Nucleophilic Selectivity in Attack at Amide Bonds. Reactivity of Oxygen and Nitrogen Nucleophiles with *N*-Acetyldehydrophenylalanyl-L-proline Diketopiperazine†

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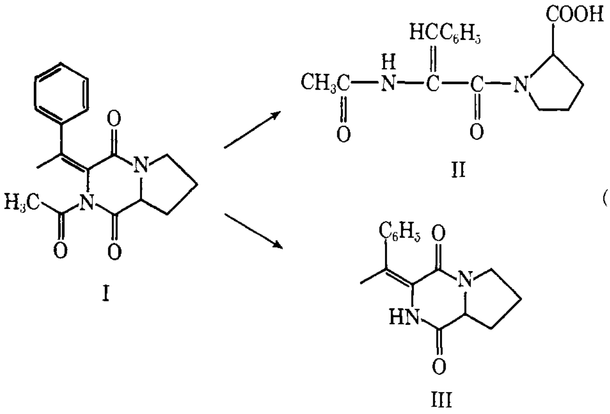
ABSTRACT: The N-acetylated diketopiperazine (*N*-acetyldehydrophenylalanyl-L-proline diketopiperazine (I)) undergoes nucleophilic attack to yield the N-acetylated dipeptide (*N*-acetyldehydrophenylalanylproline (II)) or deacylated diketopiperazine (dehydrophenylalanylproline diketopiperazine (III)). The following specificities for position of bond cleavage have been found. (1) Primary and secondary amines, hydrazine, ammonia, and imidazole ($k_r^{H_2O}/k_r^{D_2O} = 1.23$) provide III *via* unassisted nucleophilic attack (k_N[Nuc:][I]). (2) Ammonia, methoxylamine, and semicarbazide yield both II and III in reactions requiring general base and general acid assistance to nucleophilic attack [(k_{gb}[Nuc:]^2 + k_{ga}[Nuc:]·[NucH]) [I]]. (3) The oxygen bases (formate, acetate ($k_r^{H_2O}/k_r^{D_2O} = 2.23$), phosphate, trifluoroethoxide, hydroxide, as well as Tris) provide II *via* paths of general base catalyzed attack of water (k_{gb}[weak base][H_2O][I]) or direct nucleophilic attack. These observations are discussed in terms of the relative leaving tendencies in formation of II or III from I, ring strain in the diketopiperazine, and intramolecular participation of imide anion in ester hydrolysis.

In an early synthetic study, Bergmann and Tietzman (1944) found that the reaction of *N*-acetyldehydrophenylalanyl-L-proline diketopiperazine (I) with glycine ethyl ester and sodium glycinate gave exclusively dehydrophenylalanylproline diketopiperazine (III) whereas in the reaction with hydroxide ion only *N*-acetyldehydrophenylalanylproline (II) was formed.



(1)

It occurred to us that perhaps oxygen and nitrogen nucleophiles might exhibit nucleophilic specificities toward the endo- and exoamide linkages of I, respectively. To a certain degree this is so and there is described herein studies of the competition of various nucleophiles for attack at the acetyl and lactam carbonyl carbons of I.

Experimental Section

*N-Acetyldehydrophenylalanyl-*L*-proline diketopiperazine* (I) was prepared from *N*-acetyldehydrophenyl-L-proline (II) by refluxing in acetic anhydride according to the method of Bergmann and Tietzman (1944). The material melted at 170–173° (lit. mp 172–173°). *Anal.* Calcd for $C_{16}H_{16}N_2O_3$: C, 67.6; H, 5.63; N, 9.83. Found: C, 67.0; H, 5.92; N, 10.05.

*Dehydrophenylalanyl-*L*-proline diketopiperazine* (III) was prepared from I and glycine (Bergmann and Tietzman, 1944). The compound was also obtained by dissolving I in acetonitrile, adding an imidazole buffer solution (0.1 M, pH 7.04) and allowing the solution to stir for approximately 30 min. The product was then precipitated by addition of a solution of NaCl, collected by filtration, and dried *in vacuo* over P_2O_5 (mp 170–172, lit. mp 171–172). *Anal.* Calcd for $C_{14}H_{16}N_2O_3$: C, 69.40; H, 5.83; N, 11.56. Found: C, 69.24; H, 6.07; N, 11.16.

Dehydrophenylalanylproline was prepared from III by alkaline hydrolysis (Bergmann and Tietzman, 1944), mp 168–170 (lit. mp 168–171) (after one recrystallization from acetone and water). *Anal.* Calcd: C, 64.60; H, 6.20; N, 10.76. Found: C, 64.27; H, 6.36; N, 10.52.

*N-Acetyldehydrophenylalanyl-*L*-proline* (II) (mp 143–145, lit. mp 140–142) was prepared according to the procedure of Behrens *et al.* (1940).

4-Benzylidene-2-methyl-2-oxazoline-5-one was prepared from α-*N*-acetimidocinnamic acid according to the method of Darkin and West (1928): mp 152.5–153°, lit. mp 152; infrared (ir) spectrum carbonyl 1780 cm^{-1}, no NH stretch observed; nuclear magnetic resonance (nmr) spectrum (in τ values) 1.80–2.67 (5), 2.68 (1), 7.6 (3) ($CDCl_3$).

*N-Acetyldehydrophenylalanyl-*L*-prolinamide* (IV) was prepared by treatment of I with aqueous ammonia according to the method of Bergmann and Tietzman (1944): mp 234.5–235°, lit. mp 239–241°.

pK_a *Determinations.* The pK_a of dehydrophenylalanyl-L-proline diketopiperazine (III) was determined at 30°, μ = 1.0 (with KCl), by measurement of the optical density of the anion (at λ_{max} 322 nm) at various pH values. The theoretical sigmoidal curve giving the best fit to the experimental data resulted in a pK_a of 10.77.

The pK_a of II was calculated on the basis of eq 2 and 3. At the wavelength employed (322 nm) only the dianion of II ab-

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$$K_a = \frac{OD_1}{OD_{\infty 1}}(K_a + a_{H_1}) \qquad (2)$$

$$K_a = \frac{OD_2}{OD_{\infty 2}}(K_a + a_{H_2}) \qquad (3)$$

sorbed. Since the concentration of II was constant throughout the experiment, it was assumed that $OD_{\infty 1} = OD_{\infty 2}$ (OD_∞ is the absorption at 322 nm of II at pH $\gg$ pK_a). From eq 2 and 3, eq 4 is derived.

$$\frac{OD_1}{OD_2} = \frac{(K_a + a_{H_2})}{(K_a + a_{H_1})} \qquad (4)$$

The pK_a of II is an average, calculated employing eq 4 and experimental values of OD_{322nm} found at several values of a_H. This method was employed because a value for the extinction coefficient of the anion was unobtainable due to the high pK_a of the amide. The pK_a value obtained was 13.9 ± 0.2.

The pK_a values of glycylglycine and 2,2,2-trifluoroethylamine at temperatures above 30° were determined by means of half-neutralization employing a Metrohm type H high-temperature glass electrode and a Radiometer 26 pH meter used in conjunction with a constant temperature cell previously described by Bruice and Marquardt (1962). Prior to measurements, the glass electrode was calibrated at each temperature against standard buffer.

Kinetic Measurements. The rates of hydrolysis of I at 30 ± 0.1° (μ = 1.0) in water were followed by measuring the appearance of II at 278 nm or III at 292 nm (320 nm for anion) with a Gilford 2000 recording spectrophotometer. In all spectrophotometric work a stock solution of I in acetonitrile was used. For the spectrophotometric rates run on the Gilford 2000, an aliquot (20–30 μl) of stock solution of I was added to 2.5–3.0 ml of buffered solutions. The spectra of the solutions upon completion of the reaction were identical with those of II or III in the appropriate buffer solution. Rate constants for hydroxide ion catalyzed hydrolysis were determined titrimetrically (μ = 1.0 with KCl) at constant pH with a Radiometer TTT 1b autotitrator and followed spectrophotometrically with a Cary 15, RS. Pseudo-first-order rate constants were calculated with an Olivetti Underwood Programma 101 program for (log $[(A_t - A_0)/(A_0 - A_\infty)]$ *vs.* time) infinity or Guggenheim-type plots, employing least-squares analysis for slope and intercept. All pH's were determined (at 30.0 ± 0.1°) with a Radiometer Model 22 pH meter equipped with a Model 630 scale expander and a combined glass calomel electrode (Radiometer GK 2021C). The ΔpD correction (Fife and Bruice, 1961) was employed for work done in D_2O.

Product Analysis. Extinction coefficients of II, III, and *N*-acetyldehydrophenylalanyl-L-prolinamide (IV) were determined at 278, 282, and 292 nm, respectively. Stock solutions of the compounds in CH_3CN were pipetted in microliter quantities into a cuvet holding 3.0 ml of solution. The optical density was measured for each compound at at least six concentrations at each wavelength. Product analyses for the aminolysis were done at a constant concentration of I. Final values were obtained by solution of the simultaneous equations (eq 5 and 6) and the fact that (1) [IV] + [III] + [II] = [I_0] and (2) the fraction of product arising from hydroxide ion catalyzed hydrolysis can be calculated from the observed pseudo-first-order rate constant (k_{obsd}), which has kinetic terms both in ammonia and hydroxide ion.

$$OD_{278nm} = \epsilon_{278IV}[IV] + \epsilon_{278II}[II] + \epsilon_{278III}[III] \qquad (5)$$

$$OD_{292nm} = \epsilon_{292IV}[IV] + \epsilon_{292II}[II] + \epsilon_{292III}[III] \qquad (6)$$

$$k_{obsd} = k_{HO}[HO^-] + k_n[NH_3]_T + k_n{}^2[NH_3]_T{}^2 \qquad (7)$$

Results

The reaction of I with nineteen nucleophilic species has been investigated under the pseudo-first-order conditions of [N:] + [NH] = [N_T] > [I_0] (where N: represents nucleophile free base and NH its conjugate acid). The experimental conditions and derived second-order rate constants for reaction of N: with I are presented in Table I.

In this study it was found that, in general, *primary and secondary alkylamines* yield (III), whereas the *oxygen nucleophiles* studied (acetate, formate, hydroxide, and phosphate) give II. The deuterium solvent kinetic isotope effects were determined as k_{H_2O}/k_{D_2O} = 2.23 for the hydrolysis catalyzed by acetate and k_{H_2O}/k_{D_2O} = 1.23 for imidazole. The Brönsted β values found for nucleophilic attack of amines is 0.92, whereas that for the catalysis of hydrolysis by the oxygen bases is 0.62 (Figure 1).

Spectrophotometric studies with 2,2,2-trifluoroethoxide, showed that both III and II are formed. The overall rate of appearance of II in the presence of ∼0.1 M trifluoroethanol is $^1/_{20}$th that anticipated from $k_{HO}[HO^-]$ at pH 11.5. Studies with ethanol at pH 10.35 show no such similar depression of the hydroxide rate with increasing ethanol concentration. Work done in neat trifluoroethanol (1.16 N in trifluoroethoxide) shows a product whose spectrum (λ_{max} 277 nm) is very similar to that of II (λ_{max} 275 nm). The diketopiperazine, III, has a λ_{max} of 292 nm in the same trifluoroethanol solution, thereby excluding it as the product. The ir spectrum of reaction products formed on a preparative scale in neat trifluoroethanol shows the appearance of a new carbonyl stretching band at 1760 cm^{-1} and no typical carboxylic acid absorption in the 2600- to 3300-cm^{-1} range in agreement with formation of an ester of II.

Spectrophotometric studies of the reaction of I at constant pH in the presence of ammonia showed that three products II (*via* HO$^-$-catalyzed hydrolysis), III, and *N*-acetyldehydrophenylalanyl-L-prolinamide (IV) are formed. Buffer dilution studies established the disappearance of I to be both first and second order in total ammonia buffer concentration, [NH_{3T}]. Product analysis of the reaction mixture showed that formation of III is proportional to both a first- and second-order kinetic term in ammonia concentration, whereas formation of IV is proportional only to a second order term in [NH_{3T}].

$$\frac{d[III]}{dt} = k_n[NH_{3T}] + k_{n2}[NH_{3T}]^2 \qquad (8)$$

$$\frac{d[IV]}{dt} = k'_{n2}[NH_{3T}]^2 \qquad (9)$$

leading to

$$\frac{[III]}{[IV]} = \frac{k_n[NH_{3T}] + k_{n2}[NH_{3T}]^2}{k'_{n2}[NH_{3T}]^2} \qquad (10)$$

A plot of [III]/[IV] *vs.* 1/[NH_{3T}] was found to be linear (plot

TABLE I: Rate Constants of Nucleophiles in the Reaction of III ($\mu = 1.0, T = 30.0°$).

Nucleophile	pK_a	Concn (M) Range	pH Range	No. of k_{obsd} Detmd	k_1 (1 mole^{-1} min^{-1})
1. HPO_4^-	6.7	0.214–0.680	6.93–5.97	8	0.043
2. ^-OH	15.6		9.03–10.35	6	6080
3. CH_3COO^-	4.74	0.2–1.0	4.74	4	0.00504
4. $HCOO^-$	3.6	0.36–1.0	3.53	3	0.0019
5. Tris	8.10	0.02–0.10	8.05	4	0.762
6. Gly-Gly[a]	8.17	0.02–0.10	8.03–8.89	12	8.51
7. Imidazole[a]	6.97	0.1–0.5	7.0–7.2	10	6.52
8. Morpholine[a]	8.3	0.02–0.10	8.48	4	3.70
9. Ethylenediamine	7.05[a]	0.01–0.15	7.76–7.76	20	0.676
	10.1				741.0
10. $CF_3CH_2NH_2$[a]	5.60	0.12–0.25	5.55	4	0.0466
11. NH_2NH_2[a]	7.65	0.03–0.07	7.75	4	318.0
12. $HOCH_2CH_2NH_2$	9.48	0.06–0.23	9.00	4	328
13. CH_3ONH_2	4.69	0.2–1.0	3.88–4.81	16	0.318
14. NH_3[a]	9.23	0.055–0.330	9.56	5	37.1
15. Semicarbazide	3.65	0.3–1.0	3.88	4	0.032
16. Triethylenediamine	6.17	0.4–1.0	6.17	3	0.00063
17. *tert*-Butylamine	10.6	0.14–0.48	10.60	4	*b,c*
18. *N*-Ethylmorpholine	7.7	0.2–1.0	7.7	4	*b,c*

[a] $\mu = 0.5$. [b] No catalysis of hydrolysis found. [c] Compound II formed as product.

not shown), and of slope k_n/k'_{n2} and intercept k_{n2}/k'_{n2}. From the kinetic eq 11, which relates the pseudo-first-order rate

$$k_{obsd} = k_{HO}[HO^-] + k_n[NH_{3T}] + k'_{n2}[NH_{3T}]^2 + k_{n2}[NH_{3T}]^2 \qquad (11)$$

constant (k_{obsd}) for disappearance of I to the concentrations of HO^- and NH_{3T}, eq 12 is derived. From eq 12, a plot not

$$\frac{k_{obsd} - k_{HO}[HO^-]}{[NH_{3T}]} = k_n + (k'_{n2} + k_{n2})[NH_{3T}] \qquad (12)$$

shown of $(k_{obsd} - k_{HO}[HO^-])/[NH_{3T}]$ *vs.* $[NH_{3T}]$ is of slope $(k'_{n2} + k_{n2})$ and intercept k_n. From the values of k_n/k'_{n2} obtained from the plot of eq 10 and the values for $k'_{n2} + k_{n2}$ and the k_n obtained from a plot of eq 12, the apparent rate constants based on total $[NH_3] + [NH_4^+]$ at pH 9.56 were determined to be approximately $k_n = 12.9$ min^{-1} M^{-1}, $k_{n2} = 22.6$ min^{-1} M^{-2}, and $k'_{n2} = 34.3$ min^{-1} M^{-2}.

α-Effect Compounds. The reaction of hydrazine with I gives III as the only product whereas methoxylamine and semicarbazide yield both III and the corresponding amide of II. For methoxylamine, third-order rate constants were obtained from plots of $k_{obsd}/[\text{amide}_T]$ *vs.* $[\text{amide}]_T$ at several pH values. The termolecular term for methoxyaminolysis is composed of both general acid and general base assisted nucleophilic terms, such that

$$k_{n2}^{obsd} = \left[\frac{k_{ga}a_H}{(K_a + a_H)} + \frac{k_{gb}K_a}{(K_a + a_H)}\right]\frac{K_a}{(K_a + a_H)} \qquad (13)$$

From eq 13 it follows that

$$k_{n2}^{obsd}(K_a + a_H)^2 = k_{ga}K_a a_H + k_{gb}K_a^2 \qquad (14)$$

From eq 14 a plot of the left-hand side of the equation *vs.* a_H is of slope $k_{ga}K_a$ and intercept $k_{gb}K_a^2$ (Figure 2). The calculated specific constants were found to be $k_{gb} = 2.65$ min^{-1} M^{-2} and $k_{ga} = 3.08$ min^{-1} M^{-2}. Product analysis of the methoxylamine reaction carried out at pH 6.5 ($\mu = 1.0$; pH maintained constant with a pH-Stat) established the amide to be the major product (λ_{max} (final) 281 nm). Products of methoxylaminolysis at pH 4.7 contained a larger amount of III than that present at pH 6.5 (λ_{max} (final) 268 nm). These results suggest that k_{gb} is associated more with nucleophilic attack at the lactam carbonyl and k_{ga} at the *N*-acetylcarbonyl group, respectively. This is born out by comparison of product analysis and kinetic terms. If one assumes that unassisted

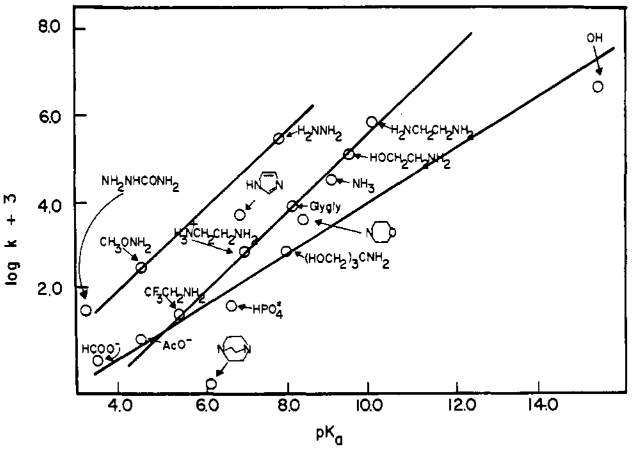


FIGURE 1: Plot of pK_a of conjugate acid of nucleophilic species *vs.* the log of the calculated second-order rate constant (M^{-1} min^{-1}) for reaction of nucleophile with *N*-acetyldehydrophenylalanyl-L-proline diketopiperazine.

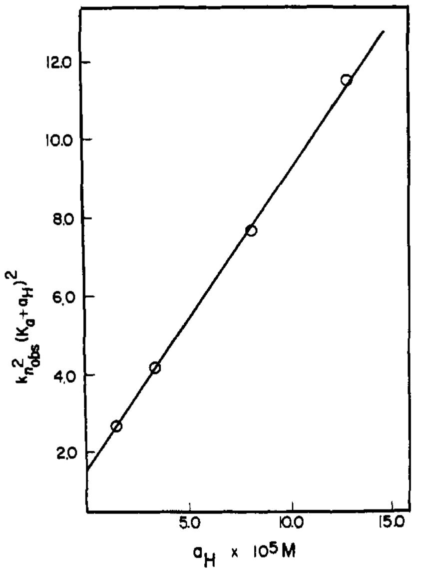


FIGURE 2: Plot of the apparent termolecular rate constants $[k_{n_2}{}^{\mathrm{obsd}}]$ times the sum squared of the dissociation constant of the conjugate acid of methoxylamine plus the hydrogen ion activity $[(K_a + a_H)^2]$ *vs.* hydrogen ion activity. From the slope and intercept the values of the termolecular constants for self-assisted general acid catalysis (k_{ga}) and general base catalysis (k_{gb}) may be derived.

nucleophilic attack (k_n) of methoxylamine yields only III (as in the case of all other amines), then it can be shown at pH 6.5 where only free methoxylamine is present that

$$\frac{[\text{amide}]}{[\text{III}]} \neq \frac{k_{gb}[\text{CH}_3\text{ONH}_2]^2}{k_n\,[\text{CH}_3\text{ONH}_2]} \qquad (15)$$

One explanation of the fact that $k_{gb}\,[\text{CH}_3\text{ONH}_2]^2/k_n[\text{CH}_3\text{ONH}_2] > [\text{amide}]/[\text{III}]$ is that the free amine assistance of nucleophilic attack is also occurring (as in the case of NH_3) at the acetyl position, such that

$$\frac{[\text{amide}]}{[\text{III}]} = \frac{k_{gb}{}^{\text{amide}}[\text{CH}_3\text{ONH}_2]}{k_{gb}{}^{\text{III}}[\text{CH}_3\text{ONH}_2] + k_n{}^{\text{III}}[\text{CH}_3\text{ONH}_2]} \qquad (16)$$

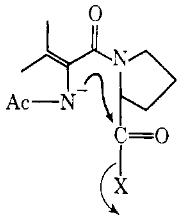
where k_{gb} determined kinetically equals $k_{gb}{}^{\text{III}} + k_{gb}{}^{\text{amide}}$; the amine assisted general base catalysis of attack of amine to provide III and the amide of II as products.

Tertiary Amines. Hydrolysis of I was catalyzed by imidazole but not by the tertiary alkyl amines *N*-ethylmorpholine and only very slightly by triethylenediamine. The product of the reaction in both *N*-ethylmorpholine and triethylenediamine buffers was II, whereas the product of the imidazole-catalyzed reaction was III.

Discussion

N-Acetyldehydrophenylalanyl-L-proline diketopiperazine I possesses two imide bonds which are susceptible to nucleophilic attack; these are an acetyl imide bond whose scission yields dehydrophenylalanylproline diketopiperazine III and an endocyclic imide bond whose hydrolytic scission yields the *N*-acetyl dipeptide, *N*-acetyldehydrophenylalanylproline (II). Our purpose has been to determine the selectivity of nucleophiles (if any) in attacking the endo- and exocyclic imide bonds and to provide a mechanistic rationale for this selectivity. Our experimental findings may be summarized as follows. (1) Primary and secondary amines, hydrazine, ammonia, and imidazole react with I *via* unassisted nucleophilic attack (k_N[N:]) at the *N*-acetyl carbonyl to yield III. (2) Ammonia, methoxylamine, and semicarbazide exhibit in their reaction with I, terms second order in buffer indicating general base (k_{gb}[N:]^2) and/or general acid (k_{ga}[N:][NH]) catalysis of nucleophilic attack and yield both II and III. (3) The oxygen bases (formate, acetate, phosphate, trifluoroethoxide, hydroxide, as well as Tris) react with I to yield the dipeptide II. In the case of trifluoroethoxide III is also obtained. The deuterium kinetic solvent isotope effect ($k_n{}^{\text{H}_2\text{O}}/k_n{}^{\text{D}_2\text{O}}$) was determined to be 2.23 for the reaction of acetate with I. (4) Trifluoroethoxide but not ethanol greatly depresses the rate constant ($k_{\text{HO}}[\text{HO}^-]$) for hydrolysis of I.

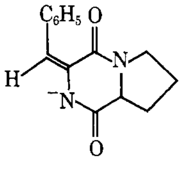
The selectivity of bases toward the endo- and exocyclic imide bonds of I to yield II or III, respectively, may be explained if one accepts the following concepts: (*i*) nucleophilic attack to yield III is favored by the lower basicity of the leaving group and the fact that production of an acyl derivative of II is difficult due to the retrograde intramolecular displacement by imide anion to regenerate I (V; intramolecular attack of imide anion upon the ester bond is a well-studied reaction (Bruice and Benkovic, 1966a,b); and (ii) formation of II is favored due to release of ring strain (the greater lability of diketopiperazines to hydrolysis, as compared to peptides, has been suggested to be due to ring strain (Corey, 1938; Edwards and Meacock, 1958; Sykes *et al.*, 1966) so that when leaving tendency is of little consequence both II and III are favored. Also, if both leaving tendency and retrograde imide anion displacement are unimportant then relief of ring strain remains and II is the sole product.



V

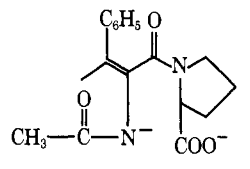
In what follows, or for that matter in any study of competing processes, it must be understood that reaction paths are identified by product analysis and that inability to detect a few percent or more of a minor product entails the assumption that the reaction path is not present. Thus, if substrate passes through path A to yield 90% of A and through path B to yield an undetectable 10% of B the assumption is made that the reaction is specific for A. Obviously, for this case, $\Delta F_B{}^{\ddagger}$ exceeds $\Delta F_A{}^{\ddagger}$ by but 1.3 kcal/mole^{-1} which hardly falls into the range of high selectivity. More importantly, this feature assures us that within a series of like nucleophiles competing for two positions on a substrate the entire series may attack at one position but in a few instances attack may occur at both positions. To rationalize these exceptions may require the explanation of physical features which must account for a difference of as little as a kilocalorie or less in free energies of activation. To attempt to do so is often unrealistic. The same considerations may apply to competing paths for attack at a single position which differ in kinetic order (*i.e.*, k_n[N:] *vs.* k_{gb}[N:]^2). Therefore, with some trepidation we proceed.

The pK_a values of the N–H bonds of III and II have been determined to be 10.8 and 13.9, respectively. Thus, III$^-$ is a better leaving group than is II$^-$. Consideration of this feature



III$^-$

pK'_a of formation 10.8



II$^-$

13.9

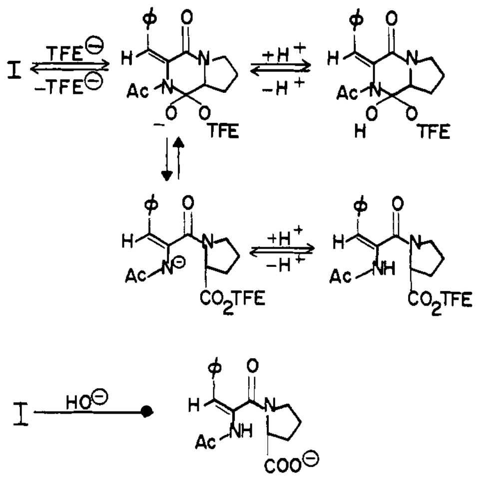
alone dictates that nucleophilic attack should be favored at the acetyl carbonyl carbon which is as observed in simple nucleophilic attack of the nitrogen bases.

As the difference in the pK_a values of the conjugate acids of the amine nucleophile and leaving group become greater it is anticipated that displacement of the leaving group by amine nucelophile should become most difficult and that general catalysis of nucleophilic attack should come into play. This expectation is based on generally known and widely appreciated experimental results (Bruice and Benkovic, 1964; Kirsch and Jencks, 1964) and is amplified by the fact that the unassisted aminolysis reaction is quite sensitive to the basicity of the amine, as shown by a Brønsted β value of 0.92 (Figure 1). In addition, it is known that the α-effect provides a greater rate enhancement to the general catalytic terms k_{gb} and k_{ga} than to the nucleophilic term k_n for amines (Bruice and Benkovic, 1964; Fedor and Bruice, 1964). It is to be expected, therefore, that k_{gb} and k_{ga} paths should favorably compete with the k_n path for α-effect amines of low pK_a as seen for methoxylamine and semicarbazide. Unlike the aminolysis of acetylimidazole free base (Jencks and Gilchrist, 1968; Fersht and Jencks, 1970), there is no evidence for general catalysis in reaction of primary and secondary amines with I to yield III. This may be attributed to the pK_a of the leaving groups in the case of acetylimidazole (14.2) (Bruice and Herz, 1964) and I (10.8). Since in general catalysis, protonation of the leaving group or proton abstraction from the attacking nucleophile is occurring in the transition state, the relative base strength of nucleophile and leaving group is of less importance and this levelling effect should lead to less selectivity. This feature explains the fact that methoxylamine, semicarbazide, and ammonia through their general catalytic paths yield with I both III and amides of II.

Nucleophilic attack of oxygen nucleophiles, save hydroxide ion, should be disfavored at the ring carbonyl group due to the expectation that the product would not be stable since intramolecular imide anion attack should regenerate I (V). Indeed, this appears to be the case for trifluoroethoxide ion and the decrease in the rate constant for hydroxide ion catalysis of the formation of II on increase in trifluoroethanol concentration finds explanation in initial attack of the alkoxide ion upon I to yield an equilibrium concentration of the ester of II (Scheme I). Since hydroxide ion yields from I only II the fact that III is formed in the presence of trifluoroethanol can only mean that the alkoxide ion can attack at both imide bonds. This may be attributed to the fact that the pK_a' of trifluoroethanol (12.36) (Ballinger and Long, 1959) is comparable to those of the leaving groups. The greater proportion of product arises from attack at the acetyl carbonyl to expel the better leaving group.

It is anticipated that general base assistance of attack of water should be the preferred mode of reaction of formate, acetate, and phosphate with I. This expectation follows from the large difference in pK_a values of the conjugate acids of the oxygen bases and the leaving group in formation of either II or III. General base assistance of the attack of H_2O (*i.e.*, k_{gb}[N:][H_2O]) has been reported for carboxyl anion nucleophiles ($RCOO^-$) to come into play in the hydrolysis of phenyl acetate when the difference in pK_a of RCOOH and pehnol are such that simple nucleophilic attack (k_n[N:]) is precluded (Gold *et al.*, 1968). That the mechanism is general base assistance of water attack is borne out by the deuterium solvent kinetic isotope effect of greater than two for acetate. Similar results were obtained in the study of the hydrolysis of *N*-acetylimidazole, where the weak oxygen bases were found to participate as general base catalysts (Oakenfall *et al.*, 1971; Oakenfall and Jencks, 1971). For general base assistance of the attack of water, bond formation in the transition state should be far toward product and in the nucleophilic attack of hydroxide ion the basicity of the entering nucleophile exceeds those for the departing groups. This leads to the expectation that leaving tendency may not have a great effect upon selectivity for the position of attack upon I by HO^- or H_2O catalyzed by weak oxygen nucleophiles. That all oxygen bases bring about hydrolysis of the ring imide bond may be related to the probability of release of strain on opening the diketopiperazine ring and to the fact that intramolecular nucleophilic attack (as in Scheme I) to regenerate, though not impossible (Hegarty and Bruice, 1969, 1970), is made energetically less likely due to the requirement of attack of imide anion upon carboxyl anion. It is interesting to note that reaction of hydroxide with I is strictly first order in hydroxide ion. This feature is shared in the HO^- catalysis of hydrolysis of acetylimidazole (leaving group 14.2). In contrast, *p*-nitroacetanilide and *p*-formylacetanilide hydrolysis (leaving group pK_a' = 13.8 and 14.1, respectively) exhibit terms second order in HO^- dictating product to arise *via* dianionic tetrahedral intermediates (Eriksson and Bratt, 1967; Pollak and Bender, 1970; Bender and Thomas, 1961; Jencks and Salvesen, 1971).

SCHEME I



An anomaly exists in the reaction of ammonia with I where it is found to be subject to self general catalysis though primary and secondary amines of greater and much less basicity do not exhibit general catalytic terms. One possible explanation for this finding may reside in the lessened steric requirement of ammonia. Thus, it has been well established that steric requirements are amplified in k_{gb} and k_{ga} terms as compared to k_n in the aminolysis of thiol and phenyl esters (Bruice *et al.*, 1967; Fedor and Bruice, 1964). That steric requirements may be particularly important in reactions at the imide bonds of I finds support in the fact that the two tertiary amines examined

(*N*-ethylmorpholine and triethylenediamine) show no and little reactivity, respectively, toward I.

Added in Proof

The structures of I and derived products have been drawn with the phenyl ring cis to the ring carbonyl group. Recent X-ray and nmr data of Brocklehurst *et al.* (1971) dictate that the structures should have a trans configuration. This in no way influences the results of this study.

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Aspartokinase I–Homoserine Dehydrogenase I of *Escherichia coli* K12 λ. Subunit Molecular Weight and Nicotinamide–Adenine Dinucleotide Phosphate Binding†

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ABSTRACT: The aspartokinase I–homoserine dehydrogenase I complex isolated from *Escherichia coli* K12 was denatured in urea and sodium dodecyl sulfate and the subunits produced were examined by dodecyl sulfate–polyacrylamide gel electrophoresis and found to be homogeneous in size with a molecular weight of 85,000. The binding of NADP$^+$ by the enzyme was investigated at pH 8.79 in the presence of 0.01 M L-threonine by gel filtration. The number of NADP$^+$ binding sites per enzyme subunit was found to be 0.50 ± 0.01 and the dissociation constant for the enzyme–NADP$^+$ complex was determined to be 5.0 ± 0.2 μM. The significance of these findings to the possible structure of the native aspartokinase I–homoserine dehydrogenase I complex is discussed.

The aspartokinase I–homoserine dehydrogenase I (AKI–HSDI) complex isolated from *Escherichia coli* possesses properties which make it particularly interesting from a functional as well as a structural standpoint. A number of its properties suggest that this enzyme complex may play a key role in the control of the rate of biosynthesis of some of the

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