induced isomerization of piperylene gives a photostationary state that contains 54–55% *trans*-piperylene, and ferrocene-sensitized dimerization of isoprene produces a mixture of dimers that contains 92% cyclobutanes and cyclooctadienes; the remaining dimers are cyclohexenes.

The evidence for the existence of complexes between ferrocene and olefin is twofold. The ultraviolet absorption spectra of mixtures of piperylene and ferrocene in *n*-heptane and *n*-hexane show new absorption between 2200 and 3000 Å. At higher wave lengths no deviation from Beer's law is observed. In the n.m.r. spectrum of *trans*-piperylene, a new methyl doublet appears on the addition of ferrocene. The new methyl doublet is at τ 8.03 ± 0.02, J = 13 ± 1 c.p.s., while the methyl doublet of *trans*-piperylene is at τ 8.25 ± 0.02, J = 13 ± 1 c.p.s.

Ferrocene quenches anthracene triplets ($T_1 \leftarrow S_0$ energy = 43 kcal./mole[9]) at a diffusion-controlled rate[10] which suggests that the lowest triplet state of ferrocene itself does not possess sufficient energy to effect the observed reactions.

Nor do we feel that the ferrocene–olefin complex functions as a sensitizer which excites an uncomplexed olefin molecule to its triplet state. The observation that solutions of ferrocene and olefin obey Beer's law above 3000 Å. implies that the $S_1 \leftarrow S_0$ transitions of ferrocene and complex are isoenergetic in this region. Molecular orbital calculations for ferrocene by Moffitt[11] and Dunitz and Orgel[12] suggest that the $S_1 \leftarrow S_0$ transition of ferrocene requires 45–55 kcal./mole. Experimentally, the longest wave length band of ferrocene is a broad band centered at 4500 Å. (64 kcal./mole).[13] Since the $S_1 \leftarrow S_0$ transitions of ferrocene and complex are probably isoenergetic (involving metal electrons that are not perturbed by complexation), neither the lowest singlet nor its associated triplet, lying at a yet lower energy level, should have sufficient energy to catalyze the observed reactions. It seems unlikely that a higher triplet state of the ferrocene–olefin complex acts as a sensitizer for another olefin molecule since a higher triplet would decay[14] before encountering a free piperylene molecule.

Another possibility is that ferrocene acts as a sensitizer because the heavy iron atom facilitates intersystem crossing of a neighboring piperylene. We have eliminated this possibility because the excitation of *trans*-piperylene to its triplet state requires only 58.8 kcal./mole (4860 Å.)[15] whereas light of much higher energy (*e.g.*, 2800–3300 Å.) is required for reaction.

In view of these considerations, we propose the following mechanism for reactions of dienes sensitized by ferrocene

$$\text{ferrocene} + \text{diene} \rightleftharpoons \text{complex} \quad (1)$$

$$\text{complex} + h\nu \longrightarrow \text{complex}^{S_n} \quad (2)$$

$$\text{complex}^{S_n} \longrightarrow \text{ferrocene}^{T_n} + \text{diene}^{T_n} \quad (3)$$

(9) G. Porter and M. W. Windsor, *Proc. Roy. Soc.* (London), **A245**, 238 (1958).
(10) A. Fry and G. S. Hammond, private communication.
(11) W. Moffitt, *J. Am. Chem. Soc.*, 76, 3386 (1954).
(12) J. D. Dunitz and L. E. Orgel, *J. Chem. Phys.*, **23**, 954 (1955).
(13) J. C. D. Brand and W. Snedden, *Trans. Faraday Soc.*, **53**, 894 (1957).
(14) M. Kasha, *Discussions Faraday Soc.*, **9**, 14 (1950).
(15) G. S. Hammond, J. Saltiel, A. A. Lamola, N. J. Turro, J. S. Bradshaw, D. O. Cowan, R. C. Counsell, V. Voght, and C. Dalton, *J. Am. Chem. Soc.*, **86**, 3197 (1964).

$$\text{diene}^{T_n} \longrightarrow \text{diene}^{T_1} \quad (4)$$

$$\text{diene}^{T_1} \longrightarrow \text{products} \quad (5)$$

Reaction 2 is included for the reasons discussed previously. Reaction 3 is proposed to give both ferrocene and the diene in triplet states to conserve multiplicity and because it is the reverse of the observed process of triplet–triplet annihilation.[16,17] Whether these triplets are in higher excited states is a completely open question; reaction 4 may be, therefore, unnecessary. Reaction 5 represents the known reactions of triplet dienes which lead either to isomerization or dimerization, depending on the circumstances.

Acknowledgments. We wish to acknowledge the financial assistance of the National Science Foundation and the Paint Research Institute.

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Spectroscopic and Kinetic Observation of a Pepsin–Inhibitor Complex

Sir:

We wish to report on the interaction of pepsin (E) with N-(α-acetaminocinnamoyl)-L-diiodotyrosine (N-acetyldehydrophenylalanyl-L-diiodotyrosine) (I). Compound I, a close analog of the excellent pepsin substrate[1] N-acetyl-L-phenylalanyl-L-diiodotyrosine, was prepared from L-diiodotyrosine and the azlactone of α-acetaminocinnamic acid,[2] m.p. 193–195° dec., $[\alpha]^{29}D$ −38° (*c* 3, 5% ammonia). *Anal.* Calcd. for $C_{20}H_{18}N_2O_5I_2$: C, 38.73; H, 2.93; I, 40.92; mol. wt., 620. Found: C, 38.94; H, 3.31; I, 40.38; mol. wt., 585 (osmometric, in ethanol). The ultraviolet absorption spectrum of I at pH 2, in 3% methanol, showed (mμ) λ_{max} 282 (ϵ 20,550), λ_{min} 252 (ϵ 10,810), λ 315 (ϵ 2770 ± 25).

CH₃CONH—C(=CH—C₆H₅)—CONH—CH(CH₂—C₆H₂X₂OH)—COOH

I, X = iodine
II, X = H

The apparent ϵ of I in pepsin-containing solutions is greater than 2770 at 315 mμ.[3,4] Forty-three determinations of the optical density ($O.D._0$) of such solutions have been made, with $[E]_0$ = 2.42–50.8 × 10^{-5} *M* and $[I]_0$ = 1.01–19.4 × 10^{-5} *M*. The experimental data

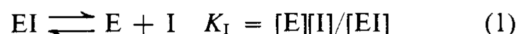
(1) L. E. Baker, *J. Biol. Chem.*, **193**, 809 (1951).
(2) The procedure was essentially that of M. Bergmann, F. Stern, and C. Witte, *Ann.*, **449**, 277 (1926), for the synthesis of II.
(3) (a) All subsequent data refer to pH 2, 35°, 315 mμ (chosen for experimental convenience). (b) In some runs a very slow change of O.D. with time occurred. The O.D. at t = 0 was used in the calculations.
(4) The environment of the cinnamoyl chromophore in cinnamoyl-α-chymotrypsin and -trypsin is such as to produce an enhanced ϵ_{315}, if simple cinnamoyl analogs in dilute aqueous solution are used as reference compounds. See M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962), and M. L. Bender and E. T. Kaiser, *ibid.*, **84**, 2556 (1962).

are not in accord with rapid hydrolysis of I to α-acetaminocinnamic acid and L-diiodotyrosine but can be explained by postulating rapid reversible formation of a 1:1 complex of I and pepsin (eq. 1). For such a scheme,[5] $O.D._0$ is defined by eq. 3, and the concentration of the EI complex, [EI], by eq. 4. Equations 3 and 4 permit calculation of $\Delta\epsilon$ for each experimental point as a function of assumed K_I values; the true K_I should provide an identical $\Delta\epsilon$ for all points. This procedure relies on the difference between two quantities of similar magnitude ($O.D._0$ and $[I]_0\epsilon_I$ of eq. 3).[6] Table I presents the results of four such calculations

Table I. Spectrophotometric Determination of the Dissociation Constant of the Complex between Pepsin and N-(α-Acetaminocinnamoyl)-L-diiodotyrosine

	No. of points	$K_I \times 10^5$, M: 30	8	3	0.3
$\Delta\epsilon$	43	1444	719	534	410
Standard deviation, %		±37	±18	±14	±24
$\Delta\epsilon$	13	1012	640	538	474
Standard deviation, %		±15	±5.2	±5.8	±12

for all 43 points and for the 13 points in which ($O.D._0 - [I]_0\epsilon_I$) was greatest.[7] A value for K_I in the vicinity of 3–8 $\times 10^{-5}$ M best fits the data but it appears unlikely that the present system can define K_I more closely.[8]



$$EI \rightleftharpoons E + I \quad K_I = [E][I]/[EI] \tag{1}$$

$$[E]_0 = [E] + [EI] \quad [I]_0 = [I] + [EI] \tag{2}$$

$$O.D._0 = [I]_0\epsilon_I + [EI](\epsilon_{EI} - \epsilon_I - \epsilon_E) \tag{3}$$

$$[EI] = ([I]_0 + [E]_0 + K_I - \sqrt{([I]_0 + [E]_0 + K_I)^2 - 4[E]_0[I]_0})/2 \tag{4}$$

$$\Delta\epsilon = \epsilon_{EI} - \epsilon_I - \epsilon_E \tag{5}$$

Solutions of N-(α-acetaminocinnamoyl)-L-tyrosine (II) in the presence of pepsin exhibit the same effects as solutions of I, but on a reduced scale.[9] The assumption that $\Delta\epsilon$ is the same[10] for II as for I permits direct calculation of K_{II} from the experimental data and eq. 3 and 4. For $\Delta\epsilon = 540$, $K_{II} = 2.7 \pm 0.5 \times 10^{-3}$ M; for $\Delta\epsilon = 640$, $K_{II} = 3.3 \pm 0.6 \times 10^{-3}$ M. The value for K_{II} is not highly sensitive to the assumed $\Delta\epsilon$ and is remarkably similar to that for the Michaelis constant ($\sim 2 \times 10^{-3}$ M) of the pepsin substrate, N-acetyl-L-phenylalanyl-L-tyrosine (AcPheTyr).[11–13]

(5) (a) In these experiments, 3.0 ml. of enzyme solution was placed in a cuvette and allowed to attain thermal equilibrium, the optical density was set at zero, 100 μl. of a methanolic solution of I added, and the $O.D._0$ recorded. (b) ϵ_{315} for pepsin is $\sim$180.

(6) The largest value for ($O.D._0 - [I]_0\epsilon_I$) was $\sim$0.1, with $O.D._0 = \sim$0.6.

(7) Calculated and observed values of $O.D._0$ were in excellent agreement for all 43 points with $K_I = 8 \times 10^{-5}$ M, $\Delta\epsilon = 640$, or $K_I = 3 \times 10^{-5}$ M, $\Delta\epsilon = 538$.

(8) (a) K. Conrow, G. D. Johnson, and R. E. Bowen, *J. Am. Chem. Soc.*, **86**, 1025 (1964), have discussed difficulties in the calculation of association constants from spectrophotometric data. (b) K_I is not small enough to make the method useful as a titration of pepsin.

(9) (a) II had m.p. 216–219°, $[\alpha]^{27}D$ +41.2° (c 3, pyridine), λ_{max} 282 (ϵ 20,650), λ_{min} 241 (ϵ 6815) (spectrum in 3% methanol, pH 2). Reference 2 gives m.p. 217–218°, $[\alpha]^{20}D$ +47.1° for II. (b) Baker (ref. 1) has shown that N-acetyl-L-phenylalanyl-L-diiodotyrosine is a much better substrate than N-acetyl-L-phenylalanyl-L-tyrosine for pepsin.

(10) The ultraviolet absorption of I at 315 mμ must arise almost exclusively from the α-acetaminocinnamoyl chromophore, since ϵ_{315} is 2630 for II and 3000 for α-acetaminocinnamic acid.

(11) L. E. Baker, *J. Biol. Chem.*, **211**, 701 (1954).

The general validity of the above interpretation of the spectroscopic studies is independently substantiated by kinetic evaluation of K_I from reactions in which I inhibits the pepsin-catalyzed hydrolysis of Ac-PheTyr or its carbobenzoxy analog, Z-PheTyr (Table II).[14] The apparent *noncompetitive* nature of the inhibition is very interesting, if substantiated by later work.

Table II. Kinetic Determination of the Dissociation Constant of the Complex between Pepsin and N-(α-Acetaminocinnamoyl)-L-diiodotyrosine[a]

Substrate	$[I] \times 10^5$, M	$K_0 \times 10^3$, M[b]	$k_0 \times 10^2$, sec.$^{-1}$[b]	$K_I \times 10^5$, M[c]
Z-PheTyr[d]	0[e]	0.214 ± 0.034[i]	1.24 ± 0.08[i]	
	3.86[f]	0.206 ± 0.040[j]	0.83 ± 0.05[j]	8.7 ± 2.5
Ac-PheTyr[d]	0[g]	1.95 ± 0.18[i]	4.66 ± 0.44[i]	
	3.86[h]	1.91 ± 0.06[j]	3.19 ± 0.11[j]	8.9 ± 0.7
	5.8[h]	1.28 ± 0.18[j]	1.96 ± 0.18[j]	10.3 ± 1.2

[a] All runs at 35°, pH 2, 3.4% methanol, $[E]_0 = 1.20–1.58 \times 10^{-5}$ M. [b] Evaluated from the equation $v = k_0[E][S]/(K_0 + [S])$. [c] Evaluated from the equation $K_I = [I]/(X/Y - 1)$, where X and Y are the slopes of the Lineweaver–Burk plots for the inhibited and uninhibited reactions, respectively, corrected for any difference in [E]. [d] All uncertainties are standard deviations. [e] $[S]_0 = 0.73–3.29 \times 10^{-4}$ M. [f] $[S]_0 = 1.02–3.05 \times 10^{-4}$ M. [g] $[S]_0 = 1.16–13.6 \times 10^{-4}$ M. [h] $[S]_0 = 2.4–9.7 \times 10^{-4}$ M. [i] Average of six independent Lineweaver–Burk plots of 7–12 points. [j] Average of three independent Lineweaver–Burk plots of 8–9 points.

We conclude that it is possible to observe spectroscopically a 1:1 complex of pepsin with N-(α-acetaminocinnamoyl)-L-diiodotyrosine, formation of which results in a diminution of the catalytic activity of the enzyme.[15] Further studies should afford valuable information on the interaction of simple dipeptides with pepsin and may provide a means of estimating the number of active sites per pepsin molecule.

Acknowledgment. This investigation was supported by Grant AM 08005-01 from the U. S. Public Health Service. Carol D. Silver carried out the computer programming. Professors Myron L. Bender and Allen Kropf provided helpful criticisms.

(12) M. S. Silver, J. L. Denburg, and J. J. Steffens, *J. Am. Chem. Soc.*, **87**, 886 (1965); see Table II.

(13) Likewise, K_I is nearly equal to K_m (7.5 $\times 10^{-5}$ M) for N-acetyl-L-phenylalanyl-L-diiodotyrosine and to K_i (8.0 $\times 10^{-5}$ M) for N-acetyl-D-phenylalanyl-L-diiodotyrosine, all at pH 2.0. The values for K_m and K_i were kindly provided to us by Dr. William T. Jackson of the University of Texas Medical Center, Houston.

(14) We suspect that the spectrophotometric method[12] employed in determining the kinetics may be breaking down in the experiments at $[I] = 5.8 \times 10^{-5}$ M, but we have not yet succeeded in pinpointing the difficulty.

(15) The present experiments indicate nothing about the nature of the binding in EI.

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Silver and Palladium Complexes of 5,6-Benzocinnoline and 3,8-Dimethyl-5,6-diaza-1,10-phenanthroline

Sir:

Evidence for the participation of the azo group in metal chelate interaction has been shown by several workers[1] for aromatic azo compounds. Substituent