TABLE IV

THE DETERMINATION OF THE SOLUBILITY OF α-AMINOPHENYLACETIC ACID IN IMIDAZOLE BUFFERS AT pH 8.6 AND 7.06 (30°; μ = 1.0 M with KCl)

pH 8.6; T = 30°; intercept gave $A = 27.9 \times 10^{-3}$ M

$(IM)(IMH^{\oplus})$ [(moles/l.)^2 × 10^2]	Solubility of amino acid—A [moles/l. × 10^2]
4.96	6.7
4.96	6.5
3.72	2.8
2.79	2.9
1.95	2.0
1.24	0.6
0.31	1.2
0.195	0.3

pH 7.06; T = 30°; intercept gave $A = 21.6 \times 10^{-3}$ M

$(IM)(IMH^{\oplus})$ [(moles/l.)^2 × 10^2]	Solubility of amino acid—A [moles/l. × 10^2]
3.98	18.7
2.99	11.4
2.24	11.3
0.25	1.9

260 mμ; Beer's law calibration curves were prepared for the amino acid in 1% HCl solutions and were used for the analyses.

The formation constants were found to be approximately independent of pH only if calculated for a complex of amino acid containing one free imidazole species and one imidazolium ion. The formation constants thus were calculated on the basis of equation 12 in which K_m^1 represents the formation constant for the complex, A_c the amino acid complex and A the free amino acid

$$K_m^1 = \frac{A_c}{A(IM)(IMH^{\oplus})} \quad (12)$$

The value of K_m^1 thus was determined from a plot of the solubility of the amino acid (in moles/l.) *vs.* the corresponding concentration of imidazole buffer (expressed as $(IM)(IMH^{\oplus})$) to give straight lines of slope $A_c/(IM)(IMH^{\oplus})$ and intercept A. The results are tabulated in Table IV.

The results of Table IV indicate the trend of greater solubility of amino acid with increasing imidazole buffer concentrations. As, however, under the conditions of the experiment, a solubility difference of only *ca.* 5 mg. of amino acid is involved between the media of lowest and highest imidazole buffer concentrations, a relatively large though unavoidable experimental error allows only an approximate assignment of the value of K_m^1. Thus the results at pH 8.6 give a value for K_m^1 of 37 and at pH 7.06, K_m^I was evaluated to be 21. The dissociation constant of the complex, $1/K_m^1$, is thus approximately 20–50 × 10^{-3} mole^2 liter^{-2} and may be seen to be at least comparable to the value of the dissociation constant (K_m = 7.2 × 10^{-3} mole^2 liter^{-2}) of the aldimine complex derived from the Michaelis–Menten kinetics. Such agreement provides further support for the postulate of complex formation on which the kinetic scheme is founded.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

Catalytic Reactions Involving Azomethines. III.[1] The Influence of Morpholine upon the Imidazole Catalysis of the Transamination of Pyridoxal by α-Aminophenylacetic Acid. The Transamination of the Morpholine Imine of Pyridoxal

BY THOMAS C. BRUICE AND RICHARD M. TOPPING[2]

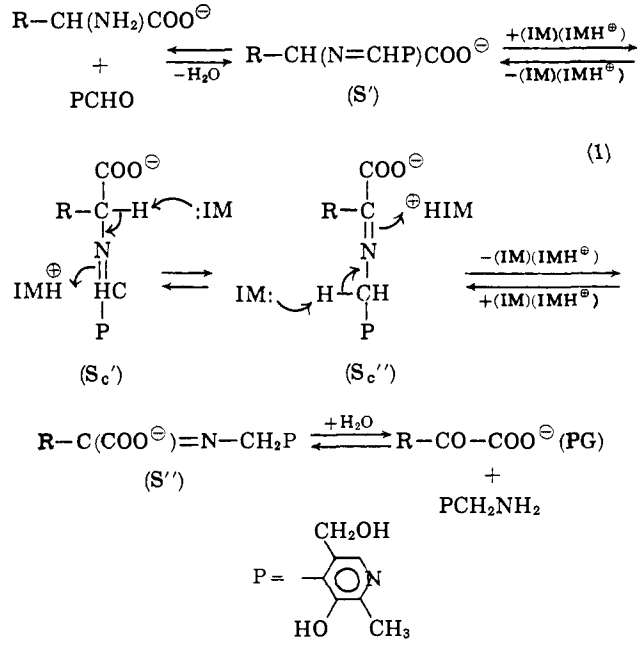
RECEIVED DECEMBER 21, 1962

The influence of morpholine upon the rate and final equilibrium position of the imidazole-catalyzed transamination of pyridoxal (and the resultant morpholine imine of pyridoxal) by α-aminophenylacetic acid has been investigated. Morpholine is found to provide no apparent catalysis under the experimental conditions and the conversion of pyridoxal to the morpholine imine is shown to result in no significant change in the kinetic characteristics of the transamination reaction. The study is suggested to provide a model for the enzymatic process in which the pyridoxal co-enzyme is bound to the enzyme surface through an azomethine link necessitating a "transimination" step prior to transamination.

Introduction

In the preceding papers[1] it has been established that imidazole catalyzes the transamination of pyridoxal by α-aminophenylacetic acid *via* a pathway involving pre-equilibrium complex formation of the intermediate imines with one molecule of imidazole and one ion of the conjugate acid of imidazole. The prototropic shift leading to the reversible interconversion of aldimine (S′) and ketimine (S″) has been postulated to occur *via* intracomplex general acid–general base catalysis (*i.e.*, in (1) $S_c' \rightleftarrows S_c''$).

In the enzymatic catalysis of the transamination of pyridoxal phosphate by amino acids, the pyridoxal phosphate is present on the enzyme surface in combination with the ε-amino group of a lysine residue as an imine.[3-5] The formation of the imine of substrate and enzyme-bound cofactor then occurs *via* a "transimination" reaction (2). It is generally known that imines are more reactive toward "carbonyl reagents" than are



the corresponding carbonyl compounds themselves.[6] The possibility thus arises that reaction 2 may facilitate

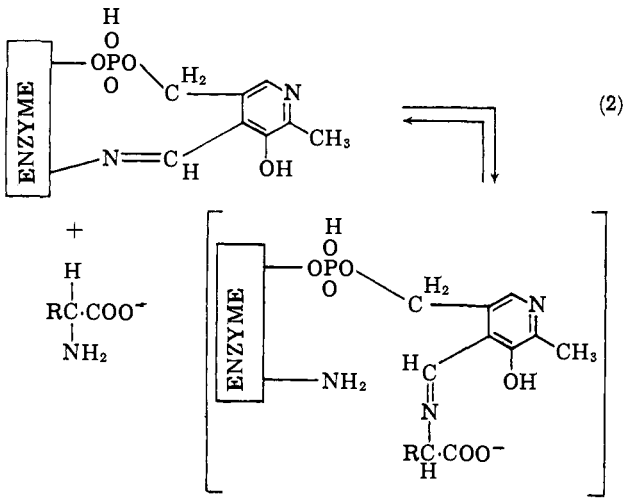
(1) Previous paper in this series, T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **85**, 1488 (1963).

(2) Post-doctoral Fellow of The Department of Chemistry, Cornell University.

(3) E. H. Fischer, A. B. Kent, E. P. Snyder and E. G. Krebs, *J. Am. Chem. Soc.*, **80**, 2906 (1958).

(4) E. H. Fischer and E. G. Krebs, Abstracts 136th National Meeting of the American Chemical Society, p. 24C.

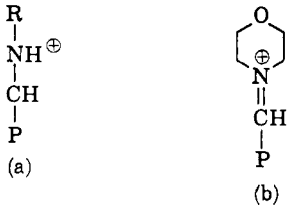
(5) W. T. Jenkins, *Fed. Proc.*, **20**, 978 (1961).



(2)

Enzyme-substrate compound

the enzymatic reaction and serve as a means of activating the carbonyl group of pyridoxal phosphate.[7] From studies of semicarbazone formation from pyridoxal phosphate in the presence of excess morpholine, Cordes and Jencks[7] have concluded that the most reactive species of pyridoxal phosphate imines toward transimination is protonated (3a) and that the morpholine imine (3b) serves as a useful model of the protonated imine of a primary amine.



(3)

(a) (b)

In this study the influence of morpholine upon the rate and final equilibrium position of the imidazole-catalyzed transamination of pyridoxal (and the resultant morpholine imine of pyridoxal) by α-aminophenylacetic acid is described.

Results[8] and Discussion

The present study was carried out under the conditions of acidity and temperature (pH 8.62 ± 0.02, T = 30°) employed in part I[9] of this series. The "apparent" formation constant (K_{Mo}) for the imine of morpholine and pyridoxal was determined from the decrease in absorbance of pyridoxal at 395 mμ upon addition of morpholine (4).

$$K'_{Mo} = \frac{S_T}{PCHO_T \cdot M_o} \qquad (4)$$

where $PCHO_T$ and S_T represent total active ($PCHO_a$ and S_a) and inactive ($PCHO_i$ and S_i) forms of pyridoxal and of the morpholine imine of pyridoxal, respectively. The term "inactive" refers to all forms (*e.g.*, hydrates, hemiacetal and carbinolamine forms) which will not react with amino acid to give the aldimine. Consider the equilibria

$$PCHO_i \overset{K_o}{\rightleftharpoons} PCHO_a$$
$$S_i \overset{K_s}{\rightleftharpoons} S_a \qquad (5)$$
$$PCHO_a + Mo \overset{K_{Mo}}{\rightleftharpoons} S_a$$

It readily may be shown that

$$K_{Mo} = \frac{K_s(1 + K_o)S_T}{K_o(1 + K_S)PCHO_T \cdot Mo} \qquad (6)$$

A summary of the equilibrium determinations is presented in Table I. The average value of 28.1 ± 5.6 M^{-1} agrees favorably with the values of 24 M^{-1} determined kinetically and 30 M^{-1} determined spectrophotometrically by Cordes and Jencks[7] under identical conditions of pH as employed in our study but at a temperature of 25°.

TABLE I

APPARENT FORMATION CONSTANT FOR THE IMINE OF MORPHOLINE AND PYRIDOXAL AT pH 8.6, T = 30°, μ = 1.0 M IN WATER

(Initial concentration of pyridoxal = 10^{-4} M)

Concn. of morpholine employed (M)	Remaining concn. of $PCHO_T$ (M)	K'_{Mo} (M^{-1})
0.00	1.0×10^{-4}	..
.01	0.8×10^{-4}	25
.05	$.5 \times 10^{-4}$	20
.2	$.116 \times 10^{-4}$	38.1
.4	$.078 \times 10^{-4}$	29.5

Using data from runs made below morpholine concentrations of 0.2 M (in which absorption at 395 mμ caused by pyridoxal is still discernible), the extinction coefficients of pyridoxal and of the ketimine (S″) both at 246 mμ were evaluated employing the method described in part I.[9] Within the limits of experimental error the determined coefficients remained constant in the presence of morpholine and had the same values as previously determined in the absence of morpholine.

In the experiments to be described, a concentration of imidazole of 1.8 M was employed. We have shown previously that at this concentration and at the pH employed the reaction is saturated with catalyst.[9] Under these conditions the influence of varying morpholine concentrations on the rate of attainment and position of equilibrium in phase one was determined. The presence of morpholine did not alter the over-all kinetic description of the reaction as detailed in part I[9] (*i.e.*, similar location of isosbestic points at 310 and 282 mμ, close adherence to first-order kinetics and virtual completion of phase one prior to the onset of phase two, see part I). The methods of calculation have been presented in part I.[9] The experimental results are provided in Table II. Examination of Table II reveals that as morpholine is added to the system the observed first-order rate constant (k_{obsd}) for attainment of equilibrium in phase one and the equilibrium constant (K_∞) for phase one correspondingly decrease. Both the observed rate constants, k_{obsd}, and the quantity $S''_{T\infty}$ are directly related to the concentration of the aldimine, S′, and thus the observation that $k_{obsd}/S''_{T\infty}$ remains constant (186 ± 5) suggests that morpholine has no effect upon the rate constant for the prototropic shift but merely influences the balance of equilibria prior to formation of S′. Experiments run also at pH 8.6, in the presence of morpholine concentrations

(6) Mme. Bruzau, *Ann. Chem.* [11], **1**, 332 (1934); E. A. Brodhag and C. R. Hauser, *J. Am. Chem. Soc.*, **77**, 3024 (1955); C. R. Hauser and D. S. Hoffenberg, *ibid.*, **77**, 4885 (1955); T. I. Crowell and F. A. Ramirez, *ibid.*, **73**, 2268 (1951); T. I. Crowell and D. W. Peck, *ibid.*, **75**, 1075 (1953); R. L. Hill and T. I. Crowell, *ibid.*, **78**, 2284 (1956); G. M. Santerre, C. J. Hansrote and T. I. Crowell, *ibid.*, **80**, 1254 (1958); R. K. McLeod and T. I. Crowell, *J. Org. Chem.*, **26**, 1094 (1961).

(7) E. H. Cordes and W. P. Jencks, *Biochemistry*, **1**, 773 (1962).

(8) Abbreviations employed for the concentrations of reactants, intermediates and products are: PCHO, pyridoxal; PCH_2NH_2, pyridoxamine; A, α-aminophenylacetic acid; PG, phenylglyoxylic acid; Mo, morpholine; S, the imine from pyridoxal and morpholine; S′, the imine from pyridoxal and α-aminophenylacetic acid; S″, the imine from phenylglyoxylic acid and pyridoxamine. The complexes of the various species with one molecule of imidazole free base (IM) and one imidazolium ion ($IMH^\oplus$) are indicated by the subscript c (S_c', A_c, S_c''). $S_T'' = S_c'' + S''$; P_T = PCHO + S + S′ + S_c'; $A_T = A + A_c$. Equilibrium concentrations at infinite times are denoted by the subscript ∞.

(9) T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **85**, 1480 (1963).

of 0.1 to 1.0 M in which imidazole buffer is omitted from the system, provide no observable reaction, again indicating the lack of catalytic activity of morpholine under the described experimental conditions.

TABLE II

THE INFLUENCE OF MORPHOLINE UPON THE RATE AND EQUILIBRIUM CONSTANTS FOR THE IMIDAZOLE (1.8 M) CATALYZED TRANSAMINATION OF PYRIDOXAL (10^{-4} M) BY α-AMINOPHENYLACETIC ACID (10^{-4} M)

(pH 8.6, T = 30°, μ = 1.0 M)

Morpholine (M)	$k_{obsd} \times 10^5$ (min.$^{-1}$)	$S_{T\infty}''$ ($M \times 10^5$)	$k_{obsd}/S_{T\infty}''$	$K_\infty \times 10^{-4}$	$c \times 10^4$	k_4K_1, l. mole^{-1} min.$^{-1}$
0.00[a]	8.79	5.40	163	2.65	0.64	158
.05	14.48	7.91	183	18.1	.68	272
.10	14.51	7.66	189	14.0	.72	283
.15	13.51	7.58	178	12.9	.76	272
.30	11.86	6.42	185	5.0	.85	265
.60	9.65	4.90	197	1.9	1.03	249
1.00	7.60	4.15	183	1.2	1.25	222

[a] Data from part I.[9]

A simplified representation of the kinetic scheme for phase one, in the presence of morpholine, is provided by

$$\begin{aligned}
&\mathrm{PCHO} + \mathrm{Mo} \overset{K_{Mo}}{\rightleftharpoons} \mathrm{S} \\
&\mathrm{PCHO} + \mathrm{A} \overset{K_1}{\rightleftharpoons} \mathrm{S}' \\
&\mathrm{S} + \mathrm{A} \overset{K_A}{\rightleftharpoons} \mathrm{S}' + \mathrm{Mo} \\
&\mathrm{A} + \mathrm{IM} + \mathrm{IMH}^{\oplus} \overset{K_2}{\rightleftharpoons} \mathrm{A_c} \qquad (7) \\
&\mathrm{S}' + \mathrm{IM} + \mathrm{IMH}^{\oplus} \overset{K_3}{\rightleftharpoons} \mathrm{S_c}' \\
&\mathrm{S_c}' \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{S_c}'' \\
&\mathrm{S_c}'' \overset{K_5}{\rightleftharpoons} \mathrm{S}'' + \mathrm{IM} + \mathrm{IMH}^{\oplus}
\end{aligned}$$

The influence of acidity need not be considered since the present study was carried out at constant pH. Assuming that the species whose concentration is measured at 395 mμ represents $PCHO_T$, $K_2 \simeq K_3$ and K_1 is between 1 and 100[10] and $A_T = 10^{-4}$ M

$$S_c' = PCHO_T A_T \left[\frac{K_1K_3(IM)(IMH^{\oplus})}{(1 + K_3(IM)(IMH^{\oplus}))(K_{Mo}M_o + 1)}\right] \qquad (8)$$

$$S_c'' = S_T'' \left[\frac{K_5(IM)(IMH^{\oplus})}{K_5(IM)(IMH^{\oplus}) + 1}\right] \qquad (9)$$

The rate expression for attainment of equilibrium in phase one is provided by the sum of the rates of the forward and reverse reactions

$$v = k_4[S_c' - S_c''/K_4] \qquad (10)$$

Substituting (8) and (9) into (10) provides (11) if we assume $K_5 \cong K_3$

$$v = \left[\frac{k_4K_1K_3(IM)(IMH^{\oplus})}{(1 + K_3(IM)(IMH^{\oplus}))(K_{Mo}Mo + 1)}\right] \times \left[\frac{PCHO_TA_T - S_T''(K_{Mo}Mo + 1)}{K_1K_4}\right] \qquad (11)$$

At equilibrium, $\frac{dS_c''}{dt} = 0$ and thus

$$K_\infty = \frac{S_{T\infty}''}{PCHO_{T\infty}A_{T\infty}} = \frac{K_1K_4}{(K_{Mo}Mo + 1)} \qquad (12)$$

The reciprocal form of equation 12 indicates that K_{Mo}/K_1K_4 may be evaluated as the slope of the line produced by plotting $^1/K_\infty$ *vs.* morpholine concentration. Examination of Fig. 1 shows that this plot

(10) D. E. Metzler, *J. Am. Chem. Soc.*, **79**, 485 (1957).

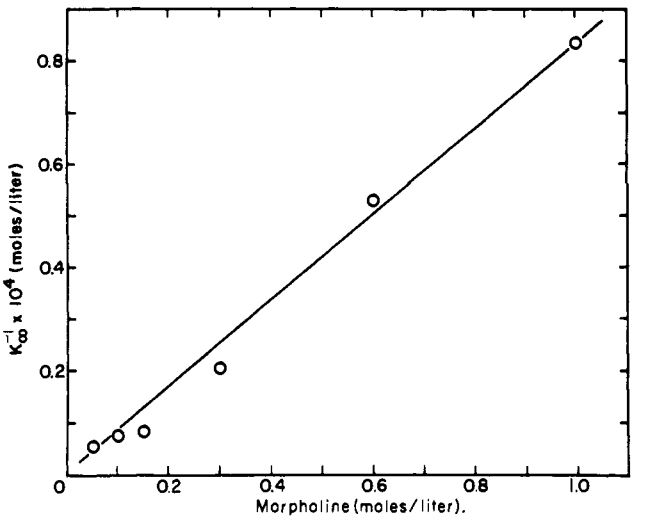


Fig. 1.—Reciprocal plot of equation 13. The slope of the line provides the quantity (K_{Mo}/K_1K_4).

does approximate to a straight line and using K_1K_4 = 2.65 × 10^4 (as determined in part I[9]) leads to a value for K_{Mo} of 2.18.

Integration of (11) leads to an expression (13) for the apparent first-order rate constant for the attainment of equilibrium in phase one

$$k_{obsd} = \frac{k_4K_1K_3(IM)(IMH^{\oplus})c}{(1 + K_3(IM)(IMH^{\oplus}))(K_{Mo}Mo + 1)1.15} \qquad (13)$$

Under the conditions of this study in which $PCHO_o = A_o = a$, the value of c is provided by equation 14

$$c = \left[\frac{a(K_{Mo}Mo + 1)}{K_1K_4} + \frac{(K_{Mo}Mo + 1)^2}{(2K_1K_4)^2}\right]^{1/2} \qquad (14)$$

Under the condition of IM_T = 1.8 M, the reaction previously has been shown to be catalytically saturated and thus (13) reduces to

$$k_{obsd} = \frac{k_4K_1c}{(K_{Mo}Mo + 1)1.15} \qquad (15)$$

Examination of column 7 of Table II reveals that the solution of equation 15 for k_4K_1 is approximately a constant (261 ± 16), showing that the kinetic scheme described by (7) in which morpholine plays no catalytic role but merely affects the over-all equilibrium by participating in certain equilibria prior to the prototropic shift is in accord with the experimental results. Experimental data, detailed in Table II, indicate that the term $k_{obsd}/S_{T\infty}''$ is independent of the concentration of morpholine added to the system and has a constant value of 186 ± 5. Division of (15) by (12) gives

$$k_{obsd}/S_{T\infty}'' = \frac{k_{-4}c}{PCHO_{T\infty}A_{T\infty} \cdot 1.15} \qquad (16)$$

Thus the constancy of $k_{obsd}/S_{T\infty}''$ is compatible with the proposed reaction scheme provided the c constant (see equation 14) bears a linear relationship to the quantity $PCHO_{T\infty}A_{T\infty}$. That this relationship does indeed approximate to linearity is shown in Fig. 2, in which c = 1.88 × 10^4 $PCHO_{T\infty}A_{T\infty}$.

It may be stated, therefore, that in the presence of morpholine, phase one of the transamination reaction is approximately described by equations 7. The addition of increasing concentrations of morpholine lowers the steady state concentration of S′ by converting S′ to the morpholine imine (S) thus displacing the over-all equilibrium by hindering formation of the ketimine. All evidence suggests that morpholine is not a catalyst in the system. The presence of morpholine provides no reaction in the absence of imidazole and the value of k_4K_1 is independent of the absolute

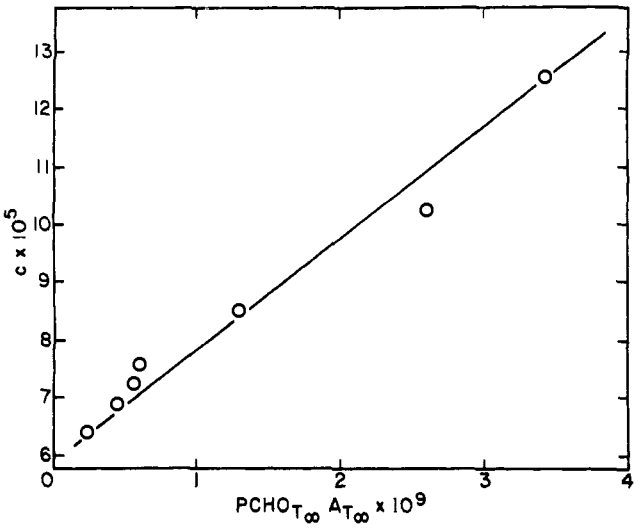


Fig. 2.—The relationship between the c constant (equation 15) and the quantity $PCHOT_{\infty}AT_{\infty}$. Linearity of the plot indicates that the constancy of $k_{obsd}/S''T_{\infty}$ is compatible with the reaction scheme described by equations 7.

morpholine concentration (see Table II). The kinetic scheme as represented by equations 7 is thus seen to predict at least qualitatively the behavior of the transamination reaction in the presence of morpholine. The fact that the value of k_4K_1 obtained in the absence of morpholine (see part I[9]) is almost 40% lower than that obtained in the presence of morpholine reflects the consideration in both treatments of only the kinetically significant rate and equilibrium steps (*i.e.*, the neglect of certain hydration equilibria, etc.). The results show, however, that the transamination reaction is at least as effective when preceded by a 'transimination' reaction in which an imine is converted into an amino acid imine which then undergoes prototropic rearrangement, as is the reaction which involves direct conversion of pyridoxal into the amino acid imine undergoing the prototropic rearrangement. This observation, therefore, suggests that the enzymatic transamination reaction would proceed at least as readily with the pyridoxal phosphate moiety attached to the enzyme surface *via* the aldehyde group and an amino group on the enzyme as with the pyridoxal co-enzyme attached to the protein residue only through the phosphate.

In summary, the "model" for the pyridoxal-requiring transamination reaction, as described in parts I,[9] II[1] and III of this series possesses the following features of enzymological interest: (1) operates in aqueous media; (2) effective at physiological pH values; (3) the reaction is facile at ambient temperatures; (4) employs the weakly basic imidazole and the weakly acidic imidazolium ion as catalysts; (5) does not require metal ions; (6) kinetically similar to enzyme systems in the formation of a complex followed by a rate-determining step affording Michaelis-Menten kinetics; (7) the ability of imidazole to form a catalyst-substrate complex results in a virtual specificity for imidazole at low reactant concentrations compared with other general bases investigated; (8) the transamination reaction in the model system is equally effective if preceded by a transimination reaction; and (9) if it is assumed (a) that the dissociation constants of all the complexes with imidazole and imidazolium ion are quite similar, (b) that the formation constant for aldimine is in the usual range of 1 to 100 and (c) that the acid dissociation constants of intermediates are similar, then it can be calculated[11] that the rate of the prototropic shift leading to the conversion of S_c' to S_c'' is only about 10 to 10^3 times slower than the corresponding step for glutamic-aspartic transaminase.[12]

Experimental

The kinetic procedures have been described in part I.[9] Morpholine (Eastman Kodak, practical) was purified by distillation, after refluxing for 24 hr. over sodium metal, under an atmosphere of dry nitrogen, b.p. 125–126° at 740 mm. (lit.,[13] b.p. 128.3°).

Acknowledgment.—This work was supported by a grant from the National Science Foundation.

(11) T. C. Bruice and R. M. Topping, Proceedings of The Symposium on Pyridoxal Catalysis (Rome, 1962), Pergamon Press, in press.
(12) G. G. Hammes and P. M. Fasella, *J. Am. Chem. Soc.*, in press.
(13) A. L. Wilson, *Ind. Eng. Chem.*, **27**, 867 (1935).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY, LAFAYETTE, IND.]

Reactions of Radicals. V. Reaction of Phenyl Radicals with Aliphatic Disulfides[1-3]

BY WILLIAM A. PRYOR AND PAUL K. PLATT

RECEIVED JANUARY 14, 1963

Phenyl radicals are generated from phenylazotriphenylmethane at 60° in a series of aliphatic disulfides as solvents. The phenyl radicals react with the disulfides both by attack on hydrogen to give benzene and by attack on sulfur to give a phenyl alkyl sulfide. The ratio of phenyl alkyl sulfide to benzene is determined using gas phase chromatography and is equal to the rate of attack by the phenyl radicals on sulfur *vs.* hydrogen for any given disulfide. The data show that phenyl radicals attack disulfides mainly on sulfur, but that the proportion of the attack on sulfur decreases as the sulfur atom becomes more hindered. Thus, 98% of the attack is on sulfur in methyl disulfide and 49% in *t*-butyl disulfide. Thiols are formed in these reactions, but data can be obtained in regions in which the thiol concentration is too low to affect the product composition. At higher thiol concentrations, an appreciable amount of the benzene is formed from the reaction of phenyl radicals with thiol, and here the relative rate of reaction of phenyl radicals with thiol and disulfide can be obtained. The data show that the phenyl radical reacts 23-fold faster with 2-propanethiol than with isopropyl disulfide, and 8-fold faster with propanethiol than with propyl disulfide. The reactivity of the phenyl radical is discussed.

Introduction

Elucidation of the factors which influence radical reactivity requires a body of data on the rate profile of various radicals with typical organic compounds, and increasing attention is being given to collecting such data. The earliest reactivity profiles were obtained from polymerization transfer studies, in which the growing polymeric radical is allowed to compete between adding another unit of its own monomer and attacking an added transfer agent. Studies of this type have led to data on the reactivity of the polystyryl radical with

(1) Part IV, W. A. Pryor, *Proc. Indiana Acad. Sci.*, in press.
(2) Taken in part from the thesis submitted by P. K. P. in partial fulfillment of the requirements for the degree of Master of Science.
(3) This work was supported in part by grant RG-9020 from the National Institutes of Health. Grateful acknowledgment is made to the donors of that fund.