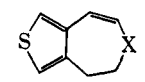
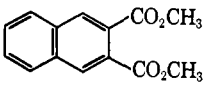
When heated above its melting point, thiepin I was found to decompose slowly to a mixture of products. However, on heating I at 150° with N-phenylmaleimide as the solvent, there was formed a single crystalline Diels–Alder adduct in nearly quantitative yield. This substance was identified as the previously reported *exo* adduct V of benzo[*c*]thiophene (IV) and N-phenylmaleimide.[10] Thiophene IV, on the other hand, gave in high yield a 1:1 mixture of the *exo* and *endo* adducts V and VI under identical conditions. Both I and IV when allowed to react with dimethyl acetylenedicarboxylate at elevated temperatures gave the naphthalene dimethyl ester VII in good yield.

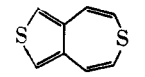
A number of explanations may be given to account for the great stability and Diels–Alder behavior of I. For example, the stability of I may be due to the contribution of charge-separated species such as VIII. The stereospecificity of I in adduct-forming reactions may be accounted for either by assuming appropriate Coulombic interaction of VIII with an electron-deficient dienophile or by assuming a nonplanar conformation for the thiepin portion of I. Additionally, the behavior of I may be rationalized in term of a common reaction intermediate such as the *o*-quinonoid episulfide IX.[11] Adducts of *exo* configuration would be expected to arise from IX, due to its unsymmetrical nature. The tetracovalent sulfur atom present in the quinonoid portion of IX should cause the extrusion of sulfur from I to be a high-energy process.[12]



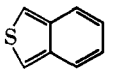
II, X = S
III, X = SO



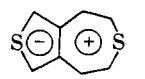
VII



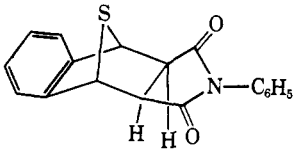
I



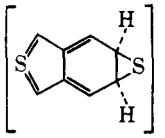
IV



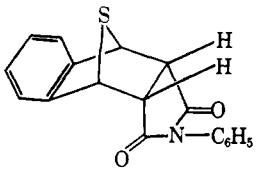
VIII



V



IX



VI

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(10) M. P. Cava and N. M. Pollack, *J. Am. Chem. Soc.*, **88**, 4112 (1966).

(11) Sulfur extrusion from thiepins long has been considered to proceed through episulfide intermediates; see J. D. Loudon in "Organic Sulfur Compounds," Vol. 1, N. Kharasch, Ed., Pergamon Press, Inc., New York, N. Y., 1961, p 299; also see ref 2a.

(12) For examples of high-energy tetracovalent sulfur species, see (a) M. P. Cava and N. M. Pollack, *J. Am. Chem. Soc.*, **89**, 3639 (1967); (b) M. P. Cava, N. M. Pollack, and D. A. Repella, *ibid.*, **89**, 3640 (1967); (c) R. H. Schlessinger and I. S. Ponticello, *ibid.*, **89**, 3641 (1967). This type of argument also may be used to account for the stability trends observed for other substituted thiepins; see B. P. Stark and A. J. Duke, "Extrusion Reactions," Pergamon Press, Inc., New York, N. Y., 1967, p 97.

The Kinetics of the *Escherichia coli* Alkaline Phosphatase Catalyzed Hydrolysis of 2,4-Dinitrophenyl Phosphate[1]

Sir:

Important progress in the elucidation of the mechanism of a multistep reaction can be achieved by observing individually the rate-limiting steps involved and by determining their kinetic parameters. In the *E. coli* alkaline phosphatase catalyzed hydrolysis of phosphate monoesters the transient formation of a phosphoryl–enzyme intermediate is well supported by experimental evidence[2] and, by using 4-nitrophenyl phosphate as a substrate, direct spectrophotometric observation of the reaction of phosphorylation of the enzyme has been accomplished.[3,4] However, in these experiments a satisfactory quantitative analysis of the results is impossible due to the unfavorable spectral properties of the substrate and the instability of the enzyme at low pH values where the reaction is slow enough to be measured by ordinary spectrophotometric techniques. These experimental difficulties can be eliminated by employing a rapid mixing technique and a substrate with negligible absorption in the spectral region of maximum absorbance of the reaction product. At pH 5.7—where the enzyme is sufficiently stable—2,4-dinitrophenyl phosphate satisfies these requirements; in the following we wish to report the results obtained by the use of this substrate.

Upon mixing an aqueous solution of an excess of the substrate at pH 5.5 (0.1 *M* acetate buffer) with a solution of alkaline phosphatase at pH 7.8 (0.01 *M* Tris-HCl, 0.1*M* NaCl) in a Durrum-Gibson stopped-flow spectrophotometer we observed at 400 mμ an initial rapid liberation of 2,4-dinitrophenolate ion for about 150 msec, followed by a slow, zero-order hydrolysis of the substrate. The kinetics of the presteady-state and the steady-state reactions were amenable to analysis by methods described earlier for this type of reactions,[5] and the appearance of 2,4-dinitrophenolate ion (P_1) has been found to obey an equation of the form: $P_1 = Vt + B(1 - e^{-bt})$, where V, B, and b are constants during the course of the reaction. By varying the initial concentration of the substrate and the enzyme and by adding an excess of phosphate ion (P_i) to the reaction mixture we found that B is proportional to the initial enzyme concentration and that the variation of b can be adequately described by $b = b_{lim}S/(S + K_S^{app})$, with S being the initial substrate concentration, b_{lim} a constant, and $K_S^{app} = K_S(1 + P_i/K_I)$.

(1) This research was supported by U. S. Public Health Service Medical Research Grant No. GM 13885. The authors wish to thank Professor J. H. Law and Professor E. A. Evans, Jr., for helpful suggestions.
(2) L. Engstrom, *Arkiv Kemi*, **19**, 129 (1962).
(3) A. Williams, *Chem. Commun.*, 676 (1966).
(4) W. K. Fife, *Biochem. Biophys. Res. Commun.*, **28**, 309 (1967).
(5) F. J. Kézdy and M. L. Bender, *Biochemistry*, 1, 1097 (1962).

Plots of $1/b$ *vs.* $1/S$ indeed yield excellent straight lines for a given constant value of P_i, and from these plots values of b_{lim}, K_S, and K_I can be calculated. Values of V have been measured more conveniently on the expanded slidewire of a Cary 15 recording spectrophotometer at 400 mμ and in a 5-cm path-length quartz cuvette. Normal Michaelis–Menten type kinetics were observed in all cases, and phosphate ions acted here too as purely competitive inhibitors, *i.e.* the rates were adequately described by $V = k_{cat}E_oS/(S + K_m(1 + P_i/K_I))$.

The data obtained in these experiments are summarized in Table I. These results are consistent with the kinetic scheme[6]

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} \underset{+\ P_1}{E{-}P_i} \xrightarrow{k_3} E\cdot P_i \overset{K_I}{\rightleftharpoons} E + P_i$$

with the following values for the individual constants: $K_s = k_{-1}/k_1 = 8 \times 10^{-7} M$; $k_2 = 24.6\ sec^{-1}$; $k_3 = 0.8\ sec^{-1}$; and $K_I = 2.4 \times 10^{-6} M$. Since k_3 is much smaller than k_2 and since in all our stopped-flow experiments the condition $S \gg K_m$ was satisfied, the amount of P_1 released in the presteady state is a direct measurement of the concentration of active sites in the reaction mixture.[7] Using $\epsilon_{400} = 11{,}130$ for the 2,4-dinitrophenolate ion and a molecular weight of 86,000 for the enzyme we found that in all our measurements on the stopped-flow instrument the ratio of the molarity of the active sites *vs.* the molarity of the enzyme was 0.9 ± 0.1. This ratio remained the same in the absence of phosphate ions and with phosphate ion concentrations as high as 1×10^{-3} M. Also, preincubation of the enzyme at pH 6.3 for 10 min with or without phosphate ions present had no effect on the ratio.

Table I. Kinetic Parameters of the *E. coli* Alkaline Phosphatase Catalyzed Hydrolysis of 2,4-Dinitrophenyl Phosphate[a]

$P_i \times 10^5$, M	b_{lim}, sec^{-1}[b]	$K_S^{app} \times 10^7$, M[b]	k_{cat}, sec^{-1}[c]	$K_m \times 10^8$, M[c]
0	25.4	8 ± 5	0.7	1.4 ± 0.7
1.5	25.3	25 ± 4	0.8	10.6 ± 3
5	25.4	91 ± 9	0.8	28.5 ± 5

[a] pH 5.7, 0.05 M acetate buffer, 25°. The enzyme used was Worthington alkaline phosphatase BAPC 71A, activity 30 units/mg. A stock solution of the enzyme was obtained by using the procedure described by Fife.[4] The enzyme concentration was determined from the absorption of that solution at 280 mμ using the value of 0.77 AU = 1 mg of protein/ml and a molecular weight of 86,000 (M. J. Schlesinger and K. J. Barrett, *J. Biol. Chem.*, **240**, 4284 (1965)). 2,4-Dinitrophenyl phosphate monolutidinium salt was used (the authors wish to thank Dr. A. Thomson and Professor M. L Bender for a sample of this compound), mp 140° (lit. mp 142° (A. J. Kirby and A. G. Vargolis, *J. Am. Chem. Soc.*, **88**, 1824 (1966)). [b] S_o = 6–50 × 10^{-6} M; E_o = 6–30 × 10^{-7} M. [c] S_o = 2–7 × 10^{-7} M; E_o = 1.8 × 10^{-9} M.

However, at pH 5.0 a reversible partial loss of activity of the enzyme is observed in the few seconds following the presteady state, thus decreasing slowly the value of V to a constant value. Since phosphate ions and excess substrate seem to protect the enzyme from this loss of activity, this phenomenon offers a possible explanation of the abnormally high titration values observed at pH 4.7 using 2-nitrophenyl phosphate as a substrate.[4]

(6) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, 37, 751 (1959).
(7) M. L. Bender, F. J. Kézdy, and F. C. Wedler, *J. Chem. Educ.*, **44**, 84 (1967).

The following conclusions are suggested by our results. (1) The *E. coli* alkaline phosphatase catalyzed hydrolysis of 2,4-dinitrophenyl phosphate is adeq iately described by a kinetic scheme involving the format on of an enzyme–substrate complex followed by a fast phosphorylation and a slower dephosphorylation of the enzyme, and no other steps involving changes in covalent bonds need be invoked. (2) In our experimental conditions phosphate ion acts as a competitive inhibitor, and it phosphorylates less than 10% of the active sites reacting with the substrate. (3) If one assumes that k_1 measures a diffusion-controlled process, then by analogy with the ribonuclease-catalyzed reactions[8] a lower limit of 10^9 M^{-1} sec^{-1} can be estimated for k_1. Then from the experimental value of K_S the lower limit for k_{-1} will be 800 sec^{-1}. Thus k_{-1} is much greater than k_2 and for all practical purposes K_S is a true equilibrium constant measuring the binding of the substrate to the enzyme. (4) The unusually low value of the binding constants K_S and K_I is suggestive of binding forces other than "hydrophobic," possibly of metal ion–ligand coordination complexes. (5) Finally, the titration of the active sites would indicate a 90% pure enzyme preparation, each molecule of mol wt 86,000 possessing one active site.[9]

(8) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4674 (1965).
(9) The specific activity of our enzyme preparation was 86 ± 5% of that measured with the most pure crystallized enzyme (M. H. Malamy and B. L. Horecker, *Biochemistry*, 3, 1893 (1964)). This then argues against the possibility of a 45% pure enzyme preparation with two active centers per molecule.

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New Cyclobutadiene Complexes of Cobalt

Sir:

The recent communication by Rausch and Genetti[1] prompts us to report some initial results of our research which is also directed at the synthesis and study of π-cyclopentadienyl-π-cyclobutadienecobalt(I) and its derivatives. Until recently only one complex of this type, π-cyclopentadienyl-π-tetraphenylcyclobutadienecobalt(I),[2] had been prepared despite the fact that considerations of electronic arrangement and symmetry lead to the expectation that these compounds should parallel ferrocene and cyclobutadieneiron tricarbonyl in reactivity and thermal stability. Since some previous syntheses of π-cyclopentadienyl-π-tetraphenylcyclobutadienecobalt(I) had involved the reaction of diphenylacetylene with π-cyclopentadienyl-π-1,5-cyclooctadienecobalt(I)[2a] or cobaltocene,[2b] it seemed desirable to investigate possible extensions of these techniques.

We find that a number of arylacetylenes bearing a variety of functional groups attached to the triple bond react with π-cyclopentadienyl-π-1,5-cyclooctadienecobalt(I) or cobaltocene to produce cyclobutadiene complexes, and we have for the first time prepared derivatives of π-cyclopentadiene-π-cyclobutadienenecobalt(I)

(1) M. D. Rausch and R. A. Genetti, *J. Am. Chem. Soc.*, **89**, 5502 (1967).
(2) (a) A. Nakamura and H. Hagihara, *Bull. Chem. Soc. Japan*, **34**, 452 (1961); (b) J. L. Boston, D. W. A. Sharpe, and G. Wilkinson, *J. Chem. Soc.*, 3488 (1962); (c) P. M. Maitlis, A. Efraty, and M. L. Games, *J. Organometal. Chem.* (Amsterdam), 2, 284 (1964).