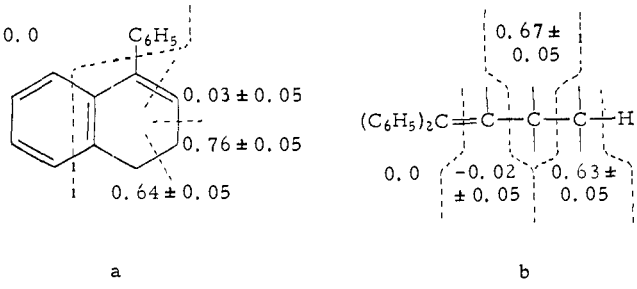

Figure 1. Distribution of the deuterium label as inferred by nmr spectroscopy in products from the decomposition of deuterium-labeled *t*-butyl (γ,γ-diphenylallyl)peracetate in (a) cyclohexane at 125°, and (b) 1.34 *M* triethyltin hydride in *n*-octane at 125°.

actually VII or a tetrahydronaphthalene believed to correspond to an as yet uncharacterized reaction product.

A detailed product analysis to be reported later indicates that relative yields of diphenylcyclopropylmethane and diphenylbutene formed from either perester in 1,4-cyclohexadiene as a function of initial perester and cyclohexadiene concentrations are compatible with VI being formed mainly through donation of hydrogen to II by cyclohexadienyl radicals while V arises similarly from I and 1,4-cyclohexadiene. In the solvents which are poor hydrogen donors, VI can be formed by hydrogen abstraction from VIII.[9]

For the decomposition of III in triethyltin hydride–*n*-octane mixtures, the ratio VI:V is independent of the tin hydride concentration. This observation indicates that the rearrangement of I to II is fast with respect to hydrogen abstraction by I from the hydride, or that there is a single "nonclassical" radical of intermediate structure which gives rise to both hydrocarbons. Thus, the degree of equivalence attained by the methylene groups in I before conversion to product is of special interest. To determine this, perester III was prepared with 1.40 g-atoms of deuterium in the α position. Following decomposition in cyclohexane at 125°, the distribution of the deuterium label in VII, the major reaction product, was determined by nmr spectroscopy. The results are summarized in Figure 1a.

It is clear that the rearrangement of I to II must be fast with respect to that of I to VIII. Here, the time during which the rearrangement may take place is limited only by the relatively slow rate at which the *ortho* ring cyclization occurs. Decomposition of III in the presence of 1.34 *M* triethyltin hydride made it possible to reduce this time by approximately a factor of 23, according to the value of k_a/k_r estimated for the tin hydride. Nonetheless, nmr analysis of the 1,1-diphenyl-1-butene (V) formed showed equilibration of the methylene groups to have occurred even in the presence of this active hydrogen donor (Figure 1b). Thus, the half-time for the isomerization of I to II must be short compared to that for the reaction of the former with the tin hydride.

At present there is no reason to postulate the existence of a "nonclassical" radical species to account for the experimental results and, on the whole, the radical system behaves more like the analogous carbanion system[3] than like similar carbonium ion systems.[4] Possible answers to the intriguing question[10] as to the magnitude of the equilibrium constant between I and II will be considered in detail later.

(9) The major difference in the amount of VI formed in the decomposition of the two peresters in cyclohexadiene (see Table I) can be understood essentially as follows. The half-life for decomposition of ring-opened perester, III, at 131° is about the same as that for IV at 35°. The steady-state cyclohexadienyl radical concentration goes roughly as the square root of the decomposition rate. Therefore, the cyclohexadienyl radical concentration will be about the same in the two cases. However, the steady-state concentration of the ring-closed radical, II, will be quite different. If, as we believe, II is energetically more stable than I, the ratio I:II will be greater at the higher temperature. The rates of hydrogen abstraction to give V and of conversion to ring-cyclized radical VIII will then be much faster at 131° due both to the temperature effect on k_a and on k_r and to the greater relative amount of I. As a result, the steady-state concentration of II will be much smaller at 131° than at 35°, and the amount of VI formed will be correspondingly less.

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pH-Dependent Proton Absorption in Chymotrypsin Binding. Evidence for a pH-Dependent Conformation Change of the Enzyme[1]

Sir:

The second-order rate constant (k_{cat}/K_m(app)) of chymotrypsin-catalyzed reactions has long been known to decrease above pH 8, with an apparent dependence on a single ionizable group of p$K \sim 9$.[2] Recently this phenomenon has been identified as a pH-dependent binding by chymotrypsin, even for neutral substrates and inhibitors.[3-5] A pH-dependent binding of a neutral molecule implies that a pH-dependent proton change of the enzyme occurs on binding. In fact, a pH-dependent absorption of one proton per mole of enzyme has been observed upon acylation of chymotrypsin.[7] Is this proton phenomenon at high pH associated with the noncovalent binding of substrate to enzyme (K_s) or with the subsequent covalent acylation step (k_2)? Recent results with competitive inhibitors of chymotrypsin[4] and with derivatized chymotrypsins[8] favor the former possibility.

The binding of the competitive inhibitor, benzyl alcohol, to α-chymotrypsin was first investigated. This substance is particularly advantageous since it is endowed with both high solubility and partial resemblance to a natural chymotrypsin substrate; solutions with $[I]_0/K_i = 20$ can easily be prepared, leading to essentially complete saturation of the enzyme by the inhibitor even at high pH. The results of a series of experiments determining proton uptake by the enzyme upon binding of excess benzyl alcohol are shown in

(1) This research was supported by grants from the National Institutes of Health.
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(3) A. Himoe and G. P. Hess, *Biochem. Biophys. Res. Commun.*, 23, 234 (1966).
(4) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Natl. Acad. Sci. U. S.*, 56, 833 (1966).
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Figure 1. At pH 7, no protons are absorbed by the enzyme on binding while at pH's near 10 almost one equivalent of proton per mole of enzyme is absorbed. The pH dependence of the proton uptake follows a theoretical curve (solid line) based on a single pK of 8.8, very similar to the pK controlling the pH-dependent binding of the enzyme in this region.[4] Thus, the shapes of the $1/K_i$ (or $1/K_s$) *vs.* pH curves and the proton uptake *vs.* pH curves are essentially identical with one another. Likewise, plots of proton absorption *vs.* inhibitor concentration show saturation curves similar to normal (V *vs.* [S]) saturation binding curves. The same results are obtained with acetonitrile, a nonspecific inhibitor, and N-acetyl-D-tryptophanamide, a specific inhibitor.

At pH 8.8 the addition of benzyl alcohol ([BzOH] $\gg K_i$) caused 0.5 proton per mole of enzyme to be absorbed, and the further addition of acetonitrile, also in saturating concentration, caused no further changes in the state of protonation of the enzyme. Thus the effect of added inhibitor, especially acetonitrile, is due to a specific enzyme–small molecule interaction and not to altered solution properties.

These results suggest that the proton uptake seen in the over-all acylation (k_2/K_s) actually occurs in the binding step (K_s) preceding acylation (k_2). In the acylation step *per se* no further proton uptake can occur, since it is all accounted for in the binding.

These results further indicate that a set of coupled equilibria involving binding and protonation of the enzyme must occur. A set of coupled equilibria was long ago used to explain the pH-dependent binding and proton release on binding of oxygen to hemoglobin, the so-called Bohr effect.[9,10] The simplest expression of this coupling in the chymotrypsin system is

$$E + H^+ \overset{K_a}{\rightleftharpoons} EH^+ \overset{I}{\underset{K_i}{\rightleftharpoons}} EHI^+ \qquad (1)$$

implying that EH^+, but not E, can bind inhibitor (or substrate) and that EH^+, but not EHI^+ (EHS^+), can lose a proton. This set of equations, related to the hemoglobin equations,[9] successfully describes the sigmoid curve of Figure 1 and the corresponding sigmoid curve of $1/K_i$ *vs.* pH. However, eq 1 suffers from two defects: (1) it gives no mechanistic explanation for the binding of a *neutral* inhibitor to a *protonated* enzyme, EH^+, but not to a *neutral* enzyme, E; and (2) it does not account for the change in state of the enzyme seen polarimetrically around pH 9.[11] In order to meet these requirements, eq 1 has been expanded to eq 2.

$$\begin{array}{ccccc} E^*H^+ & \overset{K_{TH}}{\rightleftharpoons} & EH^+ & \overset{I}{\underset{K_i}{\rightleftharpoons}} & EHI^+ \\ {\scriptstyle K_a^*}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_a} & & \downarrow\uparrow{\scriptstyle K_a} \\ E^* & \overset{K_T}{\rightleftharpoons} & E & \overset{I}{\underset{K_i}{\rightleftharpoons}} & EI \end{array} \qquad (2)$$

This scheme, which postulates two states of the enzyme, rationalizes the pH dependence of both binding and proton uptake. Provided that K_{TH} (=[EH]/[E*H]) $\geqq$ 100 and that $[H^+] \gg K_a \leqq 10^{-11}$,[12,13] sigmoidal pH dependence of both processes is predicted. Thus E is the major form of the enzyme when protonated, and E* is the major form when unprotonated.

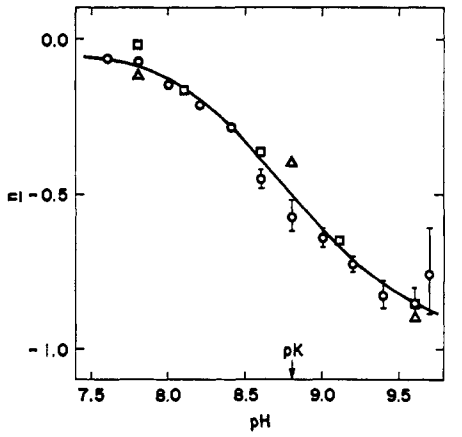

Figure 1. The pH dependence of proton absorption by α-chymotrypsin upon binding of inhibitors: O, benzyl alcohol (pH 7.9: K_i = 10 mM, competitive inhibition; 10 mM, proton absorption; pH 9.0: K_i = 17 mM, competitive inhibition; 21 mM, proton absorption; pH 9.7: K_i = 50 mM, competitive inhibition; 51 mM, proton absorption); Δ, acetonitrile (K_i = 830 mM, pH 7.9); □, N-acetyl-D-tryptophanamide (K_i = 2.3 mM, pH 7.9). $[E]_0$ = 0.86×10^{-4} M; $[I]_0$ always $5 \times > K_{i,obsd}$ at each pH; 25.0°, 0.1 M KCl. Observations of proton absorption by α-chymotrypsin were made using a recording pH meter: ordinary glass and calomel electrodes in a Faraday cage with a Radiometer TTTIc pH-Stat regulator unit used to drive a Sargent SR recorder, for which full scale could be varied from 1.0 to 0.01 pH unit. Inhibitor was added with a Gilson micrometer buret *via* a Teflon needle. Chymotrypsinogen was used as a "blank" reaction to correct for small (0–25%) pH changes due only to addition of inhibitor. The value of the pH change on addition of inhibitor to α-chymotrypsin was compared to that produced artificially by addition of 1 equiv of hydroxide ion/mole of enzyme to obtain the fraction of one proton absorbed by the enzyme per mole at the given pH. ΔpH values from 0.05 to 0.16 were observed.

The symbol n is defined as the number of protons per mole of enzyme which are released as the result of forming the enzyme–inhibitor complex (*cf.* ref 7 for a similar definition). Using the scheme of eq 2, one may then derive eq 3 and 4. Equation 4 predicts that a plot

$$\frac{1}{K_{i,\,obsd}} = \frac{(1/K_i)}{\left(1 + \dfrac{K_a}{K_T[H^+]}\right)} \qquad (3)$$

$$n = \frac{1}{\left(1 + \dfrac{K_a}{K_T[H^+]}\right)} - \frac{\left(1 + \dfrac{[I]}{K_i}\right)}{\left(1 + \dfrac{K_a}{K_T[H^+]} + \dfrac{[I]}{K_i}\right)} \qquad (4)$$

of n *vs.* [I] at a given pH will produce the usual hyperbolic saturation curve (as seen for V *vs.* [S]), leading to the same $K_{i,\,obsd}$ as that obtained by competitive inhibition experiments. Indeed this is observed and proven in experiments carried out at pH 7.8, 8.8, and 9.6.

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(10) R. A. Alberty, *J. Am. Chem. Soc.*, 77, 4522 (1955).
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(12) The assumption that $K_{TH} \geqq 100$ is borne out by the data on the relaxation of the enzyme alone.[11]
(13) Equation 2 represents two sides of a cubic array. The other corners of the cube, E^*HI^+ and E*I, have been omitted, implying that the dissociation constants of these species are infinite. Havsteen[11] has produced kinetic evidence for the top of the cube (E^*H^+, EH^+, EHI^+, and E^*HI^+) by observing at high pH one relaxation of the enzyme itself, and two relaxations of the enzyme in the presence of the inhibitor proflavin. We find that proflavin is a special case: its binding is decreased only twofold at pH 10 where the binding of many other inhibitors is decreased *ca.* tenfold, implying that proflavin can bind not only at the active site where the change of state occurs, but possibly at some second site also.

The change in state between E and E* in the native enzyme may be described as a change in hydration or, more probably, a conformational change of the native enzyme. Although the binding and proton absorption data require that E be the major form when protonated and E* be the major form when unprotonated, the introduction of inhibitor (or substrate) into the system transforms all of the enzyme to EHI^+ (or EHS^+), which explains the pH-independent catalytic steps (k_2 and k_3) in the high pH region. The conformation change thus is related to the binding of small molecules at the enzyme active site but not to the catalytic process *per se*. A pH-dependent intramolecular competitive inhibition of the active site will explain the data on the transformation given both here and elsewhere.[4] Although this (conformational) change drastically affects the activity of chymotrypsin through an inhibition of binding, it does so only in a negative way.

(14) National Institutes of Health Predoctoral Fellow.
(15) The authors thank Professor F. J. Kézdy for assistance with the mathematical formulation.

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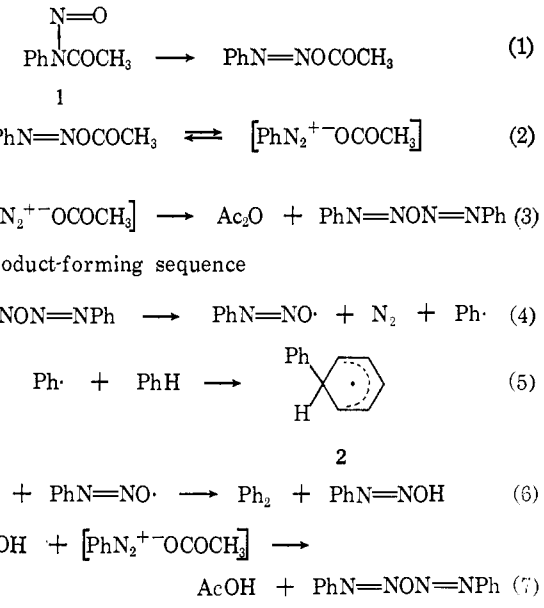
On the Mechanism of Aromatic Arylation with Nitrosoacetanilide

Sir:

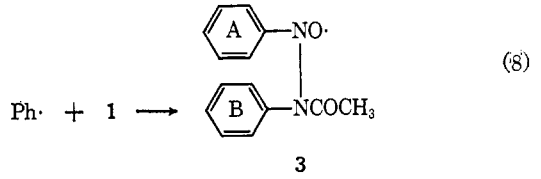
Nitrosoacetanilide (**1**) has long been known as a source of phenyl radicals[1] and has found particular application in studies of homolytic phenylation of aromatic compounds.[2,3] It has been established that the rate-determining step in these reactions is the first-order isomerization (eq 1) leading to benzenediazo acetate.[4] However, the detail of subsequent processes has been less clear. Two major difficulties challenged early mechanistic ideas. First, early schemes show the high yields of acetic acid arising by way of the acetyloxy radical, in spite of the known instability of this intermediate.[5] Second, the initial adduct of phenyl radicals to benzene (*i.e.*, the phenylcyclohexadienyl radical, **2**) is cleanly oxidized to biphenyl, while in other systems disproportionation and dimerization lead, in addition, to hydroaromatic products.[6]

The difficulties referred to have led to suggestions of concerted[4,7] or cage[8] processes. However, a more satisfactory mechanistic interpretation, which has received general acclaim,[9] was advanced recently by Rüchardt and his collaborators[10] and was successfully extended to the Gomberg reaction.[11] In this new scheme, outlined below for the phenylation of benzene, the acetic acid is formed in a nonradical process involving the ion-pair form[12] of the diazoacetate. Furthermore, rapid oxidation of radical **2** by a high stationary-state concentration of the phenyldiazotate radical



(PhN=NO·) accounts for the failure to observe disproportionation or dimerization products from **2**. This mechanism received added support when a stable radical was detected in the reaction by electron spin resonance (esr),[13] and its spectrum was interpreted in terms of the phenyldiazotate structure (a_N = 1.67, 11.61; a_H = *o*-, −2.60; *m*-, 0.89; *p*-, −2.73). The larger nitrogen splitting has subsequently been assigned to the nitrogen atom bonded to oxygen, by means of ^{15}N labeling experiments.[14]

Our interest[15] in the scavenging of phenyl radicals by C-nitroso compounds (to give nitroxide radicals) led us to propose that the radical detected by Rüchardt and Binsch[13] might, in fact, have structure **3**. Any hyperfine splitting by the protons in ring B may have been too small to have been resolved.



We have redetermined the esr spectrum of the stable radical from the decomposition of nitrosoacetanilide in benzene (Figure 1) *and have now been able to obtain*

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