Influence of Physicochemical Properties on Biodegradability of Phenylcarbamate Herbicides

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Enzymic cleavage of the phenylcarbamate herbicides is influenced by physicochemical properties of the substrates. Inductive effects exerted by meta substitution of electron-withdrawing groups and steric effects imposed by increasing the size of the alcohol group significantly altered the reaction rate. A positive relationship between the relative acidities of the phenylcarbamates, as influenced by the inductive effects of meta substituents, and hydrolytic rates was demonstrated. Resonance interactions imposed by para substitution of strong inductive groups increased the velocity of the reaction. With the nitrophenylcarbamates, the rate of reaction was inversely proportional to the basicity of the parent anilines. Increasing the over-all size of the ring portion of the molecule decreased the velocity. Molecular parameters were studied in an attempt to determine those properties of a compound that influence decomposition by microbial enzymes.

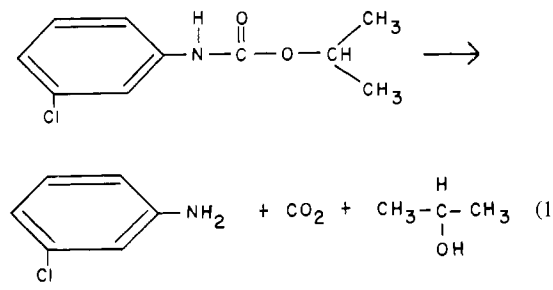
Biologists and chemists are constantly seeking empirical relationships by which to predict chemical, physical, or biochemical properties of molecules on the basis of their structure. From a pesticide residue standpoint, it would be desirable to have some information on certain physicochemical properties of a herbicide as an indicator of its general soil persistence or its biodegradability by soil microorganisms. There have been a number of problems, however, that have made structure *vs.* persistence studies difficult to perform and interpret in soils. First, the herbicide's structural and electronic properties must be well understood before any reasonable basis for predicting its susceptibility to microbial attack can be established. Second, soils are complex systems in which more than one process may affect the persistence or degradation of a herbicide; and finally, the mechanism of the degradation reaction under study is often not well understood.

Owing to these complications, it has been impossible to correlate rates of disappearance with some physical property of the herbicide. Modern physical organic chemistry, however, provides a wealth of information for studying the molecular reactivity of many herbicides. For example, detailed information on physical properties of the substituted benzenes has been obtained in an attempt to evaluate the separate contributions of induction and resonance to the electronic effects of ring substituents. A detailed consideration of steric and electronic properties of certain herbicides might yield valuable information as to their rates of breakdown under laboratory conditions and their persistence under field conditions.

Recently, the isolation and identification of several soil microorganisms capable of metabolizing the widely used herbicide CIPC [isopropyl *N*-(3-chlorophenyl)-carbamate] was reported (Kaufman and Kearney, 1965). Subsequently, the isolation and purification of the enzyme within the organism responsible for catalyzing the hydrolysis of CIPC was described (Kearney, 1965; Kearney and Kaufman, 1965). The partially purified enzyme has the capacity to hydrolyze phenylcarbamates other than CIPC. The ability of the enzyme to cleave a large number of structurally related phenylcarbamates suggested an ideal situation for studying the effect of various molecular parameters on the enzymatic rate of hydrolysis under carefully controlled conditions. Most chemical decomposition studies of the carbamates have been done by alkaline hydrolysis. Rate constants for the alkaline hydrolysis of several carbamate insecticides, including both the *N*-alkyl and *N,N*-dialkylcarbamates, have been determined (Casida *et al.*, 1960). Detailed mechanistic studies have recently been carried out on ethyl, phenoxy, and *p*-nitrophenoxy esters of carbamic acid, *N*-methylcarbamic acid, and *N,N*-dimethylcarbamic acid (Dittert, 1961). Alkaline hydrolysis of several aromatic *N*-substituted carbamates has been investigated in strongly basic or in buffered solutions and rate constants determined at three or four different temperatures (Christenson, 1964). The present paper deals with the enzymatic rate of hydrolysis of several structurally related *N*-phenylcarbamates to determine effect of various ring substituents and alcohol groups on the velocity of reaction:

Methods

The enzymatic conversion of several phenylcarbamates to their respective anilines was measured as previously described (Kearney, 1965). The enzyme catalyzes the following reaction:



(1)

The assay system used in the previous studies was modified to include some of the less soluble phenylcarbamates in the survey. The volume of the phenyl-

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carbamate substrate solution was increased from 2.5 ml. (containing 1 μmole of substrate) to 4.0 ml. The increased volume facilitated the solubilization of many phenylcarbamates less soluble than CIPC. The phenylcarbamate substrates (Table I) were obtained from commercial sources and purified by recrystallization with appropriate solvent solutions. The infrared spectra of several phenylcarbamates were examined to determine the presence of the desired functional groups.

The enzyme used was the precipitate collected from a 30 to 60% $(NH_4)_2SO_4$ fraction of a crude soluble portion of harvested, lysed cells of *Pseudomonas striata*. The rate of the reaction refers to the number of millimicromoles of aniline produced per 20 minutes (at pH 8.0 in 0.1*M* Tris buffer) with a constant number of enzyme units.

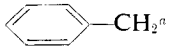
Standard curves were established for each of the respective substituted anilines by a colorimetric procedure (Pease, 1962). The identity of several of these anilines produced by the enzymatic reaction was verified as in the previous experiment (Kearney, 1965). Unfortunately, several of the corresponding anilines could not be obtained and thus precluded an examination of hydrolysis rates of the parent phenylcarbamate. For example, the isopropyl ester of *N*-(3-cyanophenyl)-carbamic acid yielded a positive aniline reaction, but 3-cyanoaniline was unavailable.

Several of the dichlorophenylcarbamates were extremely insoluble in water and could not be compared on an equal molar basis. Unfortunately, surfactants had an inhibitory effect on the enzyme, and consequently could not be used to solubilize several of the substrates.

Table I. Phenylcarbamate Substrates Examined in Enzymatic Rate Studies

Ethyl *N*-(3-chlorophenyl)carbamate
Propyl *N*-(3-chlorophenyl)carbamate
Isopropyl *N*-(3-chlorophenyl)carbamate
Benzyl *N*-phenylcarbamate
Isopropyl *N*-phenylcarbamate
Isopropyl *N*-(2-chlorophenyl)carbamate
Isopropyl *N*-(3-nitrophenyl)carbamate
Isopropyl *N*-(3-acetylphenyl)carbamate
Isopropyl *N*-(3-methoxyphenyl)carbamate
Isopropyl *N*-(4-nitrophenyl)carbamate
Isopropyl *N*-naphthylcarbamate
Isopropyl *N*-(3-carboxyphenyl)carbamate

Table II. The Effect of Increasing the Size of the Alcohol Group on the Rate of Enzyme Hydrolysis of the Various Esters of *N*-(3-Chlorophenyl)carbamic Acid

Group	Rate
C_2H_5	80
n-C_3H_7	68
iso-C_3H_7	55
 —CH_2[a]	13

[a] Hydrolysis rate for the benzyl alcohol group should be compared to the rate for the isopropyl *N*-phenylcarbamate (at 26 mμmoles) since both compounds are not chloro-substituted on the ring.

Table III. The Effect of Different Meta Substituents on the Rates of Enzyme Hydrolysis of the Isopropyl Esters of *N*-Phenylcarbamic Acid

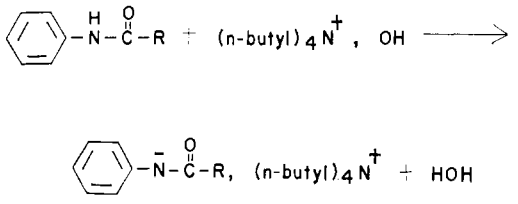
Substituent	Rate
NO_2	65
CH_3—CO	60
Cl	56
CH_3—O	39
H	26

Results and Discussion

The substrates were grouped into several classes to study the effect of certain molecular parameters on the rates of enzymatic hydrolysis. The steric effects, imposed by increasing the size of alcohol group, are shown in Table II. As the size of the alcohol group increases from ethyl to *n*-propyl to the isopropyl, the rate of the reaction steadily decreases. The bulky benzyl ester of the *N*-phenylcarbamic acid decreased the velocity by a factor of 2 when compared with the isopropyl ester of the same compound. The steric effects, imposed by the geometry of the alcohol groups, have a profound effect on the ease with which the carbonyl carbon in the molecule can be attacked by some reactive group on the enzyme surface. The unique spatial relationships, imposed by alcohol groups of varying size, suggest that the enzyme-substrate fit might be an important factor in determining the enzymatic reaction rate. Previously (Kearney, 1965), the rate of reaction was shown to decrease by introduction of chlorines into the isopropyl alcohol moiety. The reaction velocity was shown to decrease in the following order: isopropyl > 1-chloroisopropyl > 1,3-dichloroisopropyl.

The effect of various meta substituents on hydrolysis rates is shown in Table III. Since meta and para substituents are probably too far removed from the reaction site to have a noticeable steric effect on the rate, the values presented in Table III apparently must reflect the inductive or electron-withdrawing effects. The reaction rate decreases with the following meta substituents on the ring: NO_2 > CH_3—CO > Cl > CH_3—O— > H.

There are several methods by which these inductive effects can be quantitatively compared to the reaction rates. One approach is to use the well known Hammett values. Another more direct approach is the use of relative acidities of the various substrates. Cluett (1959, 1962) reported that substituted phenylamides could be titrated as acids in *n*-butylamine. The method measures the ease with which the proton is removed from the amide nitrogen (Equation 2). Camper and Moreland (1965) measured the relative acidities of many of the herbicidal phenylcarbamates used in the present enzyme studies. Relative acidities of the phenylcarbamates are obtained by a potentiometric titration and the results expressed as half-neutralization potentials or HNP values.



(2)

Benzoic acid serves as a reference standard (at 500 mv.). As the HNP decreases, the phenylcarbamate becomes more acid. The technique is of interest here, since it expresses the contribution of various ring substitutions to the intrinsic acidity of many of the phenylcarbamates used in the enzyme studies.

A previous publication shows that as the HNP decreases, the enzymatic rate of hydrolysis increases (Kearney, 1965). The HNP effect on the hydrolysis of several phenylcarbamates not previously reported is shown in Figure 1. The inductive effect of the meta substituents correlates positively with HNP or relative acidity. The *m*-nitro carbamate is the strongest acid in the meta series, followed by the acetyl, chloro, methoxy, and hydrogen. The HNP value of the methoxy carbamate was an estimate based on the ethoxy-analog value previously reported (Camper and Moreland, 1965).

There was a general tendency for the hydrolysis rate to increase with increasing relative acidity. Two other phenylcarbamates that are not meta substituted, but which show a similar relationship between HNP and hydrolysis, are found in Figure 1. The isopropyl ester of 2-chlorophenylcarbamate is a stronger acid than CIPC; however, it does not follow the expected increase in hydrolysis rate. Only one ortho-substituted carbamate was available for examination in this survey, but the single observation suggests that steric hindrance may decrease enzymic cleavage because the ortho chlorine is in close proximity to the carbon undergoing attack. In the thiono carbamate, the sulfur exerts a strong influence on the amide hydrogen, making it relatively easy for the proton to leave. Again the general tendency for the hydrolysis rate to increase with increasing acidity was noted. The thionocarbamate was the most readily cleaved compound studied. Some abnormalities arise, however, when attempts are made to interpret a strict relationship between hydrolysis rate and HNP. Still unexplained, for example, was the inability of the enzyme to attack the CIPC analog bearing a meta-carboxyl substituent.

Positional effects of certain ring substituents are also important in governing the hydrolysis rate. The hydrolysis rates for the isopropyl esters of the *m*- and *p*-nitrophenyl carbamic acids are shown in Table IV. Hydrolysis of the *p*-nitrophenyl carbamate is considerably faster than for the meta compound. This was not particularly surprising, because a resonance interaction occurs when a strong electron-withdrawing group is substituted in the para position. The ability of the nitro group to pull electrons away from the reactive site of the molecule no doubt has an influence on the relative activity of the compound, and consequently on the ease with which it is hydrolyzed. Unfortunately,

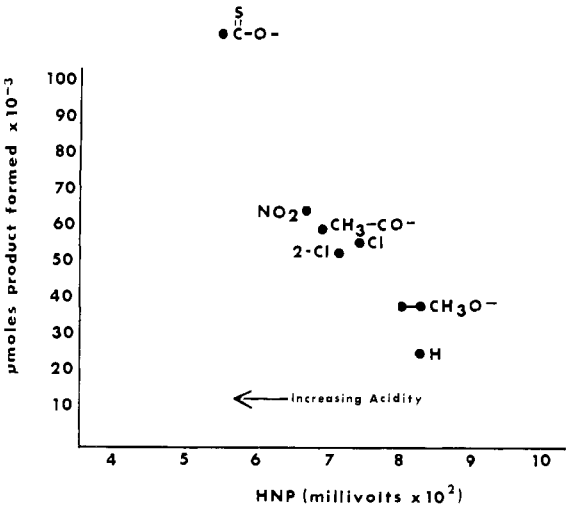
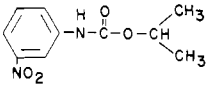
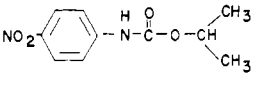


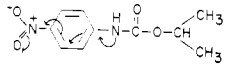
Figure 1. Rates of enzyme hydrolysis as a function of HNP for *o*-chloro and various meta-substituted isopropyl esters of the *N*-phenylcarbamic acids

Table IV. Rates of Enzyme Hydrolysis for the Isopropyl Esters of the *m*- and *p*-Nitrophenylcarbamic Acids

	Rate
	65
	85

HNP values were not available for the *p*-nitro compound.

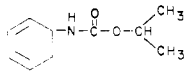
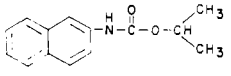
In *p*-nitrophenyl carbamates, the charge density is reduced in the vicinity of the carbonyl group by the strong electron-withdrawing power of the nitro group translated through the resonating structure.



The electron flow away from the active site is shown by a series of arrows denoting the shift in electrons away from the amide nitrogen through the aromatic system to the nitro group. In the case of the nitrophenyl carbamates, the velocity of hydrolysis is inversely proportional to the basicity of the parent aniline.

Thus far the inductive, steric, and resonance effects on the rates of hydrolysis have been discussed. The size of the molecule also has an effect on hydrolysis rate. The hydrolysis rates of the isopropyl esters of phenyl and 2-naphthyl carbamic acids were compared (Table V). The bulky naphthyl group has a profound decreasing effect on the hydrolysis rate, since only 7 mμmoles of 2-naphthylamine were produced. The

Table V. Enzymic Rates of Hydrolysis of Phenyl and 2-Naphthyl Isopropyl Esters of Carbamic Acid

	Rate
	26
	7

results strongly suggest that the actual size of the molecule may be the principal factor retarding the rate of hydrolysis.

Soil microorganisms are responsible for detoxifying many organic herbicides by reactions that, in a few cases, have been studied in detail (Kearney, 1966; Kearney *et al.*, 1967). Ester and/or amide hydrolysis is the reaction responsible for cleavage of CIPC and its related analogs. The influence of steric hindrance on this type of reaction is fairly well understood (Newman, 1956), while the electronic effects caused by ring substitutes have been discussed previously for this same enzyme (Kearney, 1965). Although studies on the enzymic rates of hydrolysis of selected phenyl carbamates may be far removed from the actual conditions in soils, an examination of relative rates of hydrolysis of model compounds may serve as an indicator of the longevity of herbicides that are largely detoxified by soil microorganisms. Conclusions drawn from this type of study, however, may have broader implications outside of a consideration of the phenylcarbamates in terms of biodegradability. An understanding of the effects of various substituents in close proximity to the site of reaction of the pesticide molecule undergoing reaction may give a keener insight into the rapidity with which other soil-applied herbicides are decomposed.

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