A Reporter Group at the Active Site of Acetoacetate Decarboxylase. II. Ionization Constant of the Amino Group[1]

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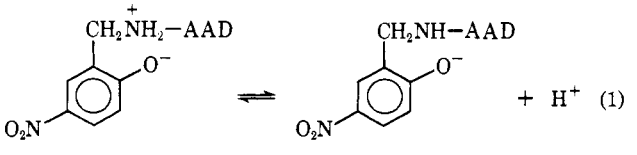
Abstract: Condensation of 5-nitrosalicylaldehyde with acetoacetate decarboxylase, followed by reduction of the resulting Schiff base with borohydride ion, introduces an N-substituted 2-hydroxy-5-nitrobenzylamino reporter group at the active site of the enzyme. The nitrophenol group is essentially completely ionized at pH's above 4.5. Titration of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase in the pH range 4.5–9.7 causes small but measurable changes in the optical absorption of the nitrophenolate chromophore, from which two pK values, 6.0 and 8.0, can be obtained. The value at 6.0 is ascribed to the ionization of the ammonium salt moiety of the reporter group and represents a decrease of 4.7 units relative to the model compound N-methyl-2-hydroxy-5-nitrobenzylamine. By implication the essential lysine residue in the enzyme has a pK of about 6. The pK of 8.0 is associated with the unusual and still unexplained absorption band of the enzyme at 320 nm. In an earlier kinetic investigation, a pK value of 5.9 was correlated with the ionization of the ϵ-ammonium ion of the essential lysine residue in acetoacetate decarboxylase. The pK value of 6 here found lends weight to the previous assignment. The enzymic environment that produces this exceptionally low pK for an ammonium salt group, and its mechanistic and evolutionary significance, are discussed.

Acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetic acid by way of a Schiff base as the essential intermediate.[3,4] In the previous paper[5] we showed that 5-nitrosalicylaldehyde condenses at the active site of the enzyme, and that reduction of the resulting Schiff base with borohydride introduces a 2-hydroxy-5-nitrobenzylamino residue as a "reporter group" into the protein. The pK of the nitrophenol residue of this reporter group is 2.4; this value is 3.5 logarithmic units less than that of the model compound, N-methyl-2-hydroxy-5-nitrobenzylamine.

In the present paper, we report our measurements directed toward a comparison of the pK of the ammonium salt group in 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase to that in the model and, by implication a comparison of the pK of the ammonium salt group of the essential lysine residue in the enzyme to that of an ordinary primary ammonium ion. Ionizations of the native enzyme and of the labeled protein have been determined by observing the changes in the absorption spectra of the nitrophenolate chromophore at pH's between 4.5 and 9.8. In this range the phenol of the reporter group is more than 99% ionized. But even though only one proton can ionize from the reporter group in alkaline solution (eq 1), two separate ionizations with pK's of 6.0 and 8.0 have been observed.



We shall show how these values were determined, and offer evidence that the lower of two pK's (6.0 or 8.0) corresponds to the ionization of the ammonium salt moiety of the reporter group.

Experimental Section

Materials. The preparations of acetoacetate decarboxylase, of acetoacetate decarboxylase labeled with the reporter group, and of the model compound (N-methyl-2-hydroxy-5-nitrobenzylamine) are presented in the previous paper.[5] Acetoacetate decarboxylase was acetylated by the treatment of the enzyme with acetic anhydride, by the method of O'Leary and Westheimer.[6] Other reagents were commercial reagent grade chemicals and were used without further purification. Distilled, deionized water was used throughout.

Methods. Spectroscopic measurements were made with a Cary 15 spectrophotometer equipped with a special cell holder thermostated at 30.0°. pH measurements were made at 30° with a Radiometer TTT1C pH meter and combination electrode using Beckman buffer solutions of pH 7.400 and 4.015 (30°) for standardization.

To obtain a spectrum, 0.80 ml of a buffer was added to each of two 1-ml quartz cells. The cells were placed in the Cary spectrophotometer and the base-line absorption determined (in duplicate). The cells were removed from the Cary and 90 μl of protein solution (13 mg/ml in 0.05 M phosphate buffer, pH 6) was delivered to the sample cell from a 100-μl Hamilton syringe equipped with a Chaney adaptor; the delivered volume was reproducible within 1–2 parts per thousand. A 90-μl sample of 0.05 M phosphate buffer, pH 6, was also delivered to the reference cell. The contents of each cell were mixed, the cells returned to the Cary, and the absorption spectrum was determined (in duplicate). Absorbance values used in our calculations are the difference between the average sample and baseline absorbances.

Buffer solutions were prepared by mixing two solutions with each other, or with either 1 N sodium hydroxide or 2 N sulfuric acid. Solution A, pH 4.7, contained 50 ml of 0.18 M KH_2PO_4, 25 ml of 1.6 M Na_2SO_4, and 20 ml of 0.05 M pH 6 potassium phosphate buffer, diluted to 200 ml. Solution B, pH 8.8, contained 50 ml of 0.18 M K_2HPO_4–0.019 M NaOH, 25 ml of 1.6 M Na_2SO_4, and 20 ml of 0.05 M pH 6 potassium phosphate buffer diluted to 200 ml.

Reversibility. An acidic (pH 4.65) and a basic (pH 9.38) solution of the labeled enzyme were prepared; these solutions contained 0.2 M sodium sulfate, and phosphate buffers at 0.05 M. The spectra of these samples were determined, and then samples of each were dialyzed at pH 4.72 and 8.42 for 24 hr at 4°, with re-

(1) This investigation has been supported by Grant No. GM-04712 from the Institute of General Medical Sciences of the National Institutes of Health.

(2) National Institutes of Health Postdoctoral Fellow 1 F02-GM 28796-01, 02, 1969–1971.

(3) G. A. Hamilton and F. H. Westheimer, *J. Amer. Chem. Soc.*, **81**, 6332 (1959).

(4) I. Fridovich and F. H. Westheimer, *ibid.*, **84**, 3208 (1962).

(5) P. A. Frey, F. C. Kokesh, and F. H. Westheimer, *ibid.*, **93**, 7266 (1971).

(6) M. H. O'Leary and F. H. Westheimer, *Biochemistry*, **7**, 913 (1968).

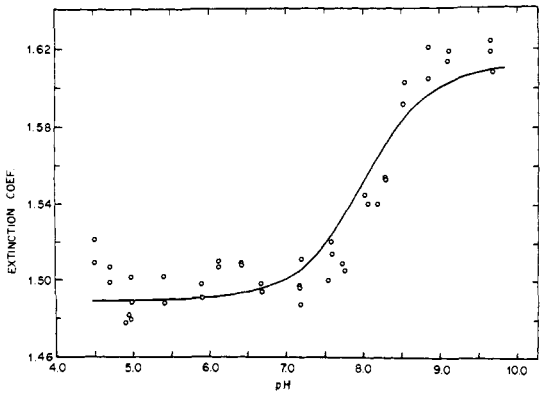


Figure 1. Absorption spectrum of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase as a function of pH at 278 nm and 30°. The curve was drawn with the value of $\epsilon_A = 1.490$ and $\epsilon_B = 1.612$ (calculated in units of centimeters²/milligram) and pK = 8.00.

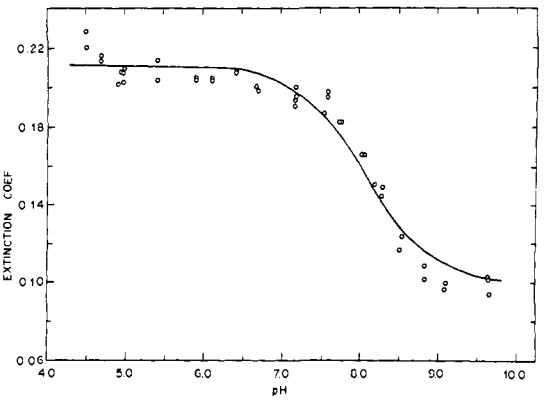


Figure 2. Absorption spectrum of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase as a function of pH at 320 nm and 30°. The curve was drawn with the values of $\epsilon_A = 0.211$ and $\epsilon_B = 0.099$ (calculated in units of centimeters²/milligram) and pK = 8.00.

newal of external buffer after 16 hr. The absorption spectra of the samples were then redetermined and normalized to $A^{280} = 1.000$ to facilitate comparisons.

Calculations. The ionization constants and extinction coefficients were obtained from the spectroscopic data by least-squares methods. The objective of the least-squares calculation is to obtain precise, rather than approximate, values of these parameters. In those instances where a single pK is involved, a computer program was devised to determine the best fit of the data to three variables: ϵ_B, the extinction coefficient of the basic form of the "indicator," ϵ_A, the extinction coefficient of the acid form, and the pK. One can write an expression for the observed optical density as a function of pH in terms of these variables. Then a function F can be set equal to the sum, at all the pH's for which measurements are available, of the squares of the differences between the observed and calculated values of the optical density; the best values of the parameters are those that make F a minimum. The process can be repeated for each wavelength. Minimization of F can be accomplished by setting the partial derivatives of F, with respect to ϵ_A, ϵ_B, and K, equal to zero, and solving the resulting three simultaneous equations in three unknowns. Unfortunately the explicit solution of these nonlinear equations is impractical. However, approximate values of all three parameters are immediately available; the two extinction coefficients correspond to the values obtained from the optical density at the most alkaline and most acid pH's used, while an estimate of the pK can be read from the titration curve. An iterative approximation method can be used to achieve the exact values of the parameters. The method here employed—the Newton–Raphson method[7]—computes successive algebraic increments to the three unknowns such as to decrease F; the program converges fairly quickly, and is terminated when further iteration fails to diminish F.

However, in much of the wavelength range for which measurements are here recorded two ionizations occur and five unknowns are involved. These are two ionization constants—one for the amino group of the benzylamino substituent, and one for the unknown ionization of the protein—and the extinction coefficients for H_2A^+, for HA, and for A^-. (The formulas H_2A^+, HA, and A^- are representative, but the total charge of the protein may really be greater or less for all of them.)

Good approximations to the extinction coefficients for H_2A^+ and for A^- can be made from the optical densities at high and at moderately low pH, and approximate values of the extinction coefficient of HA and of the two ionizations constants can be obtained from visual inspection of graphs of the data. Mathematical treatment of the data is needed, then, to refine approximate values of the desired constants, rather than to find them *ab initio*. In fact, the problem was solved by treating the two pK's as known constants, independent of wavelength, and formulating least-squares equations in terms of the three unknown extinction coefficients for H_2A^+, HA, and A^-. An iterative computer program, again based on the Newton–Raphson method, was used to produce the best values of the three extinction coefficients at each wavelength. This process was then repeated for slightly different values of the two pK's, in order to find the best fit of calculation to experiment.

The mathematics underlying the computer programs are outlined in the Appendix. Calculations were carried out on an IBM 1620 computer.

(7) A. Fox, "Fundamentals of Numerical Analysis," The Ronald Press, New York, N. Y.,1963.

Results

We obtained spectra of 42 samples of labeled enzyme at pH's between 4.5 and 9.7; the spectra for pH 4.97 and 9.68 have been presented as Figure 5 of the previous paper.[5] The spectral changes are reversible over at least most of this pH range. The spectrum of a sample adjusted from pH 4.65 to 8.42 is identical with that for a sample adjusted from pH 9.38 to 8.42, and the spectrum of a sample adjusted from pH 9.38 to 4.72 is identical with that for a sample adjusted from pH 4.65 to 4.72.

Changes in the Chromophores of the Protein. The changes in absorbance with pH at 278 and 320 nm are shown in Figures 1 and 2. These are regions where the nitrophenolate anion of a model compound, *N*-methyl-2-hydroxy-5-nitrobenzylamine, does not absorb strongly, so that changes in the absorption of our labeled protein may, to a first approximation, reasonably be assigned to changes in other chromophores. Absorption at 278 nm by tyrosine and tryptophan is typical of proteins. The absorption at 320 nm has been observed at neutral and acidic pH as a small absorption peak on the long-wave side of the 278-nm absorption band for acetoacetate decarboxylase. This band diminishes at pH 10. It is also present in the acetylated enzyme (acetylated on the essential lysine amino group) at pH 4.9 and diminishes at pH 10. Therefore this absorption, whatever its origin, is not related to that of the reporter group.

The sigmoid curves for the changes in OD with pH at 278 and 320 nm, shown in Figures 1 and 2, are best correlated with a pK of 8.22. The deviations between observed and calculated optical densities using this pK seldom exceed 0.02 OD unit, and represent a small proportional error. However, the fit of curves calculated with a pK of 8.00 is only slightly less impressive; the average deviations between calculated and observed OD are only about 50% larger.

Spectra measured as a function of pH at 278 and 320 nm for the native enzyme are quite similar to those for the enzyme marked with the reporter group, and

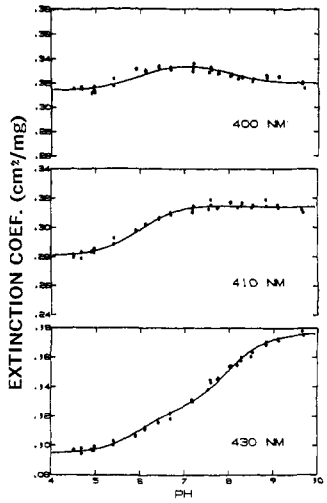


Figure 3. Absorption spectra of 2-hydroxy-5-nitrobenzylacetoacetate decarboxylase as a function of pH at 430, 410, and 400 nm. The curves are calculated for pK's of 6.00 and 8.00, using the values of the extinction coefficients determined from least-squares analysis of the data.

the pK is again about 8; a precise value of the pK for these examples has not, however, been obtained.

Changes in the Chromophore of the Reporter Group. Representative changes in absorbance with pH at 430, 410, and 400 nm for the labeled protein are shown in Figure 3. The plot of the data at 410 resembles a sigmoid titration curve for a single ionization; a pK of 5.86 can be deduced from these data by a least-squares method. Visual inspection of the graphs of the data at 430 and 400 nm shows that two ionizations are involved, and allows estimates of the corresponding pK's at about 6 and about 8. An initial fit of the data at several wavelengths calculated from pK's of 5.86 (from the data at 410 nm) and of 8.22 (from the data at 278 and 320 nm) was significantly improved by using pK's of 6.00 and 8.00. The curves in Figure 3 were drawn on the basis of two ionizations with pK's of 6.00 and 8.00, using the values of the extinction coefficients for the ionic forms H_2A^+, HA, and A^- that were determined from our least-squares program. The calculated and observed optical densities shown in Figure 3 seldom differ by more than a few thousandths of an OD unit.

The extinction coefficients for the three ionized forms of the enzyme labeled with the reporter group obtained from the least-squares program are presented graphically in Figure 4. The isobestic point for HA and A^- falls at 410, consistent with the observation that, at this wavelength, only one pK (that at 6.0) can be measured.

The scatter of the data, as shown in Figures 1–3, may appear large. However, close inspection of the graphs shows that the total variation in the optical density is quite small; the various chromophores are only marginally affected by the two ionization constants in question. On any absolute scale, the agreement between the calculated and observed optical densities is excellent.

Discussion

Assignment of the pK's. Despite the number of parameters that need to be determined, the calculations

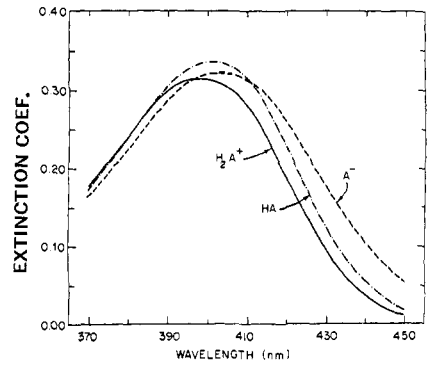


Figure 4. Extinction coefficients, in units of centimeters²/milligram, for the three ionic species that control the absorption of the nitrophenolate chromophore between pH 4.5 and 9.8. The values were determined by the least-squares analysis (discussed in the text) using pK's of 6.00 and 8.00.

of both ionization constants and extinction coefficients are reasonably secure. This follows primarily because the calculations are needed only to refine values of the various constants that can as a first approximation be obtained by visual inspection of the spectrophotometric data.

Two ionization constants,[8] corresponding to pK's of 6.0 and 8.0, were found in the pH region where only one ionization was anticipated. Presumably one of the two pK's corresponds with that shown in eq 1, and the other must be assigned to some still unknown group in the enzyme that lies close enough to the active site so that its ionization can affect the nitrophenolate chromophore of the reporter group.

We have assigned the pK of 6.0 to the ionization of the ammonium salt moiety of the reporter group on the basis of the following evidence. A pK of about 8 is observed in the native enzyme and in the acetylated enzyme, as well as in the enzyme marked with the reporter group. But acetylation of the essential amino group of the enzyme would completely alter its basic properties, so that it could not ionize in the pH range in question. It therefore follows that the pK of 8.0 does not correspond to the ionization of the ammonium salt group of the lysine residue at the active site; we are therefore led to the alternative conclusion that the pK of 6 must be assigned to this group. This represents an increase in acidity of 4.7 pK units relative to the model compound N-methyl-2-hydroxy-5-nitrobenzylamine (the ammonium group of which has a pK of 10.7). If the acidity of the essential ammonium ion of AAD is increased by the same amount relative to that of the ϵ-ammonium ion of lysine, then the active-site lysine has a pK of 6.

A prior kinetic determination gave a value of 5.9 for the pK of the active lysine in acetoacetate decarboxylase,[9] in agreement with the thermodynamic value found here. All kinetic determinations of ionization constants are suspect, since in many instances the value is not the thermodynamic constant, but that constant multiplied by a ratio of rate constants. Nevertheless,

(8) It is here assumed that the pK value of 8.22 determined at 278 and 320 nm and the value of 8.00 determined in the visible region of the spectrum refer to the same ionization. The discrepancy probably arises because the absorption of the reporter group at 278 and 320 nm is not quite negligible; ignoring it probably gave rise to a small error in calculated pK.

(9) D. Schmidt and F. H. Westheimer, *Biochemistry*, 10, 1249 (1971).

the particular reaction cited appears to be a simple one, and the kinetic determination of pK therefore is probably valid. The pK of 6.0 found for the reporter group closely approximates the value of 5.9 determined kinetically.

In addition we note that the pK for the ionization of the nitrophenolic residue in the reporter group is 3.5 units less than that for the corresponding ionization of the model compound, *N*-methyl-2-hydroxy-5-nitrobenzylamine;[5] this is consistent with the lowering of the pK of the ammonium cation by 4.7 logarithmic units, relative to the model. One would expect the lowering of the pK to be greater for the ammonium ion group than for the phenolic group, since whatever the special enzymic environment that causes the lowering of the pK's, it is likely to have its maximum impact directly at the active site.

Nothing that has so far been said helps to determine the group responsible for the absorption at 320 nm, or to explain the pK of 8.0 associated with its ionization.

The Cause of the Lowered pK's for the Ionizations of the Reporter Group. The two ionizations of the reporter group—that of the phenol and that of the substituted ammonium ion—show pK's that are 3.5–4.7 units lower than those of a nonenzymic model. These changes in pK must be ascribed to the electrostatic effect of nearby positive charges. This conclusion follows from the following considerations: changes in the pK's of groups on proteins have generally been ascribed either to the electrostatic effects of charged groups or to a change in the polarity of the environment. These effects should now be examined in turn.

A decrease in the polarity of the environment around any group will result in a smaller extent of ionization of that group regardless of its charge type. The decrease in the basicity of the "hidden" imidazole groups[10] and the decrease in the acidity of the "hidden" tyrosine residues[11] of myoglobin have been ascribed to a hydrophobic environment that inhibits solvation of ions. Similarly the pK of 2,6-di-*tert*-butylphenol[12] is increased 2–3 units relative to that of phenol presumably because the adjacent, bulky, hydrophobic *tert*-butyl groups hinder the solvation of the anion. These examples—as well as considerations of theory—suggest that a change in the environment to a more hydrophobic one will destabilize ions, and will therefore result in opposite shifts in pK for acids of differing charge type, *i.e.*, the pK's of ammonium salts will be decreased and those of phenols will be increased by substituting a more hydrophobic for a more aqueous environment.

On the other hand, electrostatic effects will move the pK's of acids in the same direction, regardless of their charge type. Specifically, adjacent positive charges will lower and negative charges will raise the pK's of acids of either the ammonium salt or phenolic type. Furthermore, the effects can be quite large. For example, the pK's for the first and second ionizations of the conjugate acid of ethylenediamine are 6.97 and 9.97;[13] the three pK's for the conjugate acid of diethylenetriamine are 4.34, 9.13, and 9.94.[14] These data show that the single positive charge of $H_3N^+CH_2CH_2NH_2$ decreases the pK_A of the conjugate acid of the adjacent amino group by 2.4 pK units (0.6 pK unit of the difference between first and second pK's is a statistical effect). The two adjacent positive charges in H_3N^+-$CH_2CH_2NHCH_2CH_2NH_3^+$ decrease the pK of the conjugate acid of the central amino group by about 5 pK units. Electrostatic effects from only one or two charges at modest distances from the reaction center can then have large effects on ionization constants.[15]

In the present instance both the pK for the nitrophenol and that for the ammonium salt in the reporter group on the enzyme have been lowered substantially. This result is inconsistent with the effects anticipated for a hydrophobic environment, but consistent with the presence at the active site of one or more positively charged groups.

A positively charged group had previously been postulated at the active site of acetoacetate decarboxylase based on the inhibition of enzymic activity by monovalent anions.[16] The pK of the group—or at least of a group—responsible for the binding of anions had been determined as about 5.9. At the time this value seemed too low for the pK of a lysine ammonium ion, and the presence of an additional positively charged group was suggested. Now, however, that the pK of the essential lysine is known to be in this region, that argument cannot be used. Other evidence, however, suggests the presence of one or more additional cationic groups. The borohydride reduction of the Schiff base salt of acetone occurs quite readily and in preference to the reduction of the Schiff base salt of acetoacetate ion.[4,17] One possible interpretation is that the negatively charged borohydride ion is attracted to a site normally occupied by the carboxylate ion group of acetoacetate. The amino acid sequence about the essential lysine[18] also suggests the possibility of an additional positively charged group near the active site. The residue adjacent to the active lysine in the amino acid sequence of acetoacetate decarboxylase is another lysine. Granted that the two ammonium salt residues could be quite widely separated in the protein, they could also be close enough to provide electrostatic lowering of the pK of the essential lysine residue.

The Evolutionary Advantage of a Low pK for the Essential Amino Group of Acetoacetate Decarboxylase. The lowered pK of the essential lysine residue in acetoacetate decarboxylase is mechanistically useful. In order for the lysine residue to react with a carbonyl group to form a Schiff base, it needs to be in the nucleophilic, unprotonated form.[19] If the lysine has a normal pK, less than one part in 10^4 would be unprotonated at pH 6, and the mechanism of decarboxyl-

(10) E. Breslow and F. R. N. Gurd, *J. Biol. Chem.*, **237**, 371 (1962).
(11) S. N. Timasheff and M. J. Gorbunoff, *Annu. Rev. Biochem.*, **36**, 40 (1967).
(12) L. A. Cohen and W. M. Jones, *J. Amer. Chem. Soc.*, **85**, 3397 (1963).
(13) G. Schwartzenbach, *Helv. Chim. Acta*, **16**, 526 (1933).
(14) J. E. Prue and G. Schwartzenbach, *ibid.*, **33**, 985 (1950).
(15) *Cf.* (a) L. Eberson, *Acta Chem. Scand.*, **13**, 203, 211 (1959); (b) J. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506, 513 (1938).
(16) I. Fridovich, *J. Biol. Chem.*, **238**, 592 (1963).
(17) S. G. Warren, B. Zerner, and F. H. Westheimer, *Biochemistry*, **5**, 817 (1966).
(18) R. A. Laursen and F. H. Westheimer, *J. Amer. Chem. Soc.*, **88**, 3426 (1966).
(19) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapter 10.

ation would be an inefficient one. The lowered pK is then reasonable on the grounds of natural selection.

The argument given above is valid only if the formation of the Schiff base from enzyme and acetoacetate is at least partially rate limiting for the enzymic process. Preliminary evidence from model studies[20] shows that even when the amine is one of low pK the rate of decarboxylation is at least of the same order of magnitude as that of decarboxylation. This aspect of the decarboxylation process, however, is still under study.

The reactive lysine residue of ribonuclease, no. 41 in the amino acid sequence, also has an anomalously low pK[21] although it is nowhere near so low as that in acetoacetate decarboxylase. The evolutionary advantage of the low pK of residue 41 of RNase is not apparent, and perhaps it constitutes merely a relatively harmless artifact. In the action of RNase, lysine 41 presumably participates in its protonated form to assist in binding the substrate at the active site.[22] The active site likewise contains other cationic groups[23] needed to bind the substrate and for catalysis, so the pK of lysine 41 is necessarily lowered electrostatically. Provided, however, that the physiological pH is less than the pK of lysine 41, most of that residue will be protonated, and the low pK, while not advantageous, may be tolerated without adverse effect.

Acknowledgments. The authors gratefully acknowledge Dr. L. David Williams' suggestions on computational methods.

Appendix

Program I. Let ϵ_{obsd} = the extinction coefficient calculated from the observed optical density, ϵ_A be the extinction coefficient of the acid form, and ϵ_B be the extinction coefficient of the basic form of the indicator. Let h_i be the hydrogen ion concentration in any solution, i, and K be the ionization constant we seek. Then the sum of the squares of the difference between observed and calculated extinction coefficients

$$F = \sum_i \left[\epsilon_{obsd(i)} - \frac{(\epsilon_B K + \epsilon_A h_i)}{(K + h_i)} \right]^2 \qquad (1A)$$

To obtain the minimum value of F

$$\partial F/\partial \epsilon_A = \partial F/\partial \epsilon_B = \partial F/\partial K = 0 \qquad (2A)$$

This provides three simultaneous equations in the three unknowns ϵ_A, ϵ_B, and K. However, explicit solution of these equations is at least difficult, and perhaps impossible, so that they have been solved by successive approximations, using the Newton–Raphson method. The values of the partial derivatives are also given by

$$\partial F/\partial x_i = \sum_{j=1}^{3} (\partial^2 F/\partial x_i \partial x_j)\Delta x_j = 0 \qquad (3A)$$

where x_1, x_2, and x_3 are the three variables, ϵ_A, ϵ_B, and K, under consideration. The mathematical expression for the second partial derivatives may be obtained from eq 1, and may be evaluated approximately by inserting into the expressions for the second partial derivatives the trial values of ϵ_A, ϵ_B, and K estimated by visual inspection of plots of the data.

The operation described above (*i.e.*, the evaluation of the terms in eq 3) will lead to three simultaneous linear equations in the three unknowns, $\Delta\epsilon_A$, $\Delta\epsilon_B$, and ΔK. These linear equations can readily be solved for the values of the increments (decrements) to the original unknowns, and using these corrections, new trial values of ϵ_A, ϵ_B, and K can be obtained.[24] The process is repeated until successive values of the least-squares function, F, differ by less than some predetermined insignificant amount.

Program II. The same rationale and the Newton–Raphson method were employed in the more complicated case of determining two ionization constants. In this calculation, the two ionization constants, K_1 and K_2, are taken as given, and the three unknowns are ϵ_A, ϵ_B, and ϵ_C, the extinction coefficients of the three ionic species previously designated as H_2A^+, HA, and A^-. Here the least-squares function has the form

$$F = \sum_i \left[\epsilon_{obsd} - \frac{(\epsilon_A h_i^2/K_2) + (\epsilon_B h_i/K_1) + \epsilon_C}{(h_i^2/K_2) + (h_i/K_1) + 1} \right]^2 \qquad (4A)$$

and F was then minimized with respect to the values of ϵ_A, ϵ_B, and ϵ_C.

(20) (a) J. P. Guthrie, Abstracts, 51st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1967, Abstract No. 1712, p 562; (b) J. P. Guthrie, Harvard University, Ph.D. Dissertation, 1968; (c) F. Jordan, 1970, unpublished data.

(21) R. P. Carty and G. H. W. Hirs, *J. Biol. Chem.*, **243**, 4254 (1968).

(22) G. C. K. Roberts, E. A. Dennis, D. H. Meadows, J. S. Cohen, and O. Jardetsky, *Proc. Nat. Acad. Sci., U. S.*, **62**, 1151 (1969).

(23) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, I. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).

(24) In order to promote sure and rapid convergence in the iteration process, wide swings in the values of the iteration variables should be avoided. Therefore, in any cycle of the iteration, each iteration variable was allowed to vary no more than about 30%. This was achieved by limiting the change permitted for A according to the equation

$$A_{i+1} = A_i + \Delta A_i/(1 + 3(\Delta A_i/A_i)^2)$$

where A_i is the initial value of the iteration variable, A, and ΔA_i is the increment to A_i calculated by the computer program.