librium.[21] Allowance of 2 kcal/mol for $T\Delta S$ (25°) is reasonable by either approach, so the heat of dissociation, $\Delta H$, $\gtrsim$ 8.5 kcal/mol. Since $D(C_6H_5-Cl) \simeq$ 93.8 kcal/mol,[22] $\pi$-chlorobenzene lies $\lesssim$85 kcal/mol above chlorobenzene ($S_0$). Transformations, energies, and lifetimes of $C_6H_5Cl$ species produced in chlorobenzene photolysis are summarized in Figure 2.

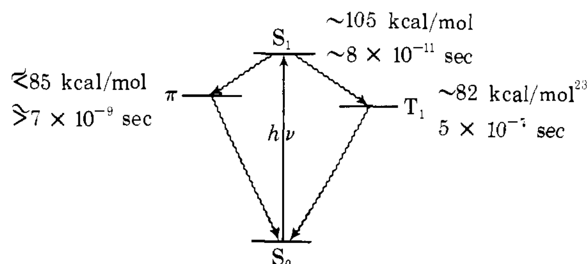
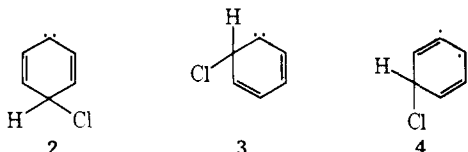



Figure 2. $C_6H_5Cl$ species formed by irradiation of chlorobenzene at 2537 Å in Freon 113.

The structure of $\pi$-chlorobenzene is not known in detail. Where the chlorine is located, whether or not it is capable of "ring whizzing," and whether the species is singlet or triplet[24] (if indeed only a single state of the biradical is involved) are all questions which remain to be answered.

There is clear evidence, however, against the possibility that the chlorine is $\sigma$ bonded to the ring as in **2**–**4**. For example, iodine quenching of **2**–**4** should




give major amounts of chloroiodobenzenes, but photolysis of chlorobenzene in the presence of iodine yielded iodobenzene uncontaminated with any of the three chloroiodobenzenes (<0.5% yield).

In principle, $\pi$-chlorobenzene could be formed by intramolecular rearrangement, but our failure to detect it unambiguously in the gas phase (even at ~12 atm pressure of Freon 12) argues that its principal, if not exclusive, origin is C–Cl homolysis followed by cage recombination. Recombination must be nearly quantitative to explain the very low $\Phi_D$ found in Freon 113 solution.[8]

Preliminary experiments indicate that photogeneration of $\pi$ isomers is a general phenomenon among more highly chlorinated benzenes and occurs with bromobenzene as well.

**Acknowledgment.** The authors are grateful to Professor C. L. Braun for valuable advice on the emission spectroscopy. They wish to thank the National Science Foundation, the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the Alfred P. Sloan Foundation for generous financial support.

(21) R. M. Keefer and L. J. Andrews, *J. Amer. Chem. Soc.*, **77**, 2164 (1955).
(22) This value is based on $\Delta H_f°$ (298°K) = 77.1 ± 3 kcal/mol for the phenyl radical (I. Szilagyi and T. Berces, *Int. J. Chem. Kinet.*, **2**, 199 (1970)).
(23) H. E. Zimmerman and G. E. Samuelson, *J. Amer. Chem. Soc.*, **91**, 5309 (1969); S. P. McGlynn, T. Azumi, and M. Kasha, *J. Chem. Phys.*, **40**, 507 (1964).
(24) Irradiation of chlorobenzene in a Fluorolube matrix at 77°K in an esr cavity failed to reveal a triplet species, but with a higher intensity source it may yet make an appearance.

**Marye-Anne Fox, William C. Nichols, Jr., David M. Lemal***
*Department of Chemistry, Dartmouth College*
*Hanover, New Hampshire 03755*
*Received June 1, 1973*

## A Phosphodiesterase from *Enterobacter aerogenes*

*Sir:*

We wish to report the purification and preliminary characterization of a phosphodiesterase from *Enterobacter aerogenes.* This diesterase is the first to be isolated that is known to catalyze the hydrolysis of simple diesters of phosphoric acid, such as diethyl phosphate and trimethylene phosphate.[1] Recently the heats of enzyme-catalyzed hydrolysis of cyclic 3′,5′-nucleotides and 2′,3′-nucleotides[2,3] were reported; since the large enthalpy of hydrolysis of cyclic AMP was unexpected, it was desirable to determine the heats of hydrolysis of simple aliphatic analogs for comparison. However, most diesters of phosphoric acid (except those containing five-membered rings) are exceedingly resistant to hydrolysis,[4] so that determinations of heats of hydrolysis were out of the question until a suitable catalyst was discovered. The enzyme here described served for the determinations of the desired heats of hydrolysis,[1] and may have other uses.

Wolfenden and Spence[5] observed that *Enterobacter aerogenes* can be grown on dimethyl phosphate as the only source of phosphorus. We have now shown that under these conditions (but not when the bacteria are grown with adequate inorganic phosphate), they produce large quantities (up to 3% of the total soluble protein) of a phosphodiesterase. The cells (American Type Culture Collection 13048) were grown at 37° on minimal medium[5] without phosphate but with 1.0–1.4 × $10^{-4}$ *M* sodium dimethyl phosphate in a rotary shaker at pH 7.5. The cells from 48 l. of culture medium were harvested and the cell paste washed with 0.01 *M* Tris buffer, pH 7.60. The cells were disrupted by a single pass through a French press[6] (Aminco) at 16,000 psi; the subsequent purification procedure is outlined briefly in Table I.

The suspension from the French press was centrifuged at 34,800*g*, and the supernatant freed of nucleic acids with protamine sulfate. After centrifugation, the supernatant was heat-treated, cooled, and again centrifuged. The supernatant was then chromatographed on Whatman DE-52 cellulose. The esterase fractions were concentrated by an Amicon ultrafilter with a PM-10 membrane, and the protein was precipitated by bringing the concentration of ammonium sulfate to

(1) J. M. Sturtevant, J. A. Gerlt, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **95**, 8168 (1973).
(2) P. Greengard, S. A. Rudolph, and J. M. Sturtevant, *J. Biol. Chem.*, **244**, 4798 (1969).
(3) S. A. Rudolph, E. M. Johnson, and P. Greengard, *ibid.*, **246**, 1271 (1971).
(4) (a) J. Kumamoto and F. H. Westheimer, *J. Amer. Chem. Soc.*, **77**, 2515 (1955); J. Kumamoto, J. R. Cox, and F. H. Westheimer, *ibid.*, **78**, 4858 (1956); (b) J. R. Cox and O. R. Ramsay, *Chem. Rev.*, **64**, 317 (1964).
(5) R. Wolfenden and G. Spence, *Biochim. Biophys. Acta*, **146**, 296 (1967).
(6) We wish to thank Professor Konrad Bloch for the use of this equipment.

Table I. Purification of the Phosphodiesterase[a]

| Procedure | Description | Total act.[b] | Protein, total mg[c] | Sp act.[d] | Purification factor |
|---|---|---|---|---|---|
| Extract from French press | 240 ml of 0.01 $M$ Tris buffer, pH 7.60 | 300 | 6400 | 0.047 | 1.0 |
| Protamine sulfate | 48 ml of 1% protamine sulfate at pH 7.5 | 290 | 4600 | 0.063 | 1.4 |
| Heat treatment | 10 min at 63° | 270 | 850 | 0.32 | 6.8 |
| Cellulose column (vol 140 ml) | 4-l. linear gradient from 0.15 to 0.55 $M$ NaCl in 0.01 $M$ Tris buffer, pH 7.60 | 120 | 145 | 0.83 | 18 |
| $(NH_4)_2SO_4$ precipitation | Ppt dissolved in 4 ml of 0.01 $M$ phosphate buffer, pH 6.8 | | | | |
| Sephadex G-200 (vol 500 ml) | 0.01 $M$ potassium phosphate buffer, pH 6.8 | 86 | 65 | 1.32 | 28 |
| Bio-Gel HT (vol 40 ml) | 400 ml of linear gradient from 0.01 to 0.1 $M$ phosphate buffer, pH 6.8 | 85 | 55 | 1.55 | 33 |

[a] The data refer to a purification starting with 72 g of *Enterobacter aerogenes* from 48 l. of culture medium. Substantially similar results were obtained with prior experiments, including a large batch where the products from the first five steps from three runs were combined for the Sephadex gel filtration procedure. [b] Micromoles phosphate per minute at 30°, in the presence of alkaline phosphatase. [c] From $OD_{280}/OD_{260}$ for the first three steps; $OD_{280}$ for the last three steps, assuming that $OD_{280} = 1.0$ for a protein concentration of 1 mg/ml. [d] Total activity/mg.

60% saturation. The resulting protein was gel filtered in 0.01 $M$ phosphate buffer (pH 6.80), on a column of Sephadex G-200. The phosphodiesterase fractions from this column were about 98% pure by polyacrylamide gel electrophoresis of the enzyme in 0.1% sodium dodecyl sulfate solution. The diesterase solution, after further concentration with the Amicon ultrafilter, could be brought to homogeneity as judged by electrophoresis on polyacrylamide gels by further chromatography over Bio-Gel HT hydroxylapatite with a linear gradient between 0.01 and 0.1 $M$ potassium phosphate buffer (pH 6.8). When the enzyme solution from this column was concentrated by ultrafiltration to an $OD_{280}$ of about 3, and a clear solution brought to 35% saturation with ammonium sulfate, the resulting suspension slowly crystallized. The enzyme was assayed by following its hydrolysis of trimethylene phosphate.[7] This results in the quantitative formation of γ-hydroxypropyl phosphate that can be hydrolyzed by alkaline phosphatase, and inorganic phosphate can then be estimated colorimetrically by a phosphomolybdate analysis. The diesterase itself does not hydrolyze γ-hydroxypropyl phosphate under the conditions of the assay. The enzyme can also be assayed conveniently by following the increase in absorption at 400 nm that accompanies the catalyzed hydrolysis of bis(*p*-nitrophenyl) phosphate, but this procedure must be used with care, as the bacteria produce more than one enzyme that will cleave the bis(nitrophenyl) ester, and the diesterase slowly attacks mono-*p*-nitrophenyl phosphate.

The diesterase has a subunit molecular weight by sodium dodecyl sulfate–polyacrylamide gel electrophoresis[8] of 29,000, and a molecular weight, by sedimentation equilibrium[9] in an ultracentrifuge,[10] of about 180,000 ± 10,000. Its activity is not affected by EDTA, but somewhat inhibited by some metal ions (*e.g.*, $Zn^{2+}$). Preliminary determinations of its kinetic properties are listed in Tables II and III. The enzyme

Table II. Kinetic Parameters for Diesterase in 0.2 $M$ KCl, pH 7.3, at 30° [a]

| Substrate | $K_M$, m$M$ | $V_m$, μmol/min × $OD_{280}$[b] | $K_i$, m$M$[c] |
|---|---|---|---|
| Ethylene phosphate[d] | 0.16 | 6.18 | 2.50 |
| Trimethylene phosphate[e] | 0.34 | 2.06 | 1.08 |
| Tetramethylene phosphate[e] | 0.24 | 1.26 | 0.46 |
| Potassium phosphate | | | 0.72 |
| Diethyl phosphate | 0.83 | 0.17 | 1.50 |

[a] Rates measured with a Radiometer TTT1b Titrator. [b] Protein concentration measured by optical density at 280 nm. [c] Inhibition for hydrolysis of bis(*p*-nitrophenyl) phosphate, in 0.05 $M$ Tris buffer, pH 7.3, by product. [d] Reference 4. [e] Reference 7.

Table III. Approximate Relative Rates of Hydrolysis for 1 m$M$ Substrates in 0.1 $M$ Tris Buffer at pH 8.0 and 30°

| Substrate | Rel act. |
|---|---|
| Ethylene phosphate | 670 |
| Trimethylene phosphate | 100 |
| 2′,3′-CMP[a] | 130 |
| 3′,5′-AMP[b] | 5.5 |
| 3′,5′-UMP[b] | 6.9 |
| 3′-AMP[a] (5′-AMP,[b] 3′-UMP,[a] 5′-UMP[b]) | <0.025 |
| Bis(*p*-nitrophenyl) phosphate[c] | 1100 |
| *p*-Nitrophenyl phosphate[d] | 8.2 |
| *p*-Nitrophenyl thymidine-3′-phosphate[b] (± alkaline phosphatase) | <0.05 |
| *p*-Nitrophenyl thymidine-5′-phosphate[b] | 1.6 |
| *p*-Nitrophenyl thymidine-5′-phosphate[b] plus alkaline phosphatase | 2.3 |

[a] P-L Biochemicals. [b] Calbiochem. [c] Sigma. [d] Aldrich.

differs sharply in its properties from a 2′,3′-cyclic nucleotide diesterase previously[11] obtained from Enterobacteriaceae since the two proteins have different molecular weights and chromatographic properties, and since the nucleotidase hydrolyzes 3′-nucleotides but not 3′,5′-cyclic nucleotides and so (presumably) is inactive against simple phosphate diesters. Some enzymes can attack diesters of phosphoric acid where a *p*-nitrophenyl residue[12] is a leaving group, and crude extracts have

(7) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *J. Amer. Chem. Soc.*, **79**, 430 (1957).
(8) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
(9) D. A. Yphantis, *Biochemistry*, 3, 297 (1964).
(10) We wish to thank Mr. Malcolm Smart for expert assistance and Professor Paul Doty for the use of this equipment.
(11) H. C. Neu, *Biochemistry*, 7, 3774 (1968); G. I. Drummond and M. Yamamoto, *Enzymes, 3rd Ed.*, **4**, 355 (1971).
(12) A. Bernardi and G. Bernardi, *Enzymes, 3rd Ed.*, **4**,329 (1971).

been reported[13] that will hydrolyze cyclic sugar diesters of phosphoric acid, but to the best of our knowledge the enzyme from *Enterobacter aerogenes* is the first purified diesterase with specificity known to include simple aliphatic diesters of phosphoric acid.

**Acknowledgment.** The authors wish to thank the Institute of General Medical Sciences of the National Institutes of Health (GM-04712) and the Petroleum Research Fund, administered by the American Chemical Society, for support of this work. One of us (J. A. G.) also wishes to thank the National Institutes of Health for support from a training grant.

(13) B. Zmudzka and D. Shugar, *Biochem. Biophys. Res. Commun.*, 23, 170 (1966).

**J. A. Gerlt,* F. H. Westheimer**
*James Bryant Conant Laboratories, Harvard University*
*Cambridge, Massachusetts 02138*
*Received August 20, 1973*

## Enthalpy of Hydrolysis of Simple Phosphate Diesters

*Sir:*

We wish to report the first determinations of the heats of hydrolysis of simple diesters of phosphoric acid. Such heats have previously been unavailable because most diesters of phosphoric acid are much too resistant to hydrolysis[1] to permit thermochemical measurements; the data are important because of the role such compounds play in biochemistry[2] and in the theory of the hydrolysis of phosphate esters.[3] We have succeeded in making appropriate measurements by utilizing a new phosphodiesterase recently isolated and purified in one of our laboratories.[4]

Although 2′,3′-cyclic nucleotides[5] and salts of ethylene phosphate[6] hydrolyze at moderate rates in strong alkali, salts of trimethylene phosphate[7] and of cyclic AMP,[8] like salts of dimethyl phosphate and of other simple diesters of phosphoric acid,[1] hydrolyze only $10^{-4}$–$10^{-6}$ times as fast. Strain has been implicated in the rapid hydrolysis of the five-membered rings; such strain is implicit in the small O–P–O bond angles found for these five-membered rings by X-ray crystallography[9] and explicit in the heats of hydrolysis of methyl ethylene phosphate[10] and of 2′,3′-cyclic nucleotides.[11] Recently, however, Hayaishi, *et al.*,[12] have found that the free energy of hydrolysis of cyclic AMP is −11.9 kcal/mol, a value in excess of the −8.9 kcal/mol for the hydrolysis of ATP.[13] Furthermore, Greengard, *et al.*,[11] found that the heat of hydrolysis of cyclic AMP is −14.1 kcal/mol and that other 3′,5′-cyclic nucleotides show similar enthalpies of hydrolysis; these values are much greater than those averaging around −8 kcal/mol for the 2′,3′-cyclic nucleotides.[11] Nevertheless, X-ray data for 3′,5′-cyclic nucleotides[14] and for trimethylene phosphate[15] yield O–P–O angles similar to those in acyclic phosphates,[16] and give no evidence of strain. No heats of hydrolysis of simple diesters of phosphoric acid or of simple cyclic diesters were previously available for comparison, for the reasons cited above.

We have now obtained the data in Table I. The heat of hydrolysis of sodium ethylene phosphate is about −6.8 ± 0.6 kcal/mol, and so is comparable to those of 2′,3′-cyclic nucleotides; the heats of hydrolysis of sodium diethyl phosphate and of sodium tetramethylene phosphate are smaller by about 4 kcal/mol, and the difference, although only a fraction of the difference in free energies of activation for the hydrolytic processes, is consistent with calculation.[17] The heat of hydrolysis of sodium trimethylene phosphate is about −3.5 kcal/mol. This result is consistent with the kinetic stability of trimethylene phosphate[7] and of cyclic AMP[8] and with calculations of their strain energies.[17,18] On the other hand, the new data for trimethylene phosphate stand in sharp contrast to the thermochemical data for 3′,5′-cyclic nucleotides,[11] which, on hydrolysis, release 7–11 kcal/mol more heat than does trimethylene phosphate. We have at present no explanation for this phenomenon, but believe that the contrast between the behavior of the simple six-membered ring phosphate ester and that of cyclic AMP is of importance.

Barium ethylene phosphate[6] was converted into its sodium salt with sodium sulfate. Trimethylene hydrogen phosphate[7,19] and tetramethylene hydrogen phosphate[7,18,19] were neutralized with sodium hydroxide. Barium diethyl phosphate and barium monoethyl phosphate were analytically pure, and were converted to their sodium salts with sodium sulfate. The buffers used were Tris (Sigma) and piperazine *N*,*N*′-bis(2-ethanesulfonic acid) (Pipes; Calbiochem). p*K* and pH values were measured with a Radiometer TTT1b Titrator. The diesterase was prepared from *Enterobacter aerogenes* grown on dimethyl phosphate as the only source of phosphorus, in accordance with recently developed procedures;[4] the sample used for calorimetry was judged to be at least 98% homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

(1) J. Kumamoto and F. H. Westheimer, *J. Amer. Chem. Soc.*, **77**, 2515 (1955); J. R. Cox and O. B. Ramsay, *Chem. Rev.*, **64**, 317 (1964).
(2) J. P. Jost and H. V. Rickenberg, *Annu. Rev. Biochem.*, **40**, 741 (1971).
(3) F. H. Westheimer, *Accounts Chem. Res.*, **1**, 70 (1968); P. Gillespie, F. Ramirez, I. Ugi, and D. Marquarding, *Angew. Chem., Int. Ed. Engl.*, **12**, 91 (1973).
(4) J. A. Gerlt and F. H. Westheimer, *J. Amer. Chem. Soc.*, **95**, 8166 (1973).
(5) S. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.*, 2708 (1952).
(6) J. Kumamoto, J. R. Cox, and F. H. Westheimer, *J. Amer. Chem. Soc.*, 78, 4858 (1956).
(7) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *J. Amer. Chem. Soc.*, **79**, 430 (1957).
(8) M. Smith, G. I. Drummond, and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 698 (1961).
(9) (a) T. A. Steitz and W. N. Lipscomb, *J. Amer. Chem. Soc.*, **87**, 2488 (1965); (b) C. L. Coulter and M. L. Greaves, *Science*, **169**, 1097 (1970); C. L. Coulter, *J. Amer. Chem. Soc.*, **95**, 570 (1973).
(10) E. T. Kaiser, M. Panar, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **85**, 602 (1963). The amount of thermochemical strain reported in this article is not certain, and the work is currently under reinvestigation.
(11) (a) P. Greengard, S. A. Rudolph, and J. M. Sturtevant, *J. Biol. Chem.*, **244**, 4798 (1969); (b) S. A. Rudolph, E. M. Johnson, and P. Greengard, *J. Biol. Chem.*, **246**, 1271 (1971).
(12) O. Hayaishi, P. Greengard, and S. P. Colowick, *J. Biol. Chem.*, **246**, 5840 (1971).
(13) R. A. Alberty, *J. Biol. Chem.*, **244**, 3290 (1969).
(14) (a) C. L. Coulter, *Acta Crystallogr., Sect. B*, **25**, 2055 (1969); (b) K. Watenpaugh, J. Dow, L. H. Jensen, and S. Furberg, *Science*, **159**, 206 (1968); (c) L. H. Jensen, private communication (cited above).
(15) Mazhar-ul-haque, C. H. Caughlan, and W. L. Moats, *J. Org. Chem.*, **35**, 1446 (1970).
(16) J. D. Dunitz and J. S. Rollett, *Acta Crystallogr.*, **9**, 327 (1956); Y. Kyogoku and Y. Iitaka, *ibid.*, **21**, 49 (1966).
(17) D. A. Usher, E. A. Dennis, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **87**, 2320 (1965).
(18) J. A. Gerlt, unpublished results.
(19) R. E. Wall, Ph.D. Thesis, Harvard University, 1961.