fenden, *J. Biol. Chem.*, **247**, 8195 (1972); elastase, R. C. Thompson, *Biochemistry*, **12**, 47 (1973); L-asparaginase, J. O'C. Westerik and R. Wolfenden, *J. Biol. Chem.*, **249**, 6351 (1974); α-chymotrypsin, E. J. Breaux and M. L. Bender, *FEBS Lett.*, **56**, 81 (1975).
(6) α-Keto analogues of substrate amino acids are effective Inhibitors of α-chymotrypsin: J. D. Geratz, *Arch. Biochem. Biophys.*, **111**, 134 (1965).
(7) H. Pauly and H. Sauter, *Ber.*, **63**, 2063 (1930).
(8) F. H. Westheimer, *J. Am. Chem. Soc.*, **58**, 2209 (1936); I. Roberts and H. C. Urey, *ibid.*, **60**, 880 (1938).
(9) H. Kwart and M. M. Baevsky, *J. Am. Chem. Soc.*, **80**, 580 (1958).
(10) The enzyme used in this study with a specific activity of 4000(μkat/L)/A_{280} and equivalent weight 67 300, was prepared as previously described: P. A. Inkerman, K. Scott, M. T. C. Runnegar, S. E. Hamilton, E. A. Bennett, and B. Zerner, *Can. J. Biochem.*, **53**, 536 (1975).
(11) Typically, $K_m \simeq 5 \times 10^{-5}$–$5 \times 10^{-4}$ M for a variety of substrates (see ref 3).
(12) The rate constant for dephosphorylation is $\sim 7.3 \times 10^{-5}$ s^{-1} at 25 °C in 0.1 M Tris-HCl buffer, pH 8.08, 2.94% v/v in CH_3CN.
(13) J. F. Riordan, *Biochemistry*, **12**, 3915 (1973).
(14) K. Takahashi, *J. Biol. Chem.*, **243**, 6171 (1968); K. Takahashi, *J. Biochem. (Tokyo)*, **81**, 395 (1977).
(15) Cf. R. M. Schultz and A. C. Cheerva, *FEBS Lett.*, **50**, 47 (1975).
(16) J. K. Stoops, S. E. Hamilton, and B. Zerner, *Can. J. Biochem.*, **53**, 565 (1975).
(17) The interaction of chloral hydrate and phenylglyoxal with the enzyme is also consistent with the formation of active-site hemiacetals.
(18) N. J. Leonard and P. M. Mader, *J. Am. Chem. Soc.*, **72**, 5388 (1950).
(19) The electron density difference map for subtilisin inhibited by L-phenylalanyl-L-alanyl-L-lysine chloromethyl ketone has been interpreted as supporting a covalent bond between the oxygen of the catalytic serine 221 and the carbon of the ketone carbonyl: T. L. Poulos, R. A. Alden, S. T. Freer, J. J. Birktoft, and J. Kraut, *J. Biol. Chem.*, **251**, 1097 (1976).
(20) The spectrum of benzil is little altered by 5 M imidazole at pH 9.4 (μ = 1, KCl): λ_{max} 264.5 nm (ϵ_{max} 18 720). This result argues against the addition of active-site histidine: P. Willadsen, J. de Jersey, and B. Zerner, *Biochem. Biophys. Res. Commun.*, **51**, 620 (1973).
(21) M. L. Bender and J. K. Stoops, *J. Am. Chem. Soc.*, **87**, 1622 (1965).
(22) M. C. Berndt, J. de Jersey, and B. Zerner, *J. Am. Chem. Soc.*, following communication in this issue.

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Ethyl Phenylglyoxylate, a Simultaneous Inhibitor and Substrate of Chicken Liver Carboxylesterase (EC 3.1.1.1). Enzyme-Catalyzed Fragmentation of (*E*)-Benzil Monoxime *O*-2,4-Dinitrophenyl Ether

Sir:

In 1930, Bamann and Schmeller[1,2] clearly showed that ethyl phenylglyoxylate is a much poorer substrate than ethyl (±)-mandelate for various liver carboxylesterases,[3] and reasonably estimated that its K_m was at least 5000 times less than the K_m for the mandelate ester. They drew attention to this very unusual result and rationalized their data in terms of the relative reactivities of the respective Michaelis complexes.

A priori, however, one might have expected ethyl phenylglyoxylate to be a good substrate for the carboxylesterases.[4] We have therefore reinvestigated the catalysis of hydrolysis of this ester by the chicken liver enzyme[5] in the light of the formation of hemiketal adducts reported in the previous communication.[2] Further, this work has led to the discovery of the catalysis of Beckmann fragmentation reactions[6] by these enzymes.

Consistently with the results of Bamann and Schmeller,[1] ethyl phenylglyoxylate is a poor substrate for chicken liver carboxylesterase: $k_{cat.(obsd)} = 1.71 \pm 0.06 \times 10^{-2}$ s^{-1}, $K_m < 5 \times 10^{-7}$ M ($[S]_0 = 1.15 \times 10^{-6}$–$1.012 \times 10^{-4}$ M, $[E]_0 = 1.02 \times 10^{-7}$–$3.15 \times 10^{-6}$ M, initial *zero-order* kinetics, 0.05 M phosphate buffer, pH 7.5). Moreover, ethyl phenylglyoxylate is a powerful inhibitor of the hydrolysis of *p*-nitrophenyl acetate, a good substrate for the chicken enzyme,[7] with a $K_i \approx 1 \times 10^{-8}$ M. Further if the hydrolysis of ethyl phenylglyoxylate is examined under conditions where $[E]_0 > [S]_0$, $k_{cat.(obsd)} = 1.36_5 \pm 0.01_5 \times 10^{-2}$ s^{-1} ($[E]_0 = 1.18 \times 10^{-5}$–$1.22 \times 10^{-5}$ M, $[S]_0 = 9.15 \times 10^{-6}$–$1.01 \times 10^{-5}$ M, *first-order* kinetics, 0.05 M phosphate buffer, pH 7.5), and these experiments provide direct spectrophotometric evidence for the formation of E-I.[8]

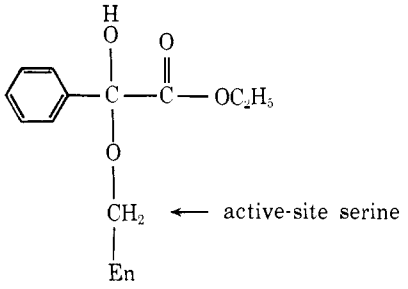
These results are consistent with the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (1)$$
$$\qquad\qquad k_2 \Big\Updownarrow k_{-2}$$
$$\qquad\qquad E\text{-}I$$

for zero-order kinetics where $k_{-1}/k_1 = K_1$, $k_{-2}/k_2 = K_2$, $k_{cat.(obsd)} = k_{cat.}K_2/(1 + K_2) \approx k_{cat.}K_2$ (K_2 small), and $K_{m(obsd)} = K_m{}^{BH}K_2/(1 + K_2) \approx K_m{}^{BH}K_2$ (K_2 small); and the scheme

$$E\text{-}I \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} ES \xrightarrow{k_{cat.}} E + P \qquad (2)$$

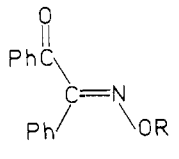
for first-order kinetics where $k_{cat.(obsd)} = k_{cat.}k_{-2}/(k_2 + k_{cat.})$, when [ES] is at steady state. E-I is the active site hemiketal adduct, and the measured K_i for the inhibition of *p*-nitrophenyl acetate hydrolysis is given by $K_1K_2/(1 + K_2)$.



E-I

The results are not only in complete accord with abortive hemiketal formation for ethyl phenylglyoxylate, but also allow the calculation of $k_{cat.}/k_2 = 0.25$, and $k_{-2} = 6.84 \times 10^{-2}$ s^{-1}. While the magnitude of K_2 is not determinable from these experiments, a not unreasonable estimate puts $K_2 = 5 \times 10^{-4}$ and consequently $k_{cat.} = 34.2$ s^{-1} and $k_2 = 137$ s^{-1}. While the absolute magnitude of these numbers may in both cases be *higher*, it is already clear that ethyl phenylglyoxylate is intrinsically a good substrate. These results constitute the first secure example of a molecule which is simultaneously a powerful inhibitor and substrate of a pure protein enzyme, by virtue of the reversible formation of an abortive active-site hemiketal adduct.[9,10]

The base-promoted fragmentation of (*E*)-benzil monoxime *O*-2,4-dinitrophenyl ether (I)[11] follows second-order kinetics



I, R = 2,4-dinitrophenyl

and yields benzoic acid, benzonitrile, and 2,4-dinitrophenolate ion.[12,13] The oxime ether is a substrate for chicken liver carboxylesterase and the above overall stoichiometry is quantitatively observed. The reaction was followed at 358 nm (0.05 M phosphate buffer, pH 7.5, 12.8% v/v CH_3CN).[14] The release of 2,4-dinitrophenolate ion is first order in the range of $[S]_0$, 2×10^{-7}–4×10^{-6} M, showing that $K_m \gg 4 \times 10^{-6}$ M. The observed first-order rate constant is proportional to enzyme concentration ($[E]_0 = 3.26$–8.01×10^{-8} M), yielding $k_{cat.}/K_m = 4.1 \times 10^5$ M^{-1} s^{-1}. The result is again consistent with active-site hemiketal formation followed by general-base-catalyzed fragmentation.[13] If the decomposition of the resulting benzoyl-enzyme is rate limiting, an estimate of K_m

may be obtained by using the $k_{cat.}$ (40 s^{-1}) for phenyl benzoate under the same conditions. This calculation gives $K_m = 9.8 \times 10^{-5}$ M.

The pH dependence of $k_{cat.}/K_m$ is not pure sigmoidal, but shows clear evidence for the involvement of a group, active in the free base form whose $pK_a' \approx 5.0$.[15]

Thus this work also provides the first example of the catalysis of carbon-carbon bond cleavage by the carboxylesterases.[16]

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References and Notes

(1) E. Bamann and M. Schmeller, *Hoppe-Seyler's Z. Physiol. Chem.*, **188**, 251 (1930).
(2) M. C. Berndt, J. de Jersey, and B. Zerner, *J. Am. Chem. Soc.*, previous communication in this issue.
(3) See footnote 2 in ref 2.
(4) k_{-OH} for ethyl mandelate at 25 °C is ~0.11 M^{-1} s^{-1}, while k_{-OH} for ethyl phenylglyoxylate is 427 M^{-1} s^{-1} at 25 °C, the temperature of the enzymatic experiments.
(5) See footnotes 4 and 10 in ref 2.
(6) C. A. Grob and P. W. Schiess, *Angew. Chem., Int. Ed. Engl.*, **6**, 1 (1967).
(7) In 0.05 M phosphate buffer, pH 7.50, at 25 °C, $k_{cat.}$ = 103 s^{-1} and K_m = 2.4 × 10^{-5} M.
(8) Spectral data obtained with $[E]_0 > [I]_0$: ϵ_{252} for ethyl phenylglyoxylate is 6600; in the presence of an excess of enzyme, ϵ_{252}, extrapolated to zero time, is 1560; the absorbance increases in a first-order reaction to a final ϵ_{252} of 13 100, in good agreement with ϵ_{252} for phenylglyoxylate ion (13 200).
(9) We have previously shown that hydroxyurea is simultaneously an inhibitor and substrate of the metalloenzyme, jack bean urease (EC 3.5.1.5): R. L. Blakeley, J. A. Hinds, H. E. Kunze, E. C. Webb, and B. Zerner, *Biochemistry*, **8**, 1991 (1969); N. E. Dixon, C. Gazzola, J. J. Watters, R. L. Blakeley, and B. Zerner, *J. Am. Chem. Soc.*, **97**, 4130 (1975); N. E. Dixon, C. Gazzola, R. L. Blakeley, and B. Zerner, *ibid.*, **97**, 4131 (1975).
(10) The $k_{cat.}$ (6.2 ± 1.7 s^{-1}) and K_m (9.1 ± 3.0 × 10^{-6} M) for *p*-nitrophenyl phenylglyoxylate at pH 6.25 are also consistent with reversible abortive hemiketal formation.
(11) K. A. Kerr, J. M. Robertson, and G. A. Sim, *Chem. Commun.*, 170 (1967).
(12) M. Green and S. C. Pearson, *J. Chem. Soc. B.*, 593 (1969).
(13) We have found that the kinetics of hydrolysis of I at 25 °C in Tris buffer (0.01–0.05 M, 12.9% v/v in CH_3CN, μ = 0.1 with KCl, pH range 8.0–9.5) follow an equation of the form

$$k_{obsd} = k_{-OH}[^-OH] + (k_{Tris} + k_b[^-OH])[K_a'/(K_a' + H_3O^+)][Tris]$$

where k_{-OH} = 707 M^{-1} s^{-1}, $k_{Tris} = 0.126_4$ M^{-1} s^{-1}, $k_b = 1.01 \times 10^4$ M^{-2} s^{-1}, and the pK_a' of Tris H^+ = 8.55 (kinetically determined).
(14) The maximum solubility of I in this system is ~2.5×10^{-6} M in the absence of enzyme.
(15) Cf. ref 2 and footnote 16 of ref 2.
(16) Cf. the desmolase activity of α-chymotrypsin: D. G. Doherty, *J. Am. Chem. Soc.*, **77**, 4487 (1955); J. Roget and F. Calvet, *Rev. Esp. Fisiol., Suppl. II*, **16**, 215 (1960).

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Hydrogenation of α-Methylstyrene by Hydridopentacarbonylmanganese(I). Evidence for a Free-Radical Mechanism

Sir:

Recent developments in organo transition metal chemistry have accorded an increasingly important and widespread role to free radical mechanisms.[1] It has been suggested, for example, that the hydrogenation of arenes and alkenes, catalyzed by metal carbonyl hydrides, may proceed through mechanisms in which intermediate free radicals are formed by H-atom transfer from the metal hydride to the substrate.[2] We now wish to present convincing evidence for such a mechanism in the case of at least one reaction, the hydrogenation of α-methylstyrene by hydridopentacarbonylmanganese(I) (eq 1). This evidence encompasses one of the only definitive applications to date of the CIDNP technique to the elucidation of the mechanism of a reaction involving a transition metal complex.

$$C_6H_5C(CH_3)=CH_2 + 2HMn(CO)_5 \rightarrow C_6H_5CH(CH_3)_2 + Mn_2(CO)_{10} \quad (1)$$

Reaction 1 was found to proceed quantitatively (confirmed by NMR, UV spectroscopy, and GLC) in benzene solution at rates conveniently measurable in the temperature range 40–75 °C. Kinetic measurements, in which the concentrations of $C_6H_5C(CH)_3=CH_2$, $C_6H_5CH(CH_3)_2$, and $HMn(CO)_5$ all were monitored by NMR, yielded the second-order rate law, eq 2, with values of (2.65 ± 0.12) × 10^{-5}, (9.0 ± 1.0) × 10^{-5}, and (20.0 ± 1.4) × 10^{-5} M^{-1} s^{-1} for k' at 45.0, 56.2, and 64.5 °C, respectively, corresponding to $\Delta H^{\ddagger}$ = 21.4 ± 0.3 kcal mol^{-1} and ΔS^{*} = −12 ± 1 cal mol^{-1} K^{-1}.[3] The kinetic results are consistent with the following mechanism (eq 3-5) for which more direct evidence is presented below and according to which $k' = k_3k'_3/(k_{-3} + k'_3)$.[4]

$$-d[C_6H_5C(CH_3)=CH_2]/dt = k'[C_6H_5C(CH_3)=CH_2][HMn(CO)_5] \quad (2)$$

$$C_6H_5C(CH_3)=CH_2 + HMn(CO)_5 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \overline{C_6H_5\dot{C}(CH_3)_2, \dot{M}n(CO)_5} \xrightarrow{k'_3} C_6H_5\dot{C}(CH_3) + \dot{M}n(CO)_5 \quad (3)$$

$$C_6H_5\dot{C}(CH_3)_2 + HMn(CO)_5 \xrightarrow{fast} C_6H_5CH(CH_3)_2 + \dot{M}n(CO)_5 \quad (4)$$

$$2\dot{M}n(CO)_5 \xrightarrow{fast} Mn_2(CO)_{10} \quad (5)$$

Definitive evidence for the proposed free-radical mechanism is provided by the observation of CIDNP effects when the reaction was followed at ~70 °C in the probe of a 60-MHz NMR spectrometer. This is illustrated by the series of stacked spectra in Figure 1, taken at ~75-s intervals over the 90-min duration of a typical reaction. The proton signals of both reactants, i.e., α-methylstyrene and $HMn(CO)_5$, as well as the product 2-phenylpropane, all exhibit polarization. The polarization of the doublet signal at 1.1 ppm due to the methyl protons of $C_6H_5CH(CH_3)_2$, which is observed in emission during the initial stages of reaction, is particularly striking. Analysis of the signal intensities (see below) also reveals polarization of the signals at 5.3, 5.0, and 2.0 ppm due to the two β and the methyl protons of $C_6H_5C(CH_3)=CH_2$, respectively, as well as the proton signal of $HMn(CO)_5$ (at −7.9 ppm, not shown in Figure 1).

The CIDNP effects can be interpreted in terms of the generally accepted "radical-pair" mechanism[6] involving competition between the back-reaction of the geminate radical pair $\overline{C_6H_5\dot{C}(CH_3)_2, \dot{M}n(CO)_5}$ produced in reaction 3 to re-form the reactants, and the separation (cage escape) of the radicals with ultimate formation of products via reactions 4 and 5. Observations on the corresponding reaction of $\dot{C}o(CN)_5^{3-}$ (which is isoelectronic with $\dot{M}n(CO)_5$) suggest that both reactions (−3 and 4) are very fast as required by the proposed interpretation.[7-9] No species, other than the reactants and products of eq 1 were detected during the course of the reactions. In particular, there was no evidence for the intermediate accumulation of detectable quantities of the cage-combination product, $(C_6H_5)(CH_3)_2CMn(CO)_5$. This compound has not