Finally, a limited configuration interaction ($CI_0$) calculation was carried out on **2** and **2**$^{\ddagger}$ for both MINDO/3 and MNDO methods, which showed only minimal lowering of the calculated heats of formation of these species.[14]

Using the above-described methods, both $H^3$ and $H^6$ in **2** were migrated to the carbene center on the MINDO/3 and MNDO potential surfaces. It was found on the latter surface that migration of $H^6$ led to **2**$^{\ddagger}$ which was the enantiomer of that derived from the migration of $H^3$; i.e., the activation enthalpies were identical ($\Delta H^{\ddagger}$ = 21.9 kcal/mol).[15] A similar situation occurred for **2** on the MINDO/3 surface with the enantiomeric transition states being only 0.7 kcal/mol above that of the starting carbene. This small activation enthalpy is not surprising, considering that the structures of carbene **2** and the transition state **2**$^{\ddagger}$ are very similar (see Table II). Similarly, migration of $H^a$ and $H^e$ in **3** led to almost identical transition states **3a**$^{\ddagger}$ and **3e**$^{\ddagger}$ which were not quite enantiomeric because the tetramethylene chain attached to the migration origin and terminus had differing conformations in the two transition states. As can be seen from Table I, the activation enthalpies for $H^a$ and $H^e$ migration are essentially equal within each method, but MNDO gives much higher $\Delta H^{\ddagger}$s than does MINDO/3.

What has been shown in this investigation is that, when calculations are carried out with no geometrical constraints, the molecules investigated have sufficient flexibility to rearrange with complete stereoelectronic control. That is, the transition state is one which has the migrating hydrogen very nearly aligned with the empty orbital, *regardless of the stereochemical origin of that hydrogen.*[16] Thus it appears unnecessary to consider other factors discussed above to be operative in the experimental system **1**. There still remains the question of why considerable selectivity has been observed with rigid bi- and tricyclic carbenes,[4] which will be the subject of a full paper.

**Acknowledgment** is made to the Robert A. Welch Foundation (F-573) for support of this work. The calculations were carried out using the CDC 6400/6600 computer at The University of Texas Computation Center. The author is indebted to Professor M. J. S. Dewar for helpful discussions and for allowing him free access to the programs used by his research group. With the exception of the force constant program, all programs are available from, or have been submitted to QCPE. Helpful discussions with Professor N. L. Bauld, Dr. H. S. Rzepa, and Mr. S. Kirschner are gratefully acknowledged.

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(15) It is interesting to note that the MNDO method gives $\Delta H_f$ (**2**) − $\Delta H_f$ (**4**) = 73.3 kcal/mol, very similar to that (72.1 kcal/mol) obtained from an ab initio method, IBMOL-IV (double $\zeta$ basis set).[3e,f] In addition, the activation enthalpy to form the transition state **2**$^{\ddagger}$ assumed by Yates group (27 kcal) is quite similar to that calculated by MNDO in this work.
(16) It is important to note that, with **2**, enantiomeric transition states could be arrived at by migrating $H^3$ directly, or rotating the methyl group 60° and migrating $H^6$, although this is not how the calculation was carried out. With **3** however, a 60° rotation would involve a chair → boat transformation, and this clearly did not occur in the reaction path calculation. Thus we have referred to "limited torsion" rather than rotation as the equatorial or equatorial-like hydrogen atom is migrated to the carbene center.

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## Inhibition of Chicken Liver Carboxylesterase (EC 3.1.1.1) by Benzils. Direct Spectrophotometric Evidence for the Reversible Formation of Active-Site Hemiketal Adducts

*Sir:*

In 1927, using a crude preparation of pig liver carboxylesterase (EC 3.1.1.1), Willstätter et al.[1] convincingly demonstrated that an induction period in the enzyme-catalyzed hydrolysis of ethyl (±)-mandelate was caused by the presence of ethyl phenylglyoxylate as an impurity. The result is consistent with the subsequent observation that ethyl phenylglyoxylate has a relatively low $k_{cat.}$ and a very low $K_m$.[1,2] This fact, coupled with the knowledge that carboxylesterases are more efficient in the hydrolysis of simple esters by a factor of $10^5$-$10^6$ than the serine proteinases as exemplified by $\alpha$-chymotrypsin,[3] led us to attempt to obtain direct evidence for the formation of addition compounds between chicken liver carboxylesterase[4] and $\alpha,\beta$-dicarbonyl compounds.[5,6]

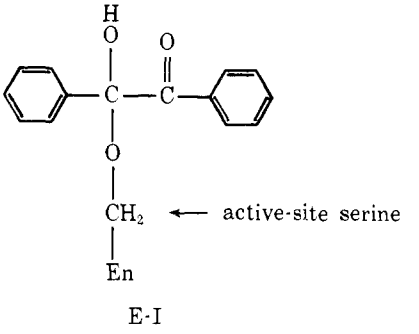
The ability of glyoxal to form addition complexes with urea[7] and of benzil, with hydroxide ion[8] and cyanide ion,[9] has long been known. Benzil (I) is a very powerful inhibitor of chicken liver carboxylesterase.[10] In 0.05 M phosphate buffer, pH 7.5, benzil has the characteristics of a classicial competitive inhibitor with $K_i = 1.0 \times 10^{-8}$ M against *p*-nitrophenyl acetate as substrate. This value is several orders of magnitude lower than that expected on the basis of a noncovalent (hydrophobic) interaction.[11]

The hemiketal (E-I) was postulated to account for the magnitude of $K_i$, and its existence is here established by the following experimental results. (1) Difference spectra of E-I obtained under conditions where [I] > [E] agreed quantitatively with spectra calculated for mixtures of I and E-I, based

**Table I.** Spectral Data on Benzils and Related Compounds[a]

| R | R′ | RC(=O)C(=O)R′ $\lambda_{max}$ (nm) | $\epsilon$ | RC(=O)CH(OH)R′ $\lambda_{max}$ (nm) | $\epsilon$ | RCHO $\lambda_{max}$ (nm) | $\epsilon$ | Enzyme-benzil compd $\lambda_{max}$ (nm) | $\Delta\epsilon$ | $10^8 K_i$[b] (M) |
|---|---|---|---|---|---|---|---|---|---|---|
| Phenyl | Phenyl | 264 | 21 280 | 251 | 11 920 | | | 252 | 11 940 | 1.0 |
| *p*-Methoxyphenyl | Phenyl | 300<br>264 | 19 110<br>14 530 | 286 | 15 370 | 285 | 16 020 | 286 | 13 500 | 1.4 |
| *p*-Dimethylaminophenyl | Phenyl | 378<br>258 | 27 730<br>15 120 | 358<br><240 | 26 750<br>6 060[c] | 353<br>246 | 29 540<br>6 030 | 346<br>244 | 23 430<br>12 000 | 116 |
| Phenyl | *p*-Nitrophenyl | 272 | 18 790 | 258[d] | 17 850[d] | 268[e,f] | 13 670[e,f] | 256 | 17 030 | 0.33 |
| Methyl | Phenyl | 258[f] | 6 640[f] | | | | | <240 | 710[c] | 75 |

[a] Spectra were determined at 25 °C in 0.05 M Tris-HCl buffer, 0.15 M in KCl, pH 7.50, 0.66% v/v $CH_3CN$. [b] $K_i$ values at 25 °C in 0.05 M phosphate buffer, pH 7.50, 1.3%, v/v $CH_3CN$, determined with *p*-nitrophenyl acetate as substrate. [c] $\epsilon$ at 240 nm. [d] *O*-Acetyl derivative. [e] R′CHO (*p*-nitrobenzaldehyde). [f] 0.05 M phosphate buffer, pH 7.50.




E-I

on a $K_i$ of $1.0 \times 10^{-8}$ M, providing evidence that the spectral changes are due solely to reaction at the active site. (2) E-I should and does have a difference spectrum very similar to that of benzoin (Figure 1). (3) Diethylphosphoryl-enzyme, E* ($1.31 \times 10^{-5}$ M, produced by the passage of paraoxon-inhibited enzyme through Sephadex G-25), under conditions where [E*] > [I], has a very small effect on the spectrum of benzil, and this effect is quantitatively accounted for by the small percentage of active dephosphorylated enzyme.[10,12] (4) The $\beta$-diketone dibenzoylmethane has a $K_i$ of $2.6 \times 10^{-5}$ M, in the range expected for noncovalent bonding. Further, the spectrum of dibenzoylmethane is unchanged on interaction with the enzyme. (5) The inhibition by benzil is completely reversible. Enzyme maintained at 25 °C for 60 h with excess benzil at pH 7.5 recovered full activity upon dialysis. Further, when paraoxon was added to enzyme inhibited by benzil, a stoichiometric burst of *p*-nitrophenol was observed, with the displacement of benzil from the active site. In addition to the spectral evidence contained in Figure 1, these results argue strongly against the possibility that reaction is occurring at an arginine residue.[13] Further, 2,3-butanedione ($1.6 \times 10^{-4}$ M) produces no observable inhibition in 3 h;[13] periodate is without effect on the reversibility of benzil inhibition;[13] and inhibition by phenylglyoxal at pH 7.5 ($K_i = 5.5 \times 10^{-6}$ M) is completely reversible.[14] (6) The $K_i$–pH profile for benzil inhibition shows a *clean* dependence on a group with $pK_a' = 5.4$,[15] a value which may be compared with the $pK_a'$ of 5.0 for the group (active in the free base form) which is involved in the enzyme-catalyzed hydrolysis of phenyl butyrate.[16] (7) The data summarized in Table I attest to the generality of the above results.[17] The spectra of the enzyme-inhibitor compounds resemble very closely the spectra of appropriate models for hemiketal structures. Spectral changes of *p*-dimethylaminobenzil on interaction with the enzyme appear less clear cut than the others, but are none the less totally consistent with a hemiketal structure. In this case, the differences observed between the enzyme-inhibitor compound and the models (shift to shorter wavelengths) are consistent with an increase in the angle between the planes of the phenyl rings on reaction with the enzyme.[18]

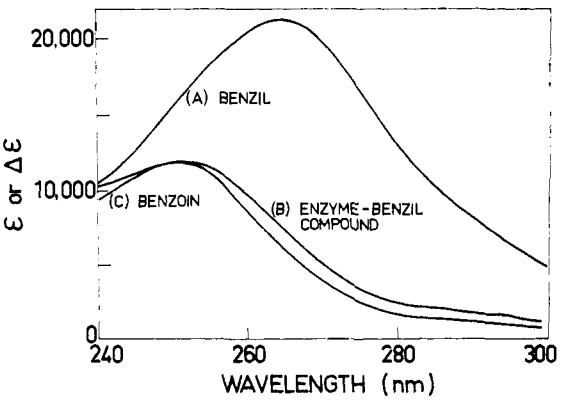



**Figure 1.** Absorption spectra at 25 °C in 0.05 M Tris-HCl buffer, 0.15 M in KCl, pH 7.5, [acetonitrile] = 2.4% v/v: (A) benzil, $1.018 \times 10^{-5}$ M; (B) [E] > [I], [enzyme] = $1.325 \times 10^{-5}$ M, [benzil] = $1.018 \times 10^{-5}$ M, vs. [enzyme] = $1.325 \times 10^{-5}$ M, ~99.7% of benzil is bound at the active site (from $K_i = 1.0 \times 10^{-8}$ M); (C) benzoin, $1.069 \times 10^{-4}$ M, in 0.05 M phosphate buffer, pH 7.5, [acetonitrile] = 0.83% v/v. Duplicate spectra agreed within 1% at $\lambda_{max}$.

Thus the present work provides the first spectrophotometric demonstration of the reversible formation of a tetrahedral adduct at the active site of a serine hydrolase.[19,20] Moreover, the similarities[3] between the carboxylesterases and acetylcholinesterase (EC 3.1.1.7)[21] suggest that suitably substituted 1-phenyl-1,2-propanediones may be very powerful inhibitors of this enzyme and therefore potentially toxic molecules. These and related systems are under active investigation in this laboratory.

In the following communication,[22] we adduce the first secure evidence for the reversible formation of an abortive covalent enzyme-substrate compound, and also demonstrate that the carboxylesterases are powerful catalysts of Beckmann fragmentation reactions.

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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]

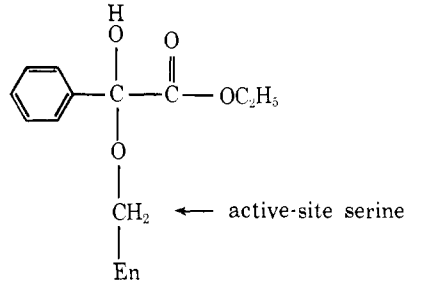
These results are consistent with the scheme

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

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

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

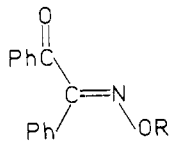
for first-order kinetics where $k_{cat.(obsd)} = k_{cat.}k_{-2}/(k_2 + k_{cat.})$, when [ES] is at steady state. E-I is the active site hemiketal adduct, and the measured $K_i$ for the inhibition of *p*-nitrophenyl acetate hydrolysis is given by $K_1K_2/(1 + K_2)$.




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




1, 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 $[S]_0$, $2 \times 10^{-7}$–$4 \times 10^{-6}$ M, showing that $K_m \gg 4 \times 10^{-6}$ M. The observed first-order rate constant is proportional to enzyme concentration ($[E]_0 = 3.26$–$8.01 \times 10^{-8}$ 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 $K_m$