retical values of $B_2^*$ for $He^4$ ($\Lambda^* = 2.74$) and for classical gases ($\Lambda^* = 0$). The agreement of the quantum values of $B_2^*$ with the experimental[10-13] results for $He^4$ is reasonable but is not as good as is the agreement of the classical values of $B_2^*$ with the experimental[14] results for Ar. In Figure 2 we have plotted the theoretical values of $B_3^*$ for $He^4$ and for classical gases. The agreement with experiment[11, 13, 15] is only fair. In Figure 3 we have plotted the theoretical values of $B_4^*$ for $He^4$ and for classical gases.

Recently, we have proposed approximate expressions for the $B_n^*(I)$.[16] It is of interest to compare these approximate results with the exact results reported in this note. In Figure 4 the approximate and exact values for $B_3^*(I)$ are plotted. In Figure 2 of our previous publication,[16] we made a comparison of the exact and approximate values of $B_3^*(I)$. That comparison is in error. Our approximate expressions are quite correct but, unfortunately, we made a slight error in what we termed our exact calculations. When the correct values are used, the agreement between the exact and approximate values of $B_3^*(I)$ is improved and becomes very good. In Figure 5 the approximate and exact values of $B_4^*(I)$ are compared.

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† Alfred P. Sloan Foundation fellow.

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## *A SPIN-LABELED SUBSTRATE FOR α-CHYMOTRYPSIN**

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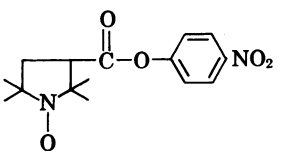
In previous work there has been introduced the technique of "spin labeling" whereby the paramagnetic resonance of synthetic organic free radicals is used to probe the structure and function of biomolecules.[1-4] The paramagnetic nitroxide radicals, RR'NO, are particularly suitable for this purpose when R and R' are bonded to the NO nitrogen atom through tertiary carbon atoms, since such radi-

cals are chemically unreactive, and show a simple nuclear hyperfine structure that is sensitive to molecular motion. In the present paper we show that the following nitroxide spin-labeled substrate can be used to study the activity of the proteolytic enzyme $\alpha$-chymotrypsin.



I

*Materials.*—The spin-labeled substrate I (DL-2,2,5,5-tetramethyl-3-carboxypyrrolidine-p-nitrophenyl ester) was prepared by mixing equimolar amounts of p-nitrophenol, 2,2,5,5-tetramethyl-3-carboxypyrrolidine-1-oxyl and dicyclohexylcarbodiimide in analogy with the methods employed by Bender for the preparation of N-acetyl-DL-tryptophan-p-nitrophenyl ester.[5] The product was purified by column chromatography on silica gel using chloroform, and crystallized from an acetone-hexane mixed solvent, mp 77.1–77.7°. Analysis: Calc. for $C_{15}H_{19}O_5N_2$: C, 58.62; H, 6.23; O, 26.03; N, 9.12. Obs.: C, 58.58; H, 6.34; O, 25.91; N, 9.17. The $\alpha$-chymotrypsin, lot CD6129-30, and phosphorylated $\alpha$-chymotrypsin, lot CD-DFP 208, were obtained from Worthington Biochemical Corp. Magnetic resonance spectra were obtained with a Varian spectrometer at 9500 Mc.

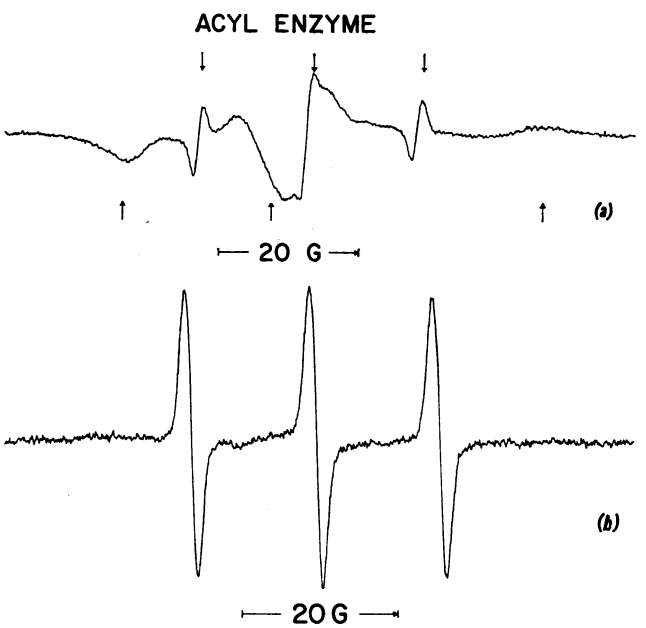
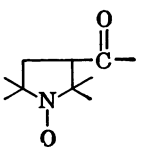



FIG. 1.—(*a*) Resonance spectrum of spin-labeled acyl enzyme at pH 3.5. Broad resonance lines indicated by *up arrows* ( ↑ ) are due to the resonance of the paramagnetic acyl group immobilized at the active site. The three narrow lines (*down arrows* ↓ ) arise from free nitroxide spins in solution due to slow deacylation at this pH. (These narrow lines are actually broadened somewhat by a high modulation amplitude.) (*b*) Resonance spectrum of the paramagnetic hydrolysis product $P_2$ at pH 4.5. This spectrum is not sensitive to pH.

*Results.*—The $\alpha$-chymotrypsin-catalyzed hydrolysis of a number of esters appears to involve the following steps.[6]

$$E + S \rightleftharpoons ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+P_1$$

In (1) it is assumed that there is a rapid reversible equilibrium between the enzyme ($E$), substrate ($S$), and complex ($ES$); the complex $ES$ decays with rate constant $k_2$ to give the alcohol $P_1$ and the acyl enzyme $ES'$, which then subsequently decays to give the acid $P_2$ and the free enzyme $E$. Several acyl enzymes $ES'$ have been isolated at acid pH where the deacylation rate $k_3$ is slow.[6] Figure 1*a* shows the resonance spectrum of a solution obtained by adding a 1.5 molar excess of I to a $1.66 \times 10^{-4}$ moles/liter solution of $\alpha$-chymotrypsin at pH 4.5, followed by dialysis. The broad spectrum seen in this figure is characteristic of an "immobilized" spin label,[2-4] and is here ascribed to the acyl enzyme, where the acyl group that contains the nitroxide spin is



At a pH of 6.8 the acyl enzyme spectrum was found to decay with a first-order rate constant $k_3 = (1.55 \pm 0.1) \times 10^{-3}$ sec$^{-1}$. This decay can be studied by the disappearance of the broad "immobilized" spin resonance characteristic of $ES'$, or by the appearance of the sharp spin resonance characteristic of the "free" spin $P_2$, illustrated in Figure 1*b*. The latter resonance gives a better signal/noise ratio, and was used to determine $k_3$. Representative data are given in Figure 2.

The following evidence demonstrates that the resonance spectrum given in Figure 1*a* is indeed the nitroxide acyl enzyme. (*a*) When a 40-fold excess of substrate I was added to a $3.86 \times 10^{-6}$ $M$ solution of $\alpha$-chymotrypsin, pH 6.8, it was found from optical absorption at 400 m$\mu$ that p-nitrophenolate ion was released at a

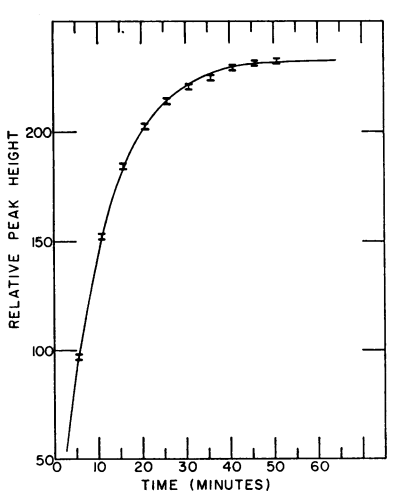



FIG. 2.—Typical plot of relative peak height versus time for the paramagnetic product $P_2$ arising from the deacylation of the spin-labeled $\alpha$-chymotrypsin, $ES'$ (pH 6.8, 0.05 $M$ phosphate buffer). Smooth curve represents theoretical first-order decay curve for $k_3 = 1.6 \times 10^{-3}$ sec$^{-1}$.

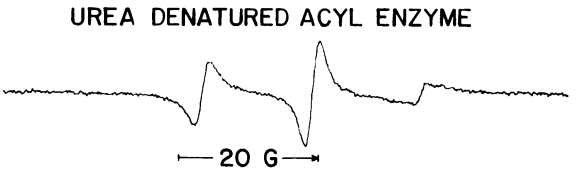



FIG. 3.—Resonance spectrum of the acyl enzyme in saturated urea solution at pH 4.5.

rate corresponding to $k_2 \gg k_3 \simeq 1.7 \times 10^{-3}$ sec$^{-1}$. Thus the rate constant for enzymatic hydrolysis of the substrate under steady-state conditions is determined by the rate-limiting step of deacylation, and the estimate of $k_3$ from the optical absorption of $P_1$ is in good agreement with the magnetic resonance determination of $k_3$ through the appearance of $P_2$. (Preliminary experiments at pH 4.5 indicate that the rate constant for acylation can also be measured by paramagnetic resonance.) (*b*) When the acyl enzyme is dissolved in saturated ($\sim$10 *M*) urea solution, pH 3.5, where the enzyme is thought to be completely unfolded, the spectrum given in Figure 3 is obtained, which is characteristic of a spin label attached to a random coil or other highly flexible site. The fact that this "mobile" spin could *not* be removed from the unfolded enzyme by dialysis demonstrates that the spin label must be attached to the enzyme by a covalent bond. (*c*) No enzymatic activity and no appearance of immobilized spin was observed when phosphorylated $\alpha$-chymotrypsin was employed.

In view of the extensive previous work on the active site of $\alpha$-chymotrypsin, it is very likely that the acyl group in the present work is attached to the active serine by a simple ester linkage. Perhaps the most significant single result of the present work is the observation that the spin-labeled acyl group is immobilized at the active site. This result is certainly consistent with Koshland's idea of an "induced fit" of substrate to enzyme.[7] That is, the local environment of the acyl group at the active site must be sufficiently rigid so as to immobilize the paramagnetic acyl group in space, but this environment must also be sufficiently flexible to admit the substrate for the acylation reaction. Preliminary urea denaturation studies indicate that when the spin label becomes mobile, the enzymatic deacylation disappears. The analysis of the data is not trivial, however, since the paramagnetic resonance spectra show evidence of an equilibrium between protein conformations that give rise to immobilized *and* mobile spins even at relatively high urea concentrations ($\lesssim$5 *M*).[8] Detailed studies of the relation between enzymatic activity and degree of substrate immobilization are in progress.

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# *CONFORMATION AND REACTION SPECIFICITY IN PYRIDOXAL PHOSPHATE ENZYMES*

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The important biochemical role of molecules with extensive pi-electron systems has long been recognized. These molecules constitute the bulk of known enzyme cofactors and, as such, participate in every variety of biochemical reaction. The mechanism of action of these cofactors often reflects the influence of their pi systems on the chemical properties of contiguous sigma bonds.

The pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP) forms of vitamin $B_6$ function in this way. The hypothesis of Snell[1] and of Braunstein[2] concerning the mechanism of action of the $B_6$ cofactors is well supported by numerous studies of the last 10 years.[3–5] This mechanistic picture emphasizes the function of the cofactor in weakening the sigma bonds around a carbon atom, usually the $\alpha$-carbon of an amino acid, which is adjacent to the cofactor pi system. This labilization can be explained in two ways, one emphasizing the equilibrium position by considering the gain in delocalization energy in the product,[6] the other emphasizing the kinetic process by focusing on the transition state for bond breaking.

An important factor in the "activation" of sigma bonds by a pi system is the stereochemical relationship of the sigma bond to the adjacent pi orbitals. Early work by Corey[7] on the stereochemistry of enolization of a steroidal ketone demonstrated a clear preference for the geometry in which the $\alpha$-carbon to hydrogen bond lies in a plane perpendicular to the plane defined by the carbonyl group and $\alpha$-carbon. Recent work has reaffirmed the validity of this principle.[8, 9] This experimental result is supported by calculations which predict maximum ground state sigma-pi interaction when the geometry is that described.[10] This interaction will increase in the transition state where the geometry approaches that of the coplanar product.

In a common step of all PLP enzyme reactions the pi system of a Schiff base formed between PLP and an amino acid is extended by loss of a group from the amino acid $\alpha$-carbon. This is accompanied by an important increase in the pi system's delocalization energy.[6] If this gain in delocalization energy is to aid the bond breaking process, the transition state must assume a geometry which places the bond to be broken in a plane perpendicular to that of the pyridoxal imine system. Thus the geometric requirements for effective sigma-pi interaction should be applicable to the whole group of $B_6$ enzymes.

The pyridoxal phosphate-amino acid imine (I) is shown in a conformation about the $\alpha$-carbon to nitrogen bond which places the $\alpha$-carbon to hydrogen bond in a