# Conformation–Reactivity Relationship for Pyridoxal Schiff's Bases. Rates of Racemization and $\alpha$-Hydrogen Exchange of the Pyridoxal Schiff's Bases of Amino Acids[†]

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ABSTRACT: The role of stereoelectronic effects in controlling the reaction specificity of biological reactions involving pyridoxal phosphate–amino acid Schiff's bases was tested with nonenzymatic models. The rates of racemization and $H_\alpha$ exchange of a series of pyridoxal–amino acid Schiff's bases were determined. The order of these rates does not parallel the predictions based solely on electronic or steric effect, but parallels the proportions of the reactive conformers (e.g., conformers with the $C_\alpha$–$H_\alpha$ bond orthogonal to the $\pi$ system) estimated by CPK models. The special reactivity of the phenylalanine Schiff's base was consistent with a special conformation in which some type of $\pi$–$\pi$ interaction increases the proportion of exchangeable conformers, thus further substantiating the role of conformation in governing the reactivity of the $C_\alpha$–$H_\alpha$ bond. Furthermore, semiempirical calculations of the conformation about the $C_\alpha$–N bond were performed using the CAMSEA conformational analysis program. The results of conformational calculations are consistent with the results of conformational analysis by nuclear magnetic resonance. The order of reactivity of the $C_\alpha$–$H_\alpha$ bond of the SB dianion, pH 12.0, predicted by calculation based on stereoelectronic effects, though not quantitatively parallel to the observed rate constants, is qualitatively in agreement with the experimental results.

$P$yridoxal phosphate–amino acid Schiff's bases are key intermediates in many important biological reactions catalyzed by enzymes which require pyridoxal phosphate as a cofactor. These include transaminases, decarboxylases, synthetases, racemases, etc. (Snell & Dimari, 1970). All of these reactions are believed to proceed via cleavage of one of three bonds to $C_\alpha$ of the amino acid.

Dunathan (1966, 1971) postulated that these enzymes control the reaction specificity by controlling the conformation about the N–$C_\alpha$ bond of the Schiff's base (SB)[1] intermediate. He suggested that the bond orthogonal to the $\pi$ system of the SB is most easily broken due to the maximum $\sigma$–$\pi$ overlap, and therefore in the enzyme active site the bond to be broken is aligned orthogonal to the $\pi$ plane. Although this model was used to relate the N–$C_\alpha$ bond conformation of the SB intermediates to the reaction specificity of a number of enzymes (Dunathan, 1966, 1971), it has not been adequately tested in nonenzymatic systems. However, the principle that bonds parallel to $\pi$ orbitals are more labile than bonds not aligned with the $\pi$ orbitals has been used to explain the 12-fold difference in the rates of exchange of the axial and equatorial protons $\alpha$ to a ketone group in $3\beta$-acetoxycholestan-7-one in the process of enolization (Corey & Sneen, 1956). This effect was attributed to stereoelectronic factors since the $\sigma$ electrons of the axial bond are more favorably located for overlap with the $\pi$ orbital of the carbonyl group. This stereoelectronic effect has also been invoked by other workers (Hine et al., 1965) and is supported by calculations which predict maximum $\sigma$–$\pi$ overlap when the orbital is parallel to the $\pi$ orbital (Dewar, 1962).

In order to probe the applicability of this stereoelectronic effect to pyridoxal–amino acid Schiff's bases as proposed by Dunathan, we have studied the effect of conformation about the N–$C_\alpha$ bond on the rates of racemization and the $H_\alpha$ exchange using a combined experimental–theoretical approach.
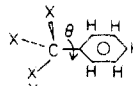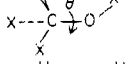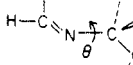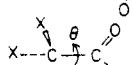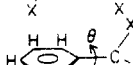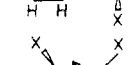
## Materials and Methods

*Materials.* The amino acids were purchased from Sigma Chemical Co. except for dihydrophenylalanine and cyclohexylalanine which were synthesized as described in the preceding paper (Tsai et al., 1978). The pyridoxal was obtained by neutralizing pyridoxal hydrochloride purchased from Sigma with NaOH and filtration. $CD_3OD$ was purchased from Koch Isotopes Inc. and Merck Sharp & Dohme Ltd. NaOD (40% in $D_2O$) and DCl (38% in $D_2O$) were purchased from Norell Chemical Co., Inc. Other chemicals used were of highest commercial grade available.

*Measurements of Exchange Rates.* The Schiff's bases for the $H_\alpha$ exchange measurement were prepared by mixing amino acid (0.5 mmol), pyridoxal (0.5 mmol), and 40% NaOD (1.25 mmol) in $CD_3OD$ solution and stirring for 30 min at room temperature under a nitrogen atmosphere. Then the pD value was adjusted to 12.3, using NaOD or DCl if necessary, and the total volume was adjusted to 1.0 mL. The rates of $H_\alpha$ exchange were measured using a Varian EM-360 60 MHz NMR spectrometer by integrating the signal of the $H_\alpha$ as a function of time. The time of mixing was set as time zero. Since the chemical shifts of the $\alpha$ protons were very close to those of $H_{5'}$ and the solvent OH, the integrations were subject to $\pm10\%$ error. Normally five integrations were taken during the period in which 70% of $H_\alpha$ was exchanged. The percentage of $H_\alpha$ left was defined relative to the integration of $H_{4'}$, e.g., $A_t = I(H_\alpha)/I(H_{4'})$. Then $\ln A_t$ was plotted against time and the least-squares analysis was applied to the data points. Usually the correlation coefficients obtained were larger than 0.90 and the resulting slopes were taken as the pseudo-first-order rate constants for $H_\alpha$ exchange. These constants were then cor-

[1] Abbreviations used: SB, Schiff's base; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; CPK, Corey–Pauling–Koltun.

TABLE I: Torsional Functions Used for Pyridoxal Schiff's Bases.

| Structure | Function | Label |
|---|---|---|
| (structure A) | $0.145(1 + \cos(6\theta))$ | A |
| (structure B) | $0.86 \sin(1.5\theta)$ | B |
| (structure C) | $1(1 + \cos(3\theta - 20))$ | C[a] |
| (structure D) | $0.58(1 + \cos(6\theta))$ | D |
| (structure E) | $0.29 \sin(3\theta)$ | E |
| (structure F) | $3.13 \sin(1.5\theta)$ | F |

[a] Note that this function is different from that used previously (Weintraub et al., 1976) and represents a refined function based on a much more extensive set of calculations.



FIGURE 1: Plots of energy vs. rotation angle for the N-CH₂ bond of $CH_2=N-CH_2COOH$, compound C in Table I.

rected for the amount of OH present in solution by multiplying $M/(M - N)$ where $M$ is the molarity of methanol (24.7) and $N$ is the concentration of OH measured by integration. However, the solvent isotope effect was not taken into account to correct for the $k_e$ value. In our experiments $M/(M - N)$ was usually 1.1–1.2.

*Measurement of Racemization Rates.* The Schiff's bases for the measurements of racemization were prepared by mixing amino acid (0.1 mmol), pyridoxal (0.1 mmol), and the calculated amount of NaOH in absolute $CH_3OH$ solution and stirring for 30 min (at higher pH) or longer (at medium or neutral pH) at room temperature under a nitrogen atmosphere. The solution was then adjusted to the desired pH using NaOH or HCl if necessary, and the total volume was adjusted to 1.0 mL. The time of mixing was set as time zero. The rates of racemization were determined by measuring the optical rotation at 546 nm as a function of time, using a Perkin-Elmer 241 polarimeter in a 10-cm glass cell thermostated at 25 °C. At least ten measurements were taken at different time intervals, depending on rates of racemization. The natural logs of these rotations were plotted against time (seconds). When the least-squares analysis was applied to the data points, the correlation coefficients obtained were larger than 0.999. The resulting slopes were therefore the pseudo-first-order rate constants for racemization $(k_e)$. The L-amino acids were used for all Schiff's bases except D-α-aminobutyric acid. No difference in the racemization rates between D and L forms has been found.

*Conformational Calculations.* The conformational analyses were performed using classical, empirical potential energy functions as employed in the CAMSEQ conformational analysis package (Weintraub & Hopfinger, 1975; Potenzone et al., 1977). The potential energy functions utilized in this study have been described in detail elsewhere (Hopfinger, 1974) but will be briefly listed at this time. Steric, nonbonded interactions are represented by sets of Lennard–Jones 6–12 potential functions. Electrostatic interactions are approximated by a Coulomb's law function. Solvation free energies were calculated using a hydration shell model proposed by Hopfinger & Battershell (1976). The calculations were performed in vacuo
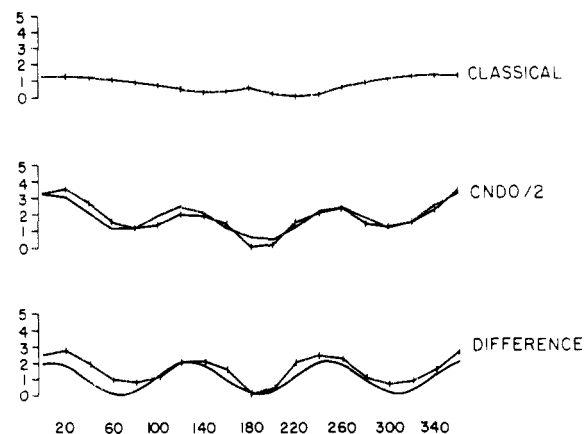
as well as in aqueous solution. A comparison study is underway to determine any differences in conformational preferences resulting from solvation in methanol as compared with an aqueous solution.

Experimentally determined torsional barriers were used where possible, while those barriers not found in the literature were determined from CNDO/2 calculations using a method described by Potenzone (1975). Torsional barriers, as utilized by CAMSEQ, are actually correction terms which are summed with the other classical potential energy functions (see above) to produce the total conformational energy. In this way the simple, efficient, classical potential energy functions reproduce the correct rotational barriers at a fraction of the computational time necessary for semiempirical calculations.

Table I lists the torsional correction functions employed in this study. As a representative sample of how the torsional barrier correction term is calculated by CNDO/2 and CAMSEQ, refer to function C, Table I. The model compound used in the calculation is illustrated in the table. A conformational scan is performed on this compound using both classical and semiempirical techniques. The resemblant energies are plotted vs. rotation angle for both methods. Figure 1 illustrates these plots. A difference curve is determined and a function fitted to this latter curve. In this manner, the sum of the classical potential energy functions and the harmonic correction function will reproduce the curve representing the semiempirical calculations. By performing this study on a small model compound, the correction function can be determined economically, eliminating the necessity of performing a complete conformational study on the entire molecule using the semiempirical techniques.

The coordinates for the compounds which are required by CAMSEQ were taken from several crystal structures. The pyridine ring system and -C=N group coordinates were taken from Barrett & Palmer (1969), while the ring substituent coordinates were taken from Hanic (1966). When necessary, standard bond lengths and angles were utilized. The partial atomic charge distributions were calculated using the CNDO/2 method for all compounds investigated. A tabulation of these charges may be found in the preliminary report (Weintraub et al., 1976).

We have employed the following techniques to determine the locations of the global and local minimum. Initially a sequential scan was performed at 20–30° resolution for all "major" torsional rotations, providing a coarse, but complete scan of conformational hyperspace. We define a "nonmajor" torsional rotation to be that of a methyl, hydroxyl, or other such

TABLE II: Pseudo-First-Order Rate Constants of $H_\alpha$ Exchange $(k_e)$ and Results of Conformational Calculations of Dianionic Schiff's Bases.
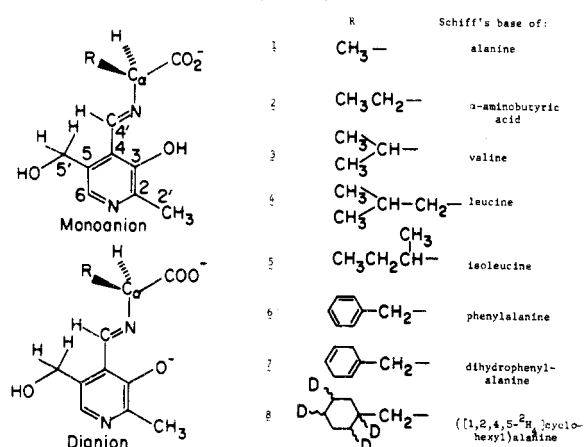
| SB of | $k_e \times 10^5$ (s$^{-1}$) | Taft's steric constant $E_s$ for R group at $C_\alpha$ | angles (deg) about the N-$C_\alpha$ bond for the lowest energy conformers (% in parentheses)[a] | |
|---|---|---|---|---|
| | | | A | B |
| Ala (1) | 8.5 | 0.00 | 70 (55) | 310 (40) |
| Phe (6) | 4.6 | -0.38 | 100 (35) | 340 (65) |
| dihydro-Phe (7) | 4.3 | | | |
| $\alpha$-aminobutyric acid (2) | 2.9 | -0.07 | | |
| Leu (4) | 0.7 | -0.93 | 80 (20) | 320 (75) |
| cyclohexyl-Ala (8) | 0.6 | -0.98 | | |
| Val (3) | <0.01 | -0.47 | 100 (60) | 320 (40) |
| Ile (5) | <0.01 | -1.13 | | |

[a] The percentage of conformer was calculated using the Boltzmann distribution and adding the percentages for conformers at 10° intervals within ±2 kcal/mol of the minimum shown. In cases where the minima were broad, the angle most favorable for exchange was listed as this would be most important during the exchange process.

group. Next, several hundred random conformation states were investigated, allowing all rotatable bonds to participate. Finally a number of starting points were selected for a multidimensional minimization involving all rotatable bonds. These starting points were chosen by examination of conformational energy maps produced at the end of the second step. This three-step procedure, sequential scan, random scan, and minimization, of course, does not guarantee that the true global energy minimum will be found, but we feel it provides the best chance to find the minimum for a molecule possessing three or more degrees of rotational freedom. The average analysis involved a sufficiently large number of conformational states to provide a reasonable statistical base for the calculation of thermodynamic probabilities and entropies (Hopfinger, 1974).

### Results and Discussion

We have determined the rates of the specific base-catalyzed racemization and $H_\alpha$ exchange of the pyridoxal Schiff's bases of a series of amino acids (1 to 8). The rates of racemization



were measured in methanol solution in the pH range for monoanions and dianions by measuring the optical rotation as a function of time. The rates of $H_\alpha$ exchange were measured by NMR integration of $H_\alpha$ of the same solution used for the NMR studies in the preceding paper (Tsai et al., 1978). The exchange rates were measured for only the dianions since the monoanions exchanged too slowly to be measured precisely by the relatively insensitive NMR integration method.

The conformations of these Schiff's bases were qualitatively investigated via examination of CPK models and by confor-

TABLE III: Pseudo-First-Order Rate Constants $(k_\alpha)$ for the Racemization of Schiff's Bases $(10^5 k_\alpha, \text{s}^{-1})$.

| amino acid | pH | | | | |
|---|---|---|---|---|---|
| | 8.5 | 9.5 | 10.5 | 11.3 | 12.0 |
| L-Ala | 0.6 | 1.1 | 4.0 | 9.4 | 13.8 |
| L-Phe | 0.04 | 0.41 | 1.86 | 4.35 | 7.62 |
| L-dihydro-Phe | 0.05 | 0.16 | 1.81 | 5.75 | 8.99 |
| D-$\alpha$-aminobuty-ric acid | <0.01 | 0.04 | 0.29 | 1.50 | 2.32 |
| L-Leu | <0.01 | 0.04 | 0.30 | 1.09 | 1.45 |
| L-cyclohexyl-Ala | <0.01 | 0.04 | 0.29 | 1.00 | 1.50 |
| L-Val | 0 | 0 | 0 | 0 | 0 |
| L-Ile | 0 | 0 | 0 | 0 | 0 |

mational calculations using the CAMSEQ conformational analysis program (Weintraub & Hopfinger, 1975). The conformational calculations utilized classical potential functions and were done assuming aqueous solvent.

In order to restrict the number of conformations we have fixed the $C_4-C_{4'}$ bond so that the C=N bond is coplanar with the pyridoxal ring and has "cis" conformation since this is the predominant conformation as suggested by the preceding paper (Tsai et al., 1978). However, the calculations were done only for dianions since in the monoanions the problem might be complicated by the tautomerism and the possible hydrogen bonding effect. Since the "trans" conformer could be present in the dianions, and since the calculation and experiments were done in different solvents, it should be realized that we have not attempted to correlate the calculated conformation with the experimental data quantitatively. Instead, we are comparing the observed "order" of the reactivity of the $C_\alpha-H_\alpha$ bond with the orders expected from CPK models and from the calculated conformation based on Dunathan's hypothesis.

Tables II and III and Figure 2 summarize the results of these experimental and theoretical studies of the rates of reaction and minimum energy conformations. In order to minimize the electronic effect, only the Schiff's bases of amino acids without ionizable groups on their side chains were chosen for comparison. Only representative Schiff's bases were calculated due to the enormous computer time involved. The fact that the "order" of $k_\alpha$ is approximately the same in the pH range from monoanions to dianions makes it feasible to use the "cis" dianion as a model for conformational calculation.
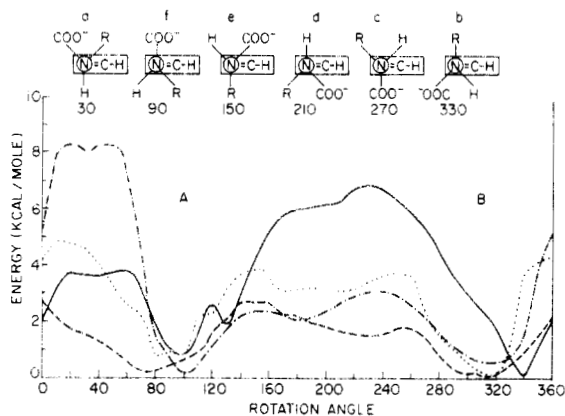
FIGURE 2: A plot of energy vs. rotation angle about the N-C$_\alpha$ bond for phenylalanine (—), alanine (- - -), leucine ($\cdots$), and valine (----) pyridoxal Schiff's bases. The curves were calculated using the CAM-SEQ program and the energies are for the N-C$_\alpha$ angle shown with all other notable bonds of the Schiff's base at their minimum energy rotation. The Newman projections at the top of the figure show the conformation of the N-C$_\alpha$ bond at the angle listed. The small letters (a-f) designate the conformation shown in the Newman projection and A and B designate the two major conformations of these compounds.

The following factors rule out the possibility that the exchange and racemization rates are controlled by only steric or electronic effects.

(1) An S$_E$1 type reaction would predict that, as the bulk of R increases, the amount of relief of crowding should increase upon going from ground state to the carbanion intermediate. Thus the rate should increase as the bulk of R increases. This is opposite to the observed order as shown in Tables II and III.

(2) An S$_E$2 type reaction would predict that, as the bulk of R increases, the rates of reaction should decrease. According to the Taft's steric constants (Taft, 1956), the predicted order of reaction rates of the Schiff's bases should be alanine (0.00) > $\alpha$-aminobutyric acid (−0.07) > phenylalanine (−0.38) > valine (−0.47) > leucine (−0.93) > isoleucine (−1.13). The observed order does not parallel this predicted order.

(3) Similarly, the order of reaction rates for the H$_\alpha$ exchange and racemization does not parallel the electronic effect of the substituent on the C$_\alpha$ carbon. The polar effect of the amino acid side chains, according to the $\sigma^*$ values (Taft, 1956) for the alkaline hydrolysis of the esters RCOOR', falls into the order C$_6$H$_5$CH$_2$ (+0.215) > CH$_3$ (0.000) > C$_2$H$_5$ (−0.100) > $i$-C$_4$H$_9$ (−0.125) > $i$-C$_3$H$_7$ (−0.190) > $sec$-C$_4$H$_9$ (−0.210). The charge densities on the C$_\alpha$ carbon (shown in parentheses) from CNDO calculations (Weintraub et al., 1976) fall into the following order: C$_6$H$_5$CH$_2$ (+0.02 esu) > $i$-C$_3$H$_7$ (0.01) $\approx$ CH$_3$ (0.01) $\approx$ $i$-C$_4$H$_9$ (0.01).

On the other hand, the experimental results support Dunathan's hypothesis that the reactivity is governed by stereoelectronic effects based on the following factors.

(4) The results in Tables II and III indicate that the reactivity of the C$_\alpha$-H$_\alpha$ bond is very sensitive to the number of $\beta$ substituents on the amino acid side chains. The Schiff's base of alanine which has no $\beta$ substituent undergoes the most rapid reactions, whereas the Schiff's bases of valine and isoleucine which have two $\beta$ substituents show no detectable racemization and H$_\alpha$ exchange. Intermediate values of $k_e$ and $K_\alpha$ were observed for the Schiff's bases of the amino acids with a single $\beta$ substituent. It is very obvious from the CPK model that in conformer a (Figure 2) in which the C$_\alpha$-H$_\alpha$ bond is orthogonal to the $\pi$ plane the $\beta$ substituent could exert a large steric effect

on the pyridoxal ring substituents. Therefore CPK models predict that the percentage of conformer a decreases as the number of $\beta$ substituents increases. Based on the Winstein-Holness equation (eq 1) (Winstein & Holness, 1955)

$$k_{obsd} = k_{conformer_1}N_1 + k_{conformer_2}N_2 + \ldots \quad (1)$$

which states that the rate constant observed depends on the rate constants of reaction of the various conformers multiplied by the mole fractions ($N$) of these conformers, the order of the reaction rates of the Schiff's bases should follow the order of the percentages of a and d if conformers a and d are far more reactive than other conformers for racemization and H$_\alpha$ exchange, as proposed by Dunathan based on the stereoelectronic effect. It is significant that the Schiff's base of leucine which has a single $\beta$ substituent but larger steric bulk ($E_s$ = −0.93) undergoes racemization and H$_\alpha$ exchange whereas that of valine which has two $\beta$ substituents but smaller steric bulk ($E_s$ = −0.47) does not.

(5) For the Schiff's bases of $\beta$-monosubstituted amino acids, CPK models predict the following order for the percentages of conformer a based on the bulk of R groups of amino acid side chains applying Taft's steric constants: $\alpha$-aminobutyric acid (R = CH$_3$CH$_2$; $E_s$ = −0.07) > phenylalanine (R = C$_6$H$_5$CH$_2$; $E_s$ = −0.38) > leucine (R = $i$-C$_4$H$_9$; $E_s$ = −0.93). The order of the observed $k_e$ and $k_\alpha$ is phenylalanine > $\alpha$-aminobutyric acid > leucine. As described in the preceding paper (Tsai et al., 1978), the conformation of phenylalanine Schiff's base was found to be influenced by a special effect, possibly the $\pi$-$\pi$ interaction between the phenyl ring and the pyridoxal $\pi$ system. In order to see if this special conformation is related to the exceptionally large $k_e$ and $k_\alpha$ of the phenylalanine Schiff's base,[2] we have determined the $k_e$ and $k_\alpha$ of the Schiff's bases of dihydrophenylalanine and cyclohexylalanine. As shown in Tables II and III, the $k_e$ and $k_\alpha$ of the dihydrophenylalanine SB are approximately equal to those of the phenylalanine SB, whereas the $k_e$ and $k_\alpha$ of the cyclohexylalanine SB are much smaller, and slightly smaller than those of the leucine SB. The results are in good agreement with the results of conformational analysis (Tsai et al., 1978) which indicates that the $\pi$-$\pi$ interaction is still present in the dihydrophenylalanine SB but completely disappears in the cyclohexylalanine SB. Since the Taft's steric constant for the R group of cyclohexylalanine (−0.98) is slightly smaller than that for the R group of leucine (−0.93), the results of cyclohexylalanine SB are in agreement with the prediction by Dunathan's hypothesis based on CPK models and Taft's steric constants.

The results of conformational calculation also substantiate the stereoelectronic effect and are consistent with the experimental data. The following comments and conclusions can be made concerning the results.

(6) The calculated conformational energy profiles (Figure 2) indicate that, as the number of $\beta$ substituents increases, the relative energy of conformer a increases substantially. This is in agreement with the prediction by CPK models based on the large steric interaction between the $\beta$ substituent and the pyridoxal ring, and also qualitatively agrees with the experimental results.

(7) The Schiff's bases calculated, share two common energy minima, one at ~90 ± 20° (A), the other at ~320 ± 20° (B). Since the calculation conditions were not exactly the same as the experimental conditions, we do not imply that the observed

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[2] Although only the special conformation of the C$_\alpha$-C$_\beta$ bond was detected by NMR, the $\pi$-$\pi$ interaction could also influence the conformation about the N-C$_\alpha$ bond, as shown in structure I and the discussions in section 9 of Results and Discussion.

$^3J(^{13}C_{4'}-H_\alpha)$ (Tsai et al., 1978) represents these two conformations exactly. However, they are at least qualitatively consistent with each other in the sense that the major conformations of the $N-C_\alpha$ bond for all the Schiff's bases studied are approximately the same and that conformers a and d are only minor conformers which can vary very much among different Schiff's bases but have little effect on the observed $^3J$ $(^{13}C_{4'}-H_\alpha)$.

(8) In the major conformer B there is a close spatial relationship between $H_{4'}$ and $H_\alpha$, in agreement with the NOE results of valine Schiff's base discussed in the preceding paper (Tsai et al., 1978).

(9) The qualitative consistency between the calculational model and the observed order of reactivity could be noted from another point of view based on stereoelectronic effect. It is known that the interaction of $\pi$ orbitals upon twisting about an essential single bond decreases as the $\cos^2\theta$ of the angle $\theta$ between the orbitals (Jaffé & Orchin, 1962). Thus exact coplanarity of the $\sigma-\pi$ orbitals gives $\cos^2\theta = 1$ (100% overlap) and when the angle is 90° a $\cos^2\theta = 0$ (no overlap) is obtained. Based on this reasoning we can now factor the rate constant into two terms: $k_{actual} = kN_A \cos^2\theta_A + kN_B \cos^2\theta_B$, where $k$ is the rate constant at 100% $\sigma-\pi$ orbital overlap, $\theta_A$ and $\theta_B$ are the angles between the $\sigma$ orbital of the major conformers A and B, respectively, and the $\pi$ orbital. $N_A$ and $N_B$ represent the percentages of conformers A and B, respectively. Using this factored equation and the data in Table II and Figure 2, which show that the $C_\alpha-H_\alpha$ bond is orthogonal to the $\pi$ system (e.g., 100% $\sigma-\pi$ overlap, $\theta = 0°$) at angles of 30° (conformer a) and 210° (conformer d), the following approximate equations can be written:
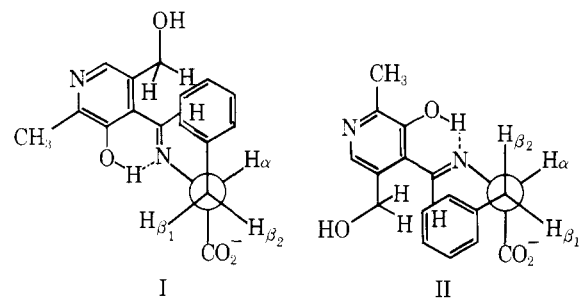
$$k_{actual} \text{ (Ala SB)} \sim k(0.55) \cos^2 40°$$
$$+ k(0.40) \cos^2 80° = 0.33k$$

$$k_{actual} \text{ (Leu SB)} \sim k(0.20) \cos^2 50°$$
$$+ k(0.75) \cos^2 70° = 0.17k$$

$$k_{actual} \text{ (Val SB)} \sim k(0.60) \cos^2 70°$$
$$+ k(0.40) \cos^2 70° = 0.12k$$

$$k_{actual} \text{ (Phe SB)} \sim k(0.35) \cos^2 70°$$
$$+ k(0.65) \cos^2 50° = 0.31k$$

The rates thus predicted are qualitatively in the same order as the experimental results. It is obvious from these data that the major contribution to the high reaction rate of alanine SB is the conformer A which has a smaller $\theta_A$ due to the smaller steric interaction between the R group and the $C_{4'}-H_{4'}$ group, compared with the other Schiff's bases. On the other hand, the main contribution to the high reaction rate of phenylalanine SB is the B conformer which has a smaller $\theta_B$ compared with the other Schiff's bases. Although the reason for the shift of $\theta_B$ of the phenylalanine SB is not entirely obvious, it is, however, consistent with the NMR results (Tsai et al., 1978). NMR shows that there is a $\pi-\pi$ interaction between the phenyl ring and the pyridoxal $\pi$ system and that $H_{4'}$ and $H_{6'}$ are shifted upfield by the phenyl ring in the phenylalanine SB. Based on CPK models, the conformations of the $N-C_\alpha$ bond most suitable for such a $\pi-\pi$ interaction are the conformation between conformers a and b (rotation angle between 330° and 30°) and the conformation between conformers e and f (rotation angle between 90° and 150°). Thus I and II show these two possible predominant conformers for the $N-C_\alpha$ bond with the phenyl ring situated above $H_{4'}$ and $H_{5'}$. Conforma-



I             II

tional analysis of the $C_\alpha-C_\beta$ bond (Tsai et al., 1978) indicates that the predominant conformation about the $C_\alpha-C_\beta$ bond is that shown in I. Thus our results from both experimental and theoretical studies indicate that I depicts the predominant solution conformation of the phenylalanine SB. The arguments based on stereoelectronic effect predict that the $N-C_\alpha$ bond conformation in I favors $H_\alpha$ exchange and racemization due to larger $\sigma-\pi$ overlap, which could explain the exceptionally large $k_e$ and $k_\alpha$ observed for the phenylalanine SB.

In conclusion, we have measured the rates of racemization and $H_\alpha$ exchange for the pyridoxal Schiff's bases of a series of amino acids with nonionizable side chains. The order of these rates does not parallel the predictions based only on electronic or steric effect. The role of the stereoelectronic effect in controlling the reactivity of the $C_\alpha-H_\alpha$ bond, as proposed by Dunathan, is supported by: (1) consistency between the experimental data and the prediction based on CPK models; (2) relationship between the special conformation and the special reactivity of the phenylalanine Schiff's base; (3) qualitative correlation between the experimental results and results of conformational calculations. However, other explanations involving a combination of several effects cannot be ruled out completely.

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# Modification of the Arginines in Parathyroid Hormone: Effect on Biological Activity[†]

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ABSTRACT: The amino-terminal region 1–34 of parathyroid hormone contains all the structural requirements necessary for full biological activity in vitro and in vivo. Carboxyl- and amino-terminal modifications and substitutions of this region have been studied extensively, leading to the synthesis of analogues of enhanced biological activity and hormone antagonists. Structural alteration of the central region of the active segment has failed to produce large changes in biological activity. Arginine is present at positions 20 and 25. Modification of these arginines was performed without undertaking total synthesis of an arginine-substituted analogue. 1,2-Cyclohexanedione reacts specifically, completely, and reversibly with arginine to form a single addition product. The 1,2-cyclohexanedione-treated analogue of parathyroid hormone, [DHCH-Arg-20, DHCH-Arg-25]hPTH-(1–34), was, at most, 16% as active as the unmodified hormone, hPTH-(1–34). Reversal of the arginine modification completely restored biopotency. Hence, the centrally positioned arginines of the active fragment may serve an important role in receptor interaction. This finding suggests that the preparation of synthetic analogues of parathyroid hormone which contain substitutions for arginine may be important to future structure-activity analyses of this hormone. Specific postsynthetic modification of arginine should prove of general utility in evaluating the role of arginine in the biological activity of peptide hormones.

Structure–activity studies utilizing synthetic fragments and analogues of parathyroid hormone (PTH)[1] have led to the successful preparation of peptides more potent than the native hormone itself (Rosenblatt and Potts, 1977; Rosenblatt et al., 1976) and inhibitors of PTH action (Goltzman et al., 1975; Rosenblatt et al., 1977). These and other studies (Tregear and Potts, 1973; Parsons et al., 1975) have explored extensively the effect on biological activity of structural modification at either the amino or carboxyl terminus of the fully active region, PTH-(1–34), of the 84 amino acid hormone. When attention turned toward other regions of the molecule, it was found that substitutions or modifications in the central portion of the active fragment were remarkable for their relative lack of effect on biological activity: substitution of norleucine for the methionines of positions 8 and 18 was well tolerated in terms of biopotency (Rosenblatt et al., 1976), and modification of the tryptophan at position 23 by attachment of the o-nitrophenylsulfenyl group to the indole nitrogen produced no change in the potency of an analogue of enhanced activity (Rosenblatt and Potts, 1977) or a hormone inhibitor (Rosenblatt et al., 1977). Interest continues in finding structural alterations of the central portion of the active region which cause either

dramatic quantitative or qualitative effects on biological properties. Such modifications, if combined with modifications at the amino or carboxyl terminus, might augment either biopotency or inhibitory activity of certain analogues. Since the synthesis, purification, chemical characterization, and biological evaluation of a 34 amino acid hormone analogue entail considerable effort, a convenient approach to screening for alterations that cause large changes in biological properties is to selectively modify particular amino acids after synthesis. This approach avoids undertaking an exclusive synthesis for each new analogue in order to determine sequence positions critical for biological activity. In PTH, arginine occurs centrally in the active fragment at positions 20 and 25. Patthy and Smith (1975a) reported a technique for selectively modifying arginine under mild conditions using 1,2-cyclohexanedione. They demonstrated this reagent to react completely with the guanidino function of arginine to form a single addition product, $N^7, N^8$-(1,2-dihydroxycyclohex-1,2-ylene)arginine (DHCH-Arg). Unmodified arginine could be regenerated quantitatively from DHCH-Arg by another set of mild reaction conditions. The method was then applied to determining the function of arginine in two enzymes, lysozyme and RNase A (Patthy and Smith, 1975b). Our study describes the use of this method to prepare an analogue of human PTH, [DHCH-Arg-20,DHCH-Arg-25]hPTH-(1–34), and the biological evaluation of this compound in vitro to assess the role of arginine in PTH action.

## Experimental Procedure

*Synthesis of hPTH-(1–34).* The active region of human PTH, hPTH-(1–34), was synthesized by a modification (Tregear et al., 1974) of the Merrifield solid-phase technique (1969) using a Beckman Model 990 automated synthesizer. Merrifield 1% cross-linked chloromethylated resin (Lab Sys-