pH, temperature, and flow rate; (iv) information relative to the influence of a progressively changing resin (resin in the process of exhaustion) upon bac-

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