Purification and Properties of an Enzyme Responsible for Hydrolyzing Phenylcarbamates

PHILIP C. KEARNEY
Crops Research Division,
Agricultural Research Service,
U. S. Department of Agriculture,
Beltsville, Md.

The purification and properties of an enzyme, isolated from a soil microorganism, which catalyzes the hydrolysis of isopropyl *N*-(3-chlorophenyl)carbamate to 3-chloroaniline are described. Purification was achieved by a combination of salt fractionation and chromatography on DEAE cellulose. The purified enzyme was characterized as to pH optimum metal ion requirement, inhibitors, and substrate specificity. Enzymic rates of hydrolysis of several phenylcarbamates are correlated to a physical-chemical property of the substrate. Differences in rates can be explained by considering certain steric and electronic characteristics of the phenylcarbamate.

THE PHENYLCARBAMATES constitute a large class of compounds, many of which exhibit herbicidal activity. Previous reports (*1, 5, 16, 17, 19, 24*) indicate that degradation of these carbamates occurs rapidly in soils, and that soil microorganisms are largely responsible for inactivation. Recently (*12*), a crude enzyme system that rapidly liberated 3-chloroaniline from isopropyl *N*-(3-chlorophenyl)carbamate [CIPC] was isolated from a *Pseudomonas* sp. A broad substrate specificity with respect to substituents on the acyl and alkyl portion of the molecule was observed in the cell-free system. Here we describe the purification and properties of the hydrolytic enzyme and examine the effects of electronic and steric properties of certain substrates on rates of enzymic hydrolysis. The enzyme catalyzes the hydrolysis of acid anilides as well as phenylcarbamates.

Methods

Growth Conditions. *Pseudomonas striata* Chester has been identified as one soil microorganism which rapidly degrades CIPC (*11*). Degradation was determined by assaying for chloride ion liberation (*8*) or $C^{14}O_2$ production from the labeled CIPC. The organism was mass cultured in 10- to 15-liter volumes of nutrient solution with the following composition: 8.0 grams of K_2HPO_4; 2.0 grams of KH_2PO_4; 2.0 grams of $MgSO_4 \cdot 7H_2O$; 50.0 grams of $NaNO_3$; 0.5 gram of $CaSO_4 \cdot 2H_2O$; 0.01 gram of $NH_4Mo_7O_{24} \cdot 4H_2O$; and 5.0 grams of yeast extract in 10 liters of distilled water. Five grams of CIPC were dissolved in 10 ml. of 95% ethanol; the ethanol solution was filtered through a sterile pad and pipetted into the growth media under aseptic conditions. The culture solutions were maintained in 18-liter carboys. If agitation with a magnetic stirrer was immediate and continuous, and if the temperature was maintained above 30° C., CIPC remained suspended as globules in the liquid phase. Cultures were vigorously aerated from the center of the solution; this prevented extensive crystallization of the CIPC near the top of the carboy. Cells were harvested near the peak of the exponential growth phase (usually after 4 to 5 days) by continuous flow centrifugation at 5° C. Harvested cells were washed with distilled water and the packed cells stored at −5° C. Whole cells or sonicated cells stored in this manner were stable for several months.

Enzyme Assay. Enzyme activity was demonstrated by incubating the enzyme preparation with CIPC in a buffered solution and then determining colorimetrically the amount of 3-chloroaniline produced (*22*). The assay procedure is based on the assumption that the following reactions are taking place (Figure 1).

The assay mixture consisted of 0.1 ml. of protein (4 to 20 units), 0.4 ml. of 0.1*M* Tris-HCl buffer (pH 8.5), and 2.5 ml. of CIPC solution (1 μmole). The reaction was initiated with the addition of substrate and terminated after 10 minutes at 30° C. by the addition of 25 ml. of an acid solution (8 parts of 1*N* HCl to 1 part of concentrated acetic acid). A boiled enzyme control was included in all assays. Figures 2 and 3 show that 3-chloroaniline production was linear with respect to time for 30 minutes and with respect to enzyme concentration up to 2.0 mg. of protein.

Reagents for the 3-chloroaniline test were prepared fresh daily. Protein was determined by the biuret procedure (*6*) for aliquots containing more than 1 mg. of protein and the Lowry procedure (*14*) for more dilute protein solutions.

The specific activity is defined as the number of micrograms of 3-chloroaniline produced per milligram of protein per 10 minutes. A unit is defined as the amount of enzyme required for the formation of 1 μg. of 3-chloroaniline under the assay conditions described.

Substrates and Reagents. All phenylamides used as substrates in these investigations were recrystallized twice in hexane or alcohol. Substrates were solubilized by dissolving the material in 5 ml. of chloroform, and then placing the chloroform solution in the desired amount of water and removing the chloroform phase by aeration. Assays were performed with CIPC near its maximum solubility (100 p.p.m.); the limited solubility of many of the phenylamides in aqueous solution prevented comparisons on an equimolar basis. Assays for 1-naphthol production from 1-naphthyl *N*-methylcarbamate [carbaryl] were conducted at pH 6.5 by the *p*-nitrobenzenediazonium fluoroborate procedure described by Johnson (*10*). Incubations at more alkaline pH's caused spontaneous hydrolysis.

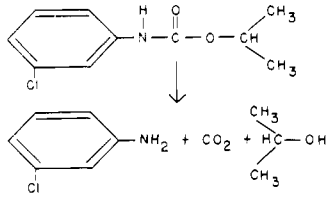

Figure 1. Proposed mechanism of phenylcarbamate hydrolyzing enzyme on CIPC

DEAE cellulose was prepared for chromatography by the procedure outlined by Peterson and Sober (*23*). Although the cellulose was screened and

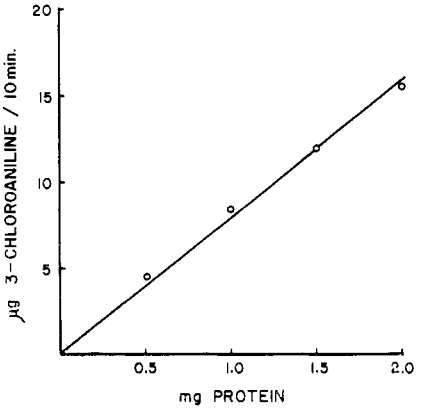

Figure 2. 3-Chloraniline formation as a function of amount of protein

washed, reasonable flow rates were achieved only after exhaustive decanting of the fine particles before preparing the column.

Results

Enzyme Purification. All manipulations with the enzyme preparation were carried out at ice-bath temperature unless otherwise stated. The harvested, packed cells collected by centrifugation were suspended in distilled water and subjected to sonic oscillation for 10 minutes at about 4° C. After centrifugation at 20,000 g for 10 minutes, 1 ml. of a 2% solution of streptomycin sulfate was slowly added to 10 ml. of the supernatant from the crude extract. After continuous stirring for 30 minutes, the precipitate collected by centrifugation was discarded. Attempts to remove nucleic acids by the addition of $MnCl_2$ (*13*) resulted in 48% loss in yield and in no purification.

Solid ammonium sulfate was added to the supernatant (10 ml.) to 30% saturation and the precipitate collected after centrifugation was discarded. The precipitate collected after a further addition of solid ammonium sulfate to 50% saturation was resuspended in 5 ml. of distilled water. The ammonium sulfate precipitate collected between 30 and 50% saturation was eluted through a column (2 × 15 cm.) of Sephadex G-25, previously equilibrated with 0.01*M* phosphate buffer or dialyzed overnight against several changes of distilled water.

The desalted protein was washed onto a column of DEAE cellulose (2 × 20 cm.) with 0.01*M* phosphate buffer. A gradient elution was performed using the apparatus described by Palmer (*21*). The ionic strength of the eluent was continuously increased by allowing 200 ml. of 0.1*M* phosphate buffer (pH 8.0) to flow gradually into the mixing flask containing 210 ml. of 0.01*M* phosphate (at the same pH). After the reservoir containing the 0.1*M* buffer were depleted, an additional 200 ml. of 1.0*M* buffer were added to the reservoir. Five-milliliter fractions were collected automatically and assayed for enzyme activity. Tubes containing the greatest activity were combined and made up to 90% saturation with respect to ammonium sulfate; a small precipitate was collected by centrifugation at 30,000 g for 30 minutes.

Figure 4 shows the elution pattern of the enzyme through DEAE cellulose. A summary of the purification scheme is shown in Table I.

pH Optimum. The pH optimum of the partially purified enzyme (step 3) is shown in Figure 5. Optimum activity was exhibited near pH 8.5, although activity was observed in the range pH 5 to 10. Attempts to purify the enzyme by acid precipitation at pH 4.5 caused complete inhibition of enzyme activity. Jakoby and Fredericks (*9*) noted two pH optima for an amidase, isolated from *Pseudomonas fluorescens*, when actamide and propionamide were used as the substrates. Hydrolysis was maximal at the basic optimum (pH 8.0), while the transfer reaction to hydroxylamine was maximal at the acidic optimum (about 6.2). No such bimodal optimum was observed with the phenylcarbamate enzyme.

Table I. Purification Scheme

Stage of Purification	Protein, Mg. per Ml.	Total Protein	Specific Activity	Total Units
1. Crude extract	30	300	10.8	3240
2. Streptomycin sulfate	21	211	10.6	2236
3. $(NH_4)_2SO_4$ precipitation	10	50	32.5	1625
4. DEAE cellulose	0.12	1.05	766.0	807

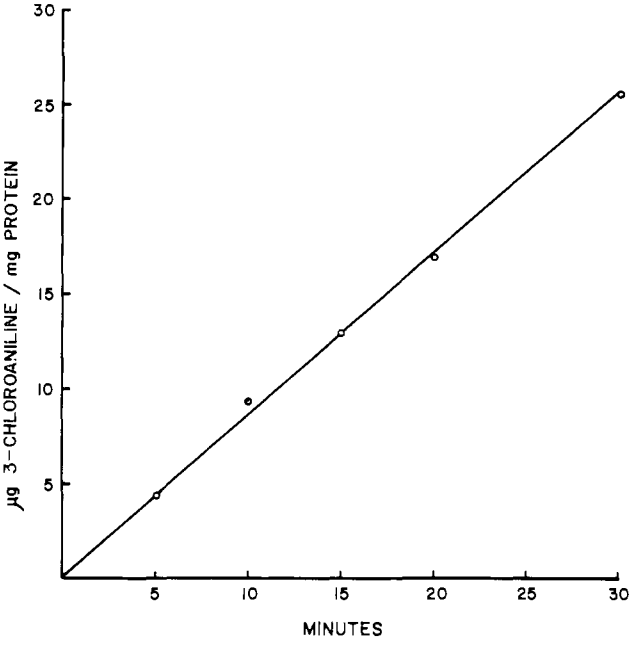

Figure 3. 3-Chloroaniline formation as a function of time

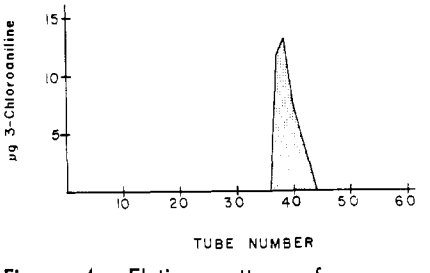

Figure 4. Elution pattern of enzyme through DEAE cellulose

Each tube assayed for 3-chloroaniline production and protein content

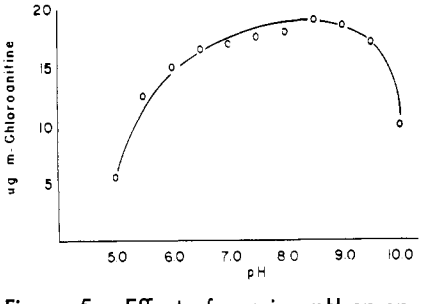

Figure 5. Effect of varying pH on enzyme activity

Results obtained by a combination of carbonate, phosphate, Tris, and acetate buffers measuring activity at every 0.5 pH unit

Metal Ions and Inhibitors. Preincubation of the enzyme with $10^{-4}M$ (ethylenedinitrilo)tetraacetic acid (EDTA) at pH 6.5 for 10 minutes caused no decrease in activity compared to a control at the same pH with no EDTA. Metal ions, Mg^{+2}, Ca^{+2}, Co^{+2}, and Mn^{+2}, at concentrations up to $10^{-3}M$ caused no stimulation in 3-chloroaniline production.

As previously observed (*12*), the enzyme is strongly inhibited by diisopropyl fluorophosphate ($10^{-6}M$ = 42% inhibition). This implies that a hydroxyl group, probably the hydroxyl group of serine, is involved at the active center (*20*). Interpretation of DFP data, however, must be viewed with some caution since some commercial preparations are contaminated with a sulfhydryl inhibitor (*7*). The enzyme under study, however, is not inhibited by *p*-hydroxymercuribenzoate at concentrations up to $10^{-3}M$ in the assay system; nor is the enzyme enhanced by the addition of reduced glutathione.

Product. The product of the reaction was verified as 3-chloroaniline by

flame ionization gas chromatography and thin-layer chromatography. A comparison of the retention times and cochromatography of authentic material with the methylene chloride extract of the assay solution established 3-chloroaniline as the end product (*12*). Gas-liquid chromatography was performed on a 6-foot column packed with ½% Ucon LB550-X on Chromosorb W (60- to 80-mesh).

The product was also verified as 3-chloroaniline utilizing thin-layer chromatography on silica gel HF_{254} (without $CaSO_4$ binder) and a solvent system of benzene–acetic acid (1 to 1) and benzene saturated with ammonia. The silica gel contains an inorganic fluorescent indicator, and CIPC and 3-chloroaniline can be detected readily. The ultraviolet indicator, 2,7-fluorescein, mixed with the silica gel works equally well as the HF_{254}. One microcurie of labeled CIPC-C^{14} (randomly labeled in the ring, specific activity 5.99 μc. per mg.) was incubated with the purified enzyme. The reaction was terminated after 10 minutes at 30° C. and the solution extracted three times with methylene chloride. The methylene chloride extract, with carrier amounts of 3-chloroaniline, was spotted on the silica gel HF_{254} and developed first in the benzene-ammonia system; the plate was removed, turned 90°, and developed in the benzene–acetic acid solvent. A scan of the plate revealed that the ultraviolet spot for 3-chloroaniline coincided exactly with the radioactive spot from the enzyme assay solution. Figure 6 shows a diagram of the chromatogram with appropriate R_f values for CIPC and 3-chloroaniline.

Substrate Specificity. The purified enzyme (Step 4) exhibits a broad substrate specificity (Table II). The phenylcarbamates were hydrolyzed, while the corresponding 3-(*p*-chlorophenyl)1,1-dimethylurea [monuron] was not attacked. Earlier investigations (*12*) indicated that the enzyme was probably only attacking the ester linkage of the phenylamides since the crude system would not hydrolyze monuron. The observed ability of the purified enzyme to catalyze the hydrolysis of the two acylanilides (compounds VI and VII), however, implies that amidase activity is also involved. The dualistic nature of many enzymes to catalyze the hydrolysis of both amides and esters is well known—for example, chymotrypsin and trypsin. The enzyme does not hydrolyze 1-naphthyl *N*-methylcarbamate. The hydrolysis of other *N*-methylcarbamates has not been studied in detail.

Table II. Substrate Specificity of Phenylcarbamate Hydrolyzing Enzyme

	Substrate	Product Formed, μmole × 10^{-3}
I	Isopropyl *N*-(3-chlorophenyl)carbamate	59
II	*n*-Propyl *N*-(3-chlorophenyl)carbamate	70
III	Isopropyl *N*-phenylthionocarbamate[a]	118
IV	Isopropyl *N*-phenylcarbamate	42
V	Ethyl *N*-(3-chlorophenyl)carbamate	82
VI	3,4-Dichloroacetanilide	62
VII	3,4-Dichloropropionanilide[a]	74
VIII	1-Naphthyl *N*-methylcarbamate[a]	0
IX	3-(*p*-Chlorophenyl)-1,1-dimethylurea	0

[a] Saturated solutions, but not equimolar to CIPC.

Discussion

Insight into the factors responsible for altering the rates of hydrolysis of certain substrates can be gained by examining a physical property of the phenylcarbamates. Camper and Moreland (*1*) have recently studied the relative acidities of many of the same phenylcarbamates used in the study of substrate specificity reported in this paper. It is of interest, then, to determine whether any correlation exists between the relative acidities of the phenylcarbamates and their susceptibility to enzymic hydrolysis.

Relative acidities of the phenylamides are determined by potentiometric titration with 0.1*N* tetrabutylammonium hydroxide methoxide in *n*-butylamine (*1*). The procedure, essentially that described by Cluett (*2*, *3*), is a measure of the ease of proton removal from the amide nitrogen. The results, expressed as half-neutralization potentials (HNP), are based on benzoic acid (HNP = 500 mv.) as a reference standard. Any inductive effect, within the molecule, which tends to increase the acidity of the amide hydrogen would be expressed by a corresponding decrease in HNP.

The enzyme responsible for hydrolyzing the phenylcarbamates could give rise to 3-chloroaniline by two closely related processes. Enzymic attack at the ester linkage would yield 3-chlorophenylcarbamic acid and isopropyl alcohol. The carbamic acid would be unstable and spontaneously yield 3-chloroaniline and CO_2. The ability of the enzyme to attack the acylanilides, however, suggests that the system possesses amidase activity. Enzymic attack at the amide linkage would yield 3-chloroaniline directly and also the isopropyl ester of carbonic acid, which would also be unstable. Apparently the mechanism of hydrolysis by esterases and amidases is similar (*15*) and the considerations that follow are valid regardless of which bond is broken first. Model systems for these reactions prescribe a nucleophilic attack at the carbonyl carbon by some group on the enzyme surface to form an intermediary acyl enzyme complex (*25*). From a purely electronic standpoint, the lower the electron density at the carbonyl carbon, the more rapid the nucleophilic attack. Electron-withdrawing substituents bonded to the acyl or alkyl portion of the carbamate ester should lower the electron density in the vicinity of the amide nitrogen, and thus enhance proton removal or acidity. These inductive effects, with respect to lowering the electron density, would also be operative in the vicinity of the carbonyl carbon. Thus, if judged by electronic effects alone, the lower the HNP, the lower the electron density in the vicinity of the carbonyl carbon, the more rapid the nucleophilic attack at the carbonyl carbon, and consequently the more rapid the rate of hydrolysis. Conversely, large bulky substituents in the vicinity of the carbonyl carbon should have a retarding effect on the rate of hydrolysis due to steric hindrance.

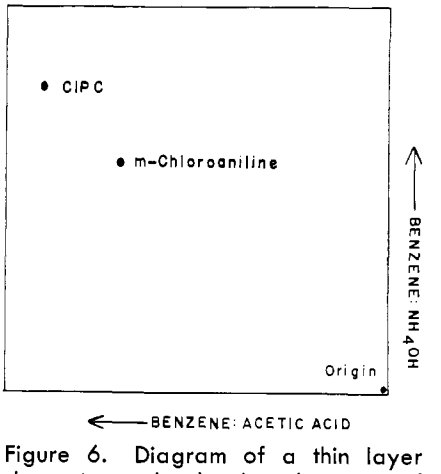

Figure 6. Diagram of a thin layer chromatograph showing location of 3-chloroaniline and CIPC

The rate of enzymic hydrolysis of nine phenylcarbamates as a function of HNP, expressed in millivolts, is shown in Figure 7. The lower the HNP, the more acidic the phenylcarbamate. Based on HNP values alone, one could predict that the rate of hydrolysis of CIPC would exceed that of IPC. The inductive effect (-I*s*) caused by ring substitution of the electronegative chlorine would increase acidity and consequently the rate of hydrolysis. The sharp increase in the rate of hydrolysis of the *n*-propyl *N*-(3-chlorophenyl)carbamate over the isopropyl analog is probably a combination of steric and electronic effects, since only a small difference in HNP (744 *vs.* 749) exists between the two carbamates. In general, esters of primary alcohols are hydrolyzed more rapidly than the corresponding secondary alcohols (*18*). The similar sharp increase in rate of hydrolysis of the ethyl analog over the *n*-propyl carbamate is probably due to the shorter chain length of the former since only small differences in HNP exist between the two compounds.

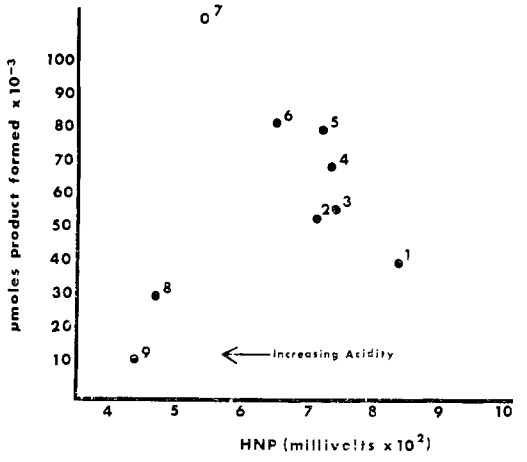

Figure 7. Rate of enzymic hydrolysis of nine *N*-phenylcarbamates as a function of HNP

Compounds listed by code numbers:
1. Isopropyl *N*-phenylcarbamate
2. Isopropyl *N*-(2-chlorophenyl)carbamate
3. Isopropyl *N*-(3-chlorophenyl)carbamate
4. Propyl *N*-(3-chlorophenyl)carbamate
5. Ethyl *N*-(3-chlorophenyl)carbamate
6. Propynyl *N*-(3-chlorophenyl)carbamate
7. Isopropyl *N*-phenylthionocarbamate[a]
8. 1-Chloroisopropyl *N*-(3-chlorophenyl)carbamate
9. 1,3-Dichloroisopropyl *N*-(3-chlorophenyl)carbamate

[a] Compound 7, designated by O, was not equimolar to CIPC in assay system

Substitution of an allylic group in the alkyl portion of the 3-chlorophenyl carbamate ester causes an increase in acidity and a corresponding increased rate of hydrolysis. From right to left in Figure 7 there is a general increase in enzymic rate as the phenylcarbamates become more acidic.

If electronic forces are the main factors governing the rate of hydrolysis, then the more acidic chloroalkyl-substituted phenylcarbamates should be the most susceptible to attack. However, the rate of hydrolysis of 1-chloroisopropyl- and 1,3-dichloroisopropyl *N*-(3-chlorophenyl)-carbamate actually decreases as the HNP decreases. One explanation for this phenomenon is that the bulky chlorine substituents sterically interfere with attack at the carbonyl carbon. At this point the steric effects appear to outweigh the electronic effects. The other alternative is that the products of the reaction—i.e., the chlorinated alcohols—are inhibiting the reaction. This possibility is under investigation.

Substitution of the chlorine into the 2 position of the ring causes a small decrease in HNP and a corresponding small decrease in rate compared to CIPC. The isopropyl *N*-(2-chlorophenyl)carbamate represents an interesting departure from the behavior of corresponding 3-chlorophenylcarbamates. The nonreactivity of 2,6-disubstituted aromatic acids toward esterification is well known (*18*), but single ortho-substituted acids are readily esterified. Also, when one or more atoms separate the carboxyl group in diortho-substituted acid, the acid is readily esterified. These same structural considerations should also apply to the rate of ester hydrolysis. The decreased rate of enzymatic hydrolysis of the 2-chloro-substituted compound as compared to the 3-chloro-substituted carbamate may be a steric effect, resulting not necessarily from a hindered nucleophilic attack alone, but also from the geometry of the binding site on the enzyme surface for the phenylcarbamate. An inspection of molecular models of the 1-chloroisopropyl *N*-(3-chlorophenyl)carbamate also suggests that enzyme substrate fit may be a reasonable explanation for the unreactivity of the chloroalkyl carbamates rather than a sterically retarded carbonyl addition reaction.

Acknowledgment

The author is indebted to D. E. Moreland, North Carolina State University, for generously supplying the recrystallized phenylcarbamates and the HNP values.

Literature Cited

(1) Camper, N. D., Moreland, D. E., *Biochim. Biophys. Acta* **94,** 383 (1965).
(2) Cluett, M. L., *Anal. Chem.* **31,** 610 (1959).
(3) *Ibid.*, **34,** 1491 (1962).
(4) DeRose, H. R., *Bot. Gaz.* **107,** 583 (1946).
(5) Freed, V. H., *Weeds* **1,** 48 (1951).
(6) Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.* **177,** 751 (1949).
(7) Gould, N., Wong, R. C., Liener, I. E., *Biochem. Biophys. Res. Commun.* **12,** 469 (1963).
(8) Iwasaki, I., Utsumi, S., Ozawa, T., *Bull. Chem. Soc. Japan* **25,** 226 (1952).
(9) Jakoby, W. B., Fredericks, Joan, *J. Biol. Chem.* **239,** 1978 (1964).
(10) Johnson, D. P., *J. Assoc. Offic. Agr. Chemists* **47,** 283 (1964).
(11) Kaufman, D. D., Kearney, P. C., *J. Appl. Microbiol.* **13,** 443 (1965).
(12) Kearney, P. C., Kaufman, D. D., *Science* **147,** 740 (1965).
(13) Korkes, S., Del Campillo, A., Gunsalus, I. C., Ochoa, S., *J. Biol. Chem.* **193,** 721 (1951).
(14) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randal, R. J., *Ibid.*, **193,** 265 (1951).
(15) Myers, D. K., "The Enzymes," P. D. Boyer, H. Lardy, K. Myrback, eds., Vol. **4,** p. 475, Academic Press, New York, 1960.
(16) Montgomery, M., Freed, V. H., J. Agr. Food Chem. **7,** 617 (1959).
(17) Newman, A. S., DeRose, H. R., DeRigo, H. T., *Soil Sci.* **60,** 393 (1948).
(18) Newman, M. S., "Steric Effects in Organic Chemistry," p. 221, Wiley, New York, 1956.
(19) Ogle, R. E., Warren, G. F., *Weeds* **3,** 257 (1954).
(20) Osterbaan, R. A., Kunst, P., van Rotterdam, J., Cohen, J. A., *Biochim. Biophys. Acta* **27,** 556 (1958).
(21) Palmer, J. K., Conn. Agr. Expt. Sta., New Haven. Bull. **589,** 7 (1955).
(22) Pease, H. L., J. Agr. Food Chem. **10,** 279 (1962).
(23) Peterson, E. A., Sober, H. A., *Biochem. Prep.* **8,** 39 (1961).
(24) Stevens, L. F., Carlson, R. F., *Weeds* **3,** 257 (1952).
(25) Wilson, I. B., Bergmann, F., Nachmanson, D., *J. Biol. Chem.* **186,** 781 (1950).

Received for review June 7, 1965. Accepted September 13, 1965.