in SbF_5–SO_2ClF solution were prepared as described previously.[2] Nmr spectra were obtained on a Varian A-56-60A nmr spectrometer equipped with a variable-temperature probe. Proton and fluorine shifts are referred to external capillary TMS and $CFCl_3$, respectively.

Registry No.—**1a**, 24154-19-6; **1b**, 24154-20-9; **1c**, 24226-22-0; **1d**, 24154-21-0; **1e**, 39982-15-5; **1f**, 39982-16-6; **1g**, 39982-17-7; **1h**, 39982-18-8; **1i**, 39982-16-6; **1j**, 39982-20-2; **1k**, 39982-21-3; **1l**, 39982-22-4; **2a**, 98-08-8; **2b**, 402-44-8; **2c**, 98-56-6; **2d**, 402-43-7; **2e**, 455-13-0; **2f**, 392-85-8; **2g**, 98-15-7; **2h**, 392-83-6; **2i**, 401-80-9; **2j**, 88-16-4; **2k**, 401-78-5; **2l**, 454-78-4.

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Effect of pK on the Rate of Amine-Catalyzed Cleavage of Diacetone Alcohol

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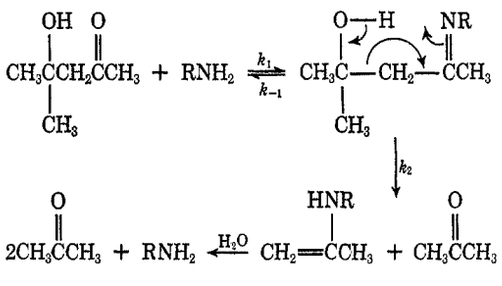
The primary amine catalyzed dealdolization of diacetone alcohol has been investigated with a series of amines whose pK's range from 5.7 to 10.9. Changing the pK of the amine from 10.9 to 5.7 has a small effect on the equilibrium constant for formation of the intermediate ketimine (fourfold decrease) and only a modest effect on the rate of cleavage of ketimine to products (25-fold decrease), indicating a relatively nonpolar transition state. The relevance of these results to the mechanism of aldolase is discussed.

A large class of enzymes, including many aldolases, appears to function *via* the formation of an imine intermediate from a carbonyl group of the substrate and a lysine residue of the enzyme.[1] The replacement of the carbonyl oxygen with the much more basic nitrogen of the enzyme allows facile protonation of the nitrogen and subsequent (or concurrent) acceptance of a pair of electrons from a leaving group to form an enamine. Hydrolysis of the enamine then leads to regeneration of the enzyme. It appears that catalysis by these enzymes is due in large part to a replacement of C=O by C=N.

In an effort to evaluate the contribution of this factor to the rate accelerations caused by these enzymes, much effort has been devoted to catalysis by simple primary amines. A convenient model system for the aldolase enzymes is the primary amine catalyzed dealdolization of diacetone alcohol (Scheme I). This reaction in-

SCHEME I



volves an analogous mechanism to that proposed for the enzymes.[2-4] For catalysis by n-propylamine (pK 10.9) we have shown that the formation of the intermediate ketimine is rapid and reversible followed by slow decomposition to products.[4] In addition we were able to evaluate the individual rate constants k_1, k_{-1}, and k_2. The rate constant for cleavage of imine to products (k_2) is of particular interest since a comparison of this rate to the rate of cleavage of the carbonyl compound itself gives a direct evaluation of the effect of replacing an oxygen by a nitrogen in this system.

The choice of n-propylamine was due to its resemblance to the lysine side chain both in structure and pK. Although the pK of n-propylamine is similar to that normally observed for lysine residues of proteins (p$K \cong 10$), it is not clear that the active site lysine of aldolase has a "normal" pK. In fact, for the related enzyme acetoacetate decarboxylase, the pK of the active site amine group has been found to be about 6.[5] Although the pK of this group in aldolase has not been determined, it is reasonable to suppose that it may be perturbed in a similar manner.

We have now extended our previous work to include a series of primary amines with widely different pK's. Our objectives in this study were threefold: (1) to establish whether there is a change in rate-determining step with changing amine pK; (2) to obtain information concerning the polarity of the transition state for cleavage of the imine; and (3) to determine what, if any, advantage could accrue to an aldolase enzyme if it were to have a lowered active site pK.

Experimental Section

Materials.—Diacetone alcohol and n-propylamine were purified as previously described.[4] Ethanolamine was distilled prior to use. Glycine and glycinamide were reagent grade chemicals used without further purification. 2,2,2-Trifluoroethylamine was prepared by the method of Bissell and Finger.[6]

Kinetic Methods.—For all catalysts except trifluoroethylamine, kinetic measurements were carried out at 260 nm for dealodolization and 235 nm (240 nm for glycinamide) for formation of the imine as described previously.[4] For 2,2,2-trifluoroethylamine, rate constants for conversion of diacetone alcohol

(1) (a) B. L. Horecker, S. Pontremli, C. Ricci, and T. Cheng, *Proc. Nat. Acad. Sci. U. S.*, **47**, 1949 (1961); (b) E. Grazi, P. T. Rowley, T. Cheng, O. Tchol, and B. L. Horecker, *Biochem. Biophys. Res. Commun.*, **9**, 38 (1962); (c) for a general review of the mechanism of action of aldolases see D. E. Morse and B. L. Horecker, *Advan. Enzymol. Relat. Subj., Biochem.*, **31**, 125 (1968).

(2) (a) F. H. Westheimer and H. Cohen, *J. Amer. Chem. Soc.*, **60**, 90 (1938); (b) F. H. Westheimer and W. A. Jones, *ibid.*, **63**, 3283 (1941).

(3) R. W. Hay and K. R. Tate, *Aust. J. Chem.*, **19**, 1651 (1966).

(4) R. M. Pollack and S. Ritterstein, *J. Amer. Chem. Soc.*, **94**, 5064 (1972).

(5) (a) D. E. Schmidt, Jr. and F. H. Westheimer, *Biochemistry*, **10**, 1249 (1971); (b) F. C. Kokesh and F. H. Westheimer, *J. Amer. Chem. Soc.*, **93**, 7270 (1971).

(6) E. Bissell and M. Finger, *J. Org. Chem.*, **24**, 1256 (1959).

TABLE I

RATE OF CLEAVAGE OF DIACETONE ALCOHOL CATALYZED BY AMINES IN AQUEOUS SOLUTION

[Amine][a]	pH[b]	Ionic strength[c]	Temp, °C	k_{obsd}, sec^{-1} [d]
			Ethanolamine	
0.200	9.70	0.4	25.1	$3.17 \pm 0.04 \times 10^{-5}$
0.100	9.70	0.4	25.1	$1.58 \pm 0.05 \times 10^{-5}$
				$k = 1.58 \pm 0.07 \times 10^{-4}\ M^{-1}$ sec^{-1}
			Glycine	
0.200	9.78	0.4	25.1	$2.45 \pm 0.3 \times 10^{-5}$
0.100	9.78	0.4	25.1	1.12×10^{-5}
				$k = 1.17 \pm 0.06 \times 10^{-4}\ M^{-1}$ sec^{-1}
			Glycinamide	
0.472	8.23	1.0	25.0	$2.49 \pm 0.07 \times 10^{-5}$
0.330	8.22	1.0	25.0	$1.66 \pm 0.01 \times 10^{-5}$
0.188	8.21	1.0	25.0	$8.71 \pm 0.26 \times 10^{-6}$
				$k = 4.98 \pm 0.20 \times 10^{-5}\ M^{-1}$ sec^{-1}
			Propylamine	
0.471	11.04	1.0	53.4	$2.22 \pm 0.07 \times 10^{-3}$
0.314	11.04	1.0	53.4	$1.62 \pm 0.07 \times 10^{-3}$
0.157	11.04	1.0	53.4	$1.07 \pm 0.06 \times 10^{-3}$
				$k = 3.63 \pm 0.40 \times 10^{-3}\ M^{-1}$ sec^{-1}
			2,2,2-Trifluoroethylamine	
0.478	5.8	1.0	53.6	1.7×10^{-5}
0.239	5.8	1.0	53.6	8.7×10^{-6}
				$k = 3.56 \times 10^{-5}\ M^{-1}$ sec^{-1}

[a] Concentration of free amine. [b] At 23°. [c] Ionic strength was maintained with either KCl or NaCl. [d] Errors are average deviations for two runs.

into the ketimine were also measured at 235 nm. The overall rate of dealdolization, however, was monitored by nmr by observing the increase of a singlet at δ 2.1 owing to formation of acetone and the decrease of a singlet at δ 1.2 owing to diacetone alcohol. The nmr tubes were removed at appropriate time intervals from a constant-temperature bath, measurements were taken, and the tubes were returned to the bath. It was assumed that no time passed during the measurement since the temperature of measurement was about 20° lower than that of the bath and the tubes were only out of the bath for about 15 min. (For the fastest run, 15 min corresponds to less than 2% reaction at 53.6° and certainly less than 1% reaction at room temperature). These reactions were followed to greater than 90% completion and were strictly first order in diacetone alcohol.

Equilibrium constants for formation of the ketimine were measured at 235 nm for all amines except glycinamide. For glycinamide measurements were made at 240 nm because of a large background absorbance due to the amine at 235 nm. Control experiments with glycine and ethanolamine showed that the change in absorbance at 235 nm upon formation of the ketimine was identical with the absorbance change at 240 nm. Equilibrium constants were calculated as before, assuming an extinction coefficient of 192 for all imines.[4]

Results

The dealdolization of diacetone alcohol (4-hydroxy-4-methyl-2-pentanone) was followed spectrally by monitoring the appearance of acetone at 260 nm for all catalysts except 2,2,2-trifluoroethylamine. With this amine, the dealdolization reaction was complicated by the formation of a species absorbing at 243 nm, presumably mesityl oxide, and first-order kinetics could not be obtained spectrally. Since mesityl oxide has an extinction coefficient about 100 times greater than that of acetone at 260 nm, it is apparent that formation of a small amount of mesityl oxide is enough to preclude a uv method of analysis. (We estimate that about 1% mesityl oxide formed under our conditions.) Instead we chose to follow the reaction by nmr. Diacetone alcohol shows a spectrum consisting of three singlets at δ 2.6 (2 H), 2.1 (3 H), and 1.1 (6 H). All three peaks are easily observable in an aqueous solution of 2,2,2-trifluoroethylamine. Upon heating the solution, the peaks at δ 2.6 and 1.1 disappear with concurrent enhancement of the peak at δ 2.1. We attribute this enhancement to the formation of acetone, which also has a peak at δ 2.1. The reaction rate was followed by determining the per cent acetone in the medium at various time intervals. No products other than acetone were detected by nmr.

Each catalyst was studied in aqueous solution at 1:1 buffer ratios of amine:amine hydrochloride at two or more amine concentrations. All reactions were first order in amine concentration with a negligible contribution to the rate from hydroxide ion catalysis, except for propylamine. Rate constants were obtained at either 25° or 53.5° at constant ionic strength (either 0.4 or 1.0). Observed pseudo-first-order rate constants, along with calculated second-order rate constants, are given in Table I.

In addition to the change in absorbance due to the overall dealdolization at 260 nm, a much more rapid increase in absorbance may be observed at 235 or 240 nm. We have previously attributed this change to the formation of the imine intermediate from diacetone alcohol and the amine.[4] Other workers have also shown that imines absorb in this region of the spectrum.[7,8] Rate constants for imine formation were measured at 25° by following the increase in absorbance at either 235 or 240 nm. Equilibrium constants were calculated from the total change in absorbance due to this

(7) A. Williams and M. L. Bender, *J. Amer. Chem. Soc.*, **88**, 2508 (1966).

(8) (a) J. Hine, C. Y. Yeh, and F. C. Schmalsteig, *J. Org. Chem.*, **35**, 340 (1970); (b) J. Hine and C. Y. Yeh, *J. Amer. Chem. Soc.*, **89**, 2669 (1967).

reaction, with the assumption that there is no change in diacetone alcohol concentration. Measurements were all made at 0.2 M free amine in 1:1 buffers of amine:amine hydrochloride. Values of equilibrium constants for imine formation are known to be independent of both the concentration of amine and the pH.[4,7,8] On the other hand, rate constants for imine formation and hydrolysis do depend on pH,[9] so that any comparison of our observed rates for ketimine formation must be done with caution. Equilibrium constants were calculated using an assumed extinction coefficient of 192 for all imines. This value is that observed for the imine from acetone and n-butylamine in acetonitrile solution at 235 nm.[7] Although the absolute value of the extinction coefficient may be somewhat in error, it is reasonable to suppose that the extinction coefficient is independent of the pK of the amine precursor. Support for this assumption may be found in the work of Hine, *et al.*,[8] in which they found that for N-isobutylidenealkylamines neither the position of the absorption maximum nor the extinction coefficient is significantly altered for a wide variety of alkyl groups.

Observed values are given in Table II. There appears to be little variation of either rate or equilibrium

TABLE II

RATE AND EQUILIBRIUM CONSTANTS FOR THE FORMATION OF KETIMINE FROM DIACETONE ALCOHOL[a]

Amine	pH	k_{ket}^{obsd},[b] sec^{-1}	K, M^{-1} [b,c]
n-Propylamine[d]	10.9	$7.0 \pm 0.5 \times 10^{-2}$	0.15 ± 0.02
Glycine	9.78	$4.42 \pm 0.09 \times 10^{-2}$	0.048 ± 0.001
Ethanolamine	9.70	$4.25 \pm 0.17 \times 10^{-2}$	0.069 ± 0.014
Glycinamide	8.13	$6.25 \pm 0.20 \times 10^{-3}$	0.037 ± 0.001
2,2,2-Trifluoroethylamine	5.67	$2.03 \pm 0.29 \times 10^{-2}$	0.037 ± 0.004

[a] 25.0°, ionic strength 0.4. [b] Errors are standard deviations for four or more runs. [c] The equilibrium constant does not include a term in water concentration. [d] Values from ref 4 (μ 0.2).

constants with the basicity of the amine. Both are slightly higher for the more basic n-propylamine but the range is only a factor of 10 in rate and 4 in equilibrium constant.

Discussion

Nature of the Rate-Determining Step.—In order to compare rate constants for each of the catalysts, it is necessary to extrapolate the rates to common conditions. Overall rates were determined at 25° for ethanolamine, glycine, and glycinamide. In addition, we previously measured the rate for n-propylamine catalysis at 25°. In order to extrapolate the rate constant for 2,2,2-trifluoroethylamine to 25°, we use the fact that the second-order rate constant for catalysis by n-propylamine shows an increase in rate of 7.3-fold on going from 25.0 to 53.6°. Application of this correction factor to 2,2,2-trifluoroethylamine catalysis gives an extrapolated rate constant for 25°. Rate constants for each of the catalysts can now be compared under similar conditions.[10]

The individual rate constants for the dealdolization reactions can be readily calculated from the following steady-state equations, where k is the second-order rate constant for the overall dealdolization reaction, k_{ket}^{obsd} is the observed rate of ketimine formation, and k_1, k_{-1},

$$k = k_2K \quad (1)$$

$$K = k_1/(k_{-1} + k_2) \quad (2)$$

$$k_{ket}^{obsd} = k_1[\text{amine}] + k_{-1} + k_2 \quad (3)$$

and k_2 are the rate constants of Scheme I.[11] These values are given in Table III.

TABLE III

VALUES OF RATE CONSTANTS FOR AMINE-CATALYZED DEALDOLIZATION OF DIACETONE ALCOHOL IN WATER AT 25.0°

Amine	pK[a]	k_1, M^{-1} sec^{-1}	k_{-1}, sec^{-1}	k_2, sec^{-1}
n-Propylamine[b]	10.9	1.1×10^{-2}	6.7×10^{-2}	3.3×10^{-3}
Glycine	9.8	2.1×10^{-3}	4.2×10^{-2}	2.5×10^{-3}
Ethanolamine	9.7	2.9×10^{-3}	4.0×10^{-2}	2.3×10^{-3}
Glycinamide	8.1	2.3×10^{-4}	5.0×10^{-3}	1.3×10^{-3}
2.2.2-Trifluoroethylamine	5.7	7.5×10^{-4}	2.0×10^{-2}	1.4×10^{-4}[c]

[a] pH of 1:1 buffer at μ 0.4. [b] Reference 4, pK at μ 0.2. [c] Extrapolated value.

The relative rates of cleavage of ketimine to products (k_2) and reversal to reactants (k_{-1}) show that for all amines investigated the rate-determining step is cleavage of the ketimine (k_2) at the pH values investi-

$$\text{amine} + \text{diacetone alcohol} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ketimine} \xrightarrow{k_2} \text{products}$$

gated. The ratio k_{-1}/k_2 varies from 4 for glycinamide to about 150 for trifluoroethylamine. It should be emphasized that, since k_{-1} is probably pH dependent,[9] the k_{-1}/k_2 ratios given here apply only to the pH values at which the measurements were made. k_2, however, is independent of pH,[4] which allows comparisons to be made of cleavage rates for all of the amine catalysts.

Polar effects have little effect on the equilibrium constant for ketimine formation. Over a range in basicity of greater than 10^5, K changes by only a factor of 4. The small variation in equilibrium constants may be compared with the work of Hine, *et al.*,[8] in which they found that for the formation of imines from isobutyraldehyde and primary amines, electron-withdrawing substituents on the amine decrease the equilibrium constant. A plot of log K *vs.* amine pK for their data was linear with a total change in the equilibrium constant of 18-fold in going from n-propylamine (pK 10.9) to 2,2,2-trifluoroethylamine (pK 5.7). Over this same range of pK we observe a similar trend but a somewhat lower sensitivity to polar effects (fourfold).

Polarity of the Transition State.—We have previously proposed that the amine-catalyzed dealdolization of diacetone alcohol involves a transition state with little or no charge separation.[4] This interpretation was based upon the small solvent effect observed on the cleavage step in going from water to 80% ethanol ($k_{H_2O}/k_{80\%\ ethanol} = 8$). One possible structure consistent with this result is a cyclic transition state involving a six-membered ring. On the basis of a low solvent isotope effect on the cleavage step ($k_{H_2O}/k_{D_2O} = 1.4$) we suggested that this structure would involve the hydrogen in a potential well while the carbon–carbon

(9) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapter 10.

(10) Although the ionic strength varied between catalysts, these effects have been shown to be negligible (R. M. Pollack, unpublished results).

(11) Implicit in the deviation of eq 3 is the assumption that $k_{-1} \gg k_1$.

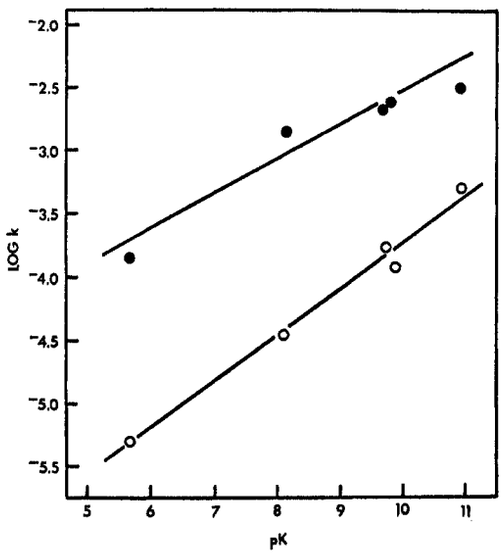
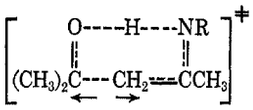


Figure 1.—Variation of the rate of amine-catalyzed cleavage of diacetone alcohol with amine pK at 25°. Closed circles are values for k_2 (cleavage of the imine intermediate). Open circles are overall rates of cleavage.

bond is undergoing cleavage. In other words, the asymmetric stretching vibration of the O–H–N system is a genuine vibration with a restoring force and zero-point energy. One could regard the hydrogen as acting to stabilize the transition state by forming a hydrogen bond.



The variation of the cleavage rate (k_2) with amine polarity allows an independent assessment of the extent of charge separation in the transition state. A plot of log k_2 *vs.* pK of the amine (Figure 1) gives a slope of 0.27 ± 0.04, indicating that this rate is about 25% as sensitive to polar effects as the equilibrium constant for protonation of the amine. This result suggests that about 25% of a full positive charge is generated on the nitrogen at the transition state. A partial negative charge, presumably on the hydroxyl oxygen, must also be formed to maintain electrical neutrality. A charge separation of this magnitude is consistent with a model in which the hydrogen acts to stabilize the transition state by formation of a hydrogen bond. Two other mechanisms which are also compatible with all the available data are (1) rate-determining proton transfer followed by rapid cleavage of the carbon–carbon bond, with the transition state occurring early along the reaction coordinate, and (2) preequilibrium proton transfer followed by rate-determining carbon–carbon bond cleavage, with a product-like transition state.

Relevance to the Aldolases.—Although the pK of the active site amine group of any of the aldolases has not as yet been determined, it is interesting to speculate on the effect that a lowered pK might have for these enzymes. It does not appear unreasonable to suppose that the active pK is perturbed in the aldolases. The cause of the perturbation in acetoacetate decarboxylase has been shown to be the presence of one or more positive charges at the active site,[5b] and one might expect the same type of interaction in the aldolases. In fructose diphosphate aldolase, at least, the substrate is negatively charged and is thought to be bound to the enzyme by electrostatic interaction with a positive charge (or charges).[12] The positive charge on the enzyme could perturb the pK of the active lysine residue of aldolase as it does in acetoacetate decarboxylase. Other evidence of similarity between the two types of enzymes also exists.[1c] In fact, Rutter has suggested that imine-forming aldolases may actually have evolved from the β-decarboxylases.[13]

The advantage to an enzyme of having a lowered pK is that at pH values near neutrality an amine of pK 11 will have about one part in 10^4 unprotonated, whereas a large fraction of an amine with a pK near 6 or 7 will be free. If the rate-determining step for enzymatic aldol cleavage is formation of the imine, a lowered pK is clearly favored since attack on the substrate carbonyl must occur through the free amine.[9] However, if the slow step is cleavage of the imine, as it is in our model system, then it is necessary to look at the effect of an altered pK on both steps of the reaction. Since the amount of free amine in solution at physiological pH's is much greater for a weakly basic amine and the equilibrium constants are only moderately sensitive to pK, a weakly basic amine will form a much larger (*ca.* 10^4) amount of reactive intermediate. However, a lowering of the amine pK by four units results in a diminished rate of cleavage by a factor of about 10 on the basis of the diacetone alcohol system. Consequently, a lowered pK value, while highly favoring imine formation, gives a more modest rate acceleration (*ca.* 10^3) to the overall reaction. It is noteworthy that the effect of changing amine pK at neutral pH is much greater on the equilibrium concentration of imine than on the rate of cleavage. Even though the decomposition step is retarded, the overall effect is still a rate acceleration.

Registry No.—Diacetone alcohol, 123-42-2; ethanolamine, 141-43-5; glycine, 56-40-6; glycinamide, 598-41-4; propylamine, 107-10-8; 2,2,2-trifluoroethylamine, 753-90-2.

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(12) See ref 1c, p 145 ff.
(13) W. J. Rutter, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **23**, 1248 (1964).