BIOORGANIC CHEMISTRY 3, 163–175 (1974)

Kinetics of Formation of the 4,4′-Bis-Dimethylaminodiphenyl Carbonium Ion (BDC^+) and its Reaction with Sulfhydryl Residues[1]

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Received September 14, 1973

The use of 4,4′-bis-dimethylaminodiphenylcarbinol (BDC-OH) as an analytical reagent for sulfhydryl residues and as a specific chemical modification reagent for proteins is dependent upon the unique properties of the BDC^+ cation present in aqueous buffers below a pH of 6.5. In the presence of aqueous buffers, pH 5.1, BDC^+ exhibits a λ_{max} of 606 nm with an apparent molar absorption coefficient of 10,000 M^{-1} cm^{-1}. Upon the addition of 4M guanidine hydrochloride this apparent coefficient is enhanced to 70,800 M^{-1} cm^{-1}. The true molar extinction coefficient for BDC^+ was determined to be 128,000 M^{-1} cm^{-1}. The reaction of BDC^+ with sulfhydryl residues of proteins or simple thiols is rapid and leads to a complex devoid of visible color. In the pH range 3.0–7.0, a complex equilibrium is established among the three species BDC-OH, BDC^+, $BDCH^{++}$. The formation of this equilibrium is proton mediated, and is discussed in terms of the equilibrium, rate, and acid dissociation constants.

We have previously reported the use of 4,4′-bis-dimethylaminodiphenylcarbinol (BDC-OH)[3] as an analytical reagent for the determination of sulfhydryl residues in simple thiols or in proteins (*1*). While the technique as described is easily usable without modification, certain aspects of the kinetic and spectral properties of the BDC^+ cation intentionally were not dealt with in order to present the technique in a simplified manner. In the following work we have attempted to describe these above-mentioned parameters, in order that modifications of the basic technique can easily be made if desired, and in order to more fully define the specific equilibria and interactions which occur in aqueous buffers containing BDC-OH both in the absence and presence of guanidine hydrochloride.

Investigations with several sulfhydryl-dependent enzymes have also indicated the potential usefulness of BDC-OH as a highly specific chemical modifying reagent. This laboratory has previously reported the interaction of BDC^+ with mitochondrial malate dehydrogenase from porcine heart as selectively labeling one cysteine per enzymatic active center (*2*). This reaction was concomitant with a total loss of enzymatic activity

[1] This investigation was supported by Grant HL-12585 from the National Institutes of Health, United States Public Health Service.

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[3] The abbreviations used are: BDC-OH, 4,4-*bis*-dimethylaminodiphenylcarbinol; BDC^+, carbonium-immonium ion; BDC_{total}, total of all species of reagent present in any given solution; BDC-SR, *S*-(4,4′-*bis*-dimethylaminodiphenylmethyl-) derivatives.

and could be protected against by the addition of the coenzyme, NAD^+. In more recent investigations, BDC^+ was shown to be effective in selectively labeling an active-center cysteine residue in both yeast alcohol dehydrogenase and horse liver alcohol dehydrogenase (*3*). It was also demonstrated that isolation of BDC-labeled peptides, from a tryptic digestion, was easily accomplished due to the unique micelle-forming phenomena attributed to the BDC moiety. Therefore, the kinetic parameters of BDC^+ in the absence of guanidine hydrochloride are also of interest for future work with this reagent.

EXPERIMENTAL PROCEDURE

Materials

Guanidine hydrochloride was purchased from Heico Inc., Delaware Water Gap, Pa. Porcine heart mitochondrial malate dehydrogenase was isolated and purified as previously described (*4*). All other chemicals were of reagent grade and used without further purification.

Preparation of 4,4'-bis-Dimethylaminodiphenylcarbinol (BDC-OH)

To 60 ml absolute ethanol was added 12.0 g 50% aqueous potassium hydroxide, 2.0 g (7.46 mmole) 4,4'-bis-dimethylaminobenzophenone, and 6 g (0.092 g-atom) zinc dust. The reaction mixture was stirred at reflux for 24 hr and filtered (paper) into ice water, producing a yellow precipitate which was collected under nitrogen, washed with cold water, and dried in vacuo at room temperature. The pale-yellow solid was recrystallized twice from cyclohexane, affording a white solid (1.09 g), 54% with melting point 103–104°C (*1*).

Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tn.

Anal.. Calcd for $C_{17}H_{22}N_2O$: C (75.52); H (8.20); N (10.36); Found: C (75.24); H (8.28); N (10.40).

Determination of the Molar Absorption Coefficient for BDC^+

Samples of BDC-OH ranging in weight from 20 to 23 mg were dissolved in 500 μl of concentrated sulfuric acid–water (9 : 1 by volume). One microliter of this solution was transferred into 15 ml of 0.1 M sodium phosphate, pH 7.2, containing either 2 M guanidine hydrochloride or 4 M guanidine hydrochloride. The final pH of these solutions after addition of the aliquot of sulfuric acid was determined to be 6.65.

Each solution was rapidly stirred and a 3-ml aliquot transferred to the sample cell of a Unicam SP1800 recording spectrophotometer, and the decrease in absorbance at 612 nm was followed as a function of time. For determinations of ε_M in the 4 M guanidine solutions, the blank cell contained a 0.1 M sodium phosphate buffer, pH 6.65, containing 4 M guanidine hydrochloride. For determination of ε_M in the 2 M guanidine solutions, the blank cell contained a solution of 0.1 M sodium phosphate, pH 6.65, containing 2 M guanidine hydrochloride. In this case the decrease in absorbance at 609 nm was followed as a function of time.

The absorbance at zero time for the BDC^+ cation was determined from the y intercept of a plot of $-\ln(A\text{-}A_e)$ versus time, where A is the absorbance of the solution of any time and A_e is the absorbance at equilibrium. The molar absorption coefficient was cal-

culated by dividing the absorbance at zero time by the molarity of BDC_{total} in the solution, assuming that at zero time all of the reagent was in the form of BDC^+.

Determination of Equilibrium Constants

Samples of BDC-OH ranging in weight from 5 to 10 mg were dissolved in 10 ml of reagent grade acetone. One hundred microliter aliquots were added to a series of test tubes and diluted with either a 0.1 M sodium acetate buffer or a 0.1 M sodium acetate buffer containing 4 M guanidine hydrochloride. The pH of these solutions were adjusted to range from pH 3 to 7. After the solutions were thoroughly mixed and allowed to stand for 20 min, their absorbances were determined on the Unicam SP 800 recording spectrophotometer. The 0.1 M sodium acetate solutions were blanked against 0.1 M sodium acetate and their absorbance at 606 nm recorded. The 0.1 M sodium acetate, 4 M guanidine solutions were blanked against 0.1 M sodium acetate containing 4 M guanidine hydrochloride and their absorbance at 612 nm determined.

The determination of the two equilibrium constants, K_1 and K_2, were calculated by fitting Eq. 1 to the experimental pH curves through use of a computer.

$$[BDC^+] = \frac{[BDC_{total}]}{(1 + 1/K_1\,[H^+] + K_2\,[H^+])} \tag{1}$$

In the above equation K_1 is taken as the equilibrium constant between BDC-OH and BDC^+, and K_2 is the equilibrium constant between BDC^+ and $BDCH^{++}$.

Determination of Rate Constants

A 6-mg sample of BDC-OH was dissolved in 10 ml of reagent grade acetone. Solutions of 0.1 M sodium acetate, pH 5.1 or 0.1 M sodium acetate, pH 5.1 containing 4 M guanidine hydrochloride were prepared, and thermostated at 25°C.

Three milliliters of the 0.1 M sodium acetate buffer was placed in both cells of a Unicam SP1800 recording spectrophotometer and the absorbance adjusted to zero. Ten microliters of the BDC-OH solution was introduced into the sample cell with rapid stirring and the increase in absorbance at 606 nm was followed as a function of time.

The same procedure was followed with the 0.1 M sodium acetate buffer containing 4 M guanidine hydrochloride solutions with the exception that the wavelength monitored as a function of time was 612 nm.

k_{obs} was determined from the slope of a plot of $\ln\left(\frac{A_0 - A_e}{A - A_e}\right)$ versus time. k_{obs} was also determined from the slope of a plot of $-\ln(A - A_e)$ versus time used for calculation of the molar absorption coefficient. The rate constants k_1 and k_{-1} were calculated from from Eqs. 2 and 3.

$$k_{obs} = (k_1[H^+] + k_1k_2[H^+]^2 + k_{-1}) \tag{2}$$

$$k_1 = \frac{k_1}{k_{-1}}.$$

Rate of Reaction of the Sulfhydryl Residues of a Protein with BDC$^+$

A 6.5-mg sample of BDC-OH was dissolved in 10 ml of reagent grade acetone. One hundred microliters of this solution were added to a 10-ml volumetric flask and

diluted to volume with 0.04 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. After 20 min, 3 ml of this solution were added to the sample cell of a Unicam SP1800 blanked against 3 ml of 0.04 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. Fifty microliters of protein solution were introduced into the sample cell and the decrease in absorbance at 612 nm was monitored as a function of time. The protein solution was prepared by dissolving 8.8 mg of lyophilized mitochondrial malate dehydrogenase from porcine heart in 2 ml of 0.04 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride.

RESULTS AND DISCUSSION

When 4,4′-*bis*-dimethylaminodiphenylcarbinol (BDC-OH) is dissolved in aqueous buffers in the pH range 3–6, an equilibrium is established in which at least three species are present. The actual concentration of each species present is determined by the hydrogen ion concentration of the solution. This equilibrium is illustrated in Fig. 1. The

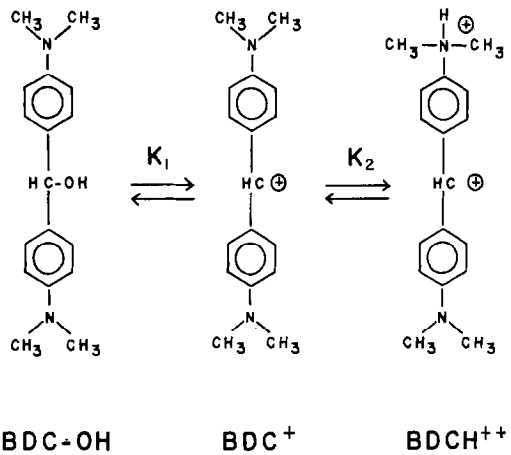

FIG. 1. Equilibria among the three species, BDC-OH, BDC^+, and $BDCH^{++}$ present in aqueous buffers.

three species present are BDC-OH, 4,4′-*bis*-dimethylaminodiphenylmethylcation, (BDC^+), and a dication $BDCH^{++}$ which we postulate has the structure indicated in which one amino group is protonated.[4] The only species which exhibits a visible absorption is BDC^+ with a λ_{max} of 606–612 nm, while both BDC-OH and $BDCH^{++}$ are transparent in this region.

Table 1 demonstrates the effect on the $\varepsilon_{m,app}$ by increasing only the concentration of guanidine hydrochloride. The absorption maxima were determined for samples of BDC^+ prepared in 0.04 M sodium acetate, pH 5.10, containing 0, 2, 4, and 6 M guanidine hydrochloride. As noted, a small red shift in λ_{max} was observed in the samples as the

[4] The pK_a (5.48) determined (See Table 2) for the $BDCH^{++}$ species supports the postulate of an N-protonated species based on other *N,N* dimethyl-substituted anilines. For example, the pK_a for *N,N*-dimethylaniline is 5.1 (6).

TABLE 1

ABSORPTION MAXIMUM AND APPARENT MOLAR ABSORPTION COEFFICIENTS OF BDC^{+a}

Conditions	Absorption maximum (nm)	Apparent[b] molar absorption coefficient (M^{-1} cm^{-1})
0.04 M sodium acetate, pH 5.1	606	10,000
0.04 M sodium acetate, pH 5.1, 2 M guanidine HCl	609	35,000
0.04 M sodium acetate, pH 5.1, 4 M guanidine HCl	612	70,800
0.04 M sodium acetate, pH 5.1, 6 M guanidine HCl	614	100,000

[a] A sample of BDC-OH (6.5 mg) was prepared in 10 ml of reagent-grade acetone. One hundred microlitres of this stock solution were transferred to each of the four volumetric flasks which contained sodium acetate buffer and guanidine hydrochloride as noted in the left-hand column. The solutions were allowed to equilibrate for 20 min and then scanned over the 600 to 620-nm region. Absorbance maxima and apparent molar absorption coefficients were determined by use of these scans.

[b] Apparent molar absorption coefficient refers to that value determined if one assumes that all of the BDC_{total} is present as the BDC$^+$ cation under a given set of conditions. This value is the only value required for thiol determinations.

concentration of guanidine hydrochloride was increased from 0 to 6M. The apparent molar absorption of BDC$^+$ in 0.04 M sodium acetate, pH 5.1, was determined to be 10,000 M^{-1} cm^{-1}; upon the addition of 6 M guanidine hydrochloride a value of 100,000 M^{-1} cm^{-1} was obtained—a 10-fold enhancement.

To obtain a true molar absorption coefficient of the BDC$^+$ cation, it was necessary to attain conditions under which the BDC_{total} concentration was present as the BDC$^+$ cation. Deno *et al.* (*5*) has reported the formation and stabilization of similar cations in solutions of 90% sulfuric acid. Therefore, samples of BDC-OH were dissolved in 90% sulfuric acid so that all the reagent, BDC_{total}, will exist in the dication forms. Spectrophotometric analysis of the sulfuric acid solutions indicates that no detectable BDC$^+$ is present in such solutions, i.e., absences of absorbance over 606 to 612-nm region. In addition to the N-protonated dication BDCH^{++}, it seems likely that other species, such as ring-protonated dications, may also exist under these strong acid conditions. However, upon addition of the sulfuric acid solutions to aqueous buffer solutions, final pH 6.65, rapid deprotonation of all the dication forms occurs to yield solutions in which initially the BDC_{total} exists exclusively as BDC$^+$. At a slower rate which can be followed spectrophotometrically, the BDC$^+$ reacts with water to yield an equilibrium mixture of BDC-OH and BDC$^+$. As discussed below, the concentration of BDCH^{++} will be less than 0.1% of the BDC_{total} concentration at this pH and the concentration of BDC_{total} will, therefore, be the sum of the concentrations of BDC$^+$ and BDC-OH.

The true molar absorption coefficient of BDC$^+$ can be determined from the absorbance at zero time (A_0). A_0 can be determined through application of Eq. 4 and extrapolation to zero time of a plot of $-\ln(A - A_e)$ versus time

$$-\ln(A - A_e) = -\ln(A_0 - A_e) + k_{obs}t. \tag{4}$$

Figure 2 is a plot of Eq. 4 using the data obtained in one determination of the molar

absorption coefficient in 0.1 M sodium phosphate, pH 6.65, containing 4 M guanidine hydrochloride.

A molar absorption coefficient of 128,000 ± 4,000 M^{-1} cm^{-1} for BDC^+ was determined from the average of five independent weighings of BDC-OH. Molar absorption coefficients determined in 0.1 M sodium phosphate buffers which were either 2 M or 4 M in guanidine hydrochloride also yielded identical values. Thus, this finding suggests the absence of an effect on ionic strength on the molar absorption coefficient of the BDC^+ cation.

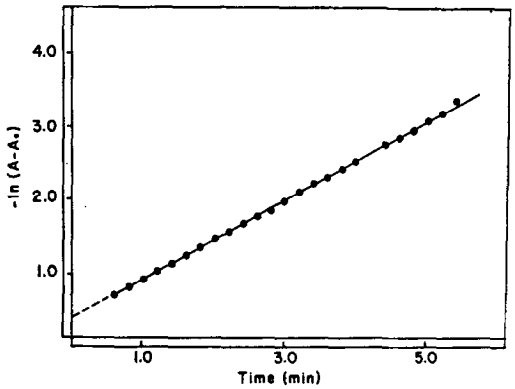

FIG. 2. Determination of the molar absorption coefficient of BDC^+. BDC-OH (21.60 mg) was dissolved in sulfuric acid and diluted into 0.1 M sodium phosphate, pH 6.65, containing 4 M guanidine hydrochloride. The decrease in absorbance at 612 nm is shown as a function of time. The molar absorption coefficient was determined as described in 'Experimental Procedure.'

Having determined the molar absorption coefficient for BDC^+, it was possible to determine the equilibrium constants, K_1 and K_2, (see Fig. 3) from the pH profile. The absorbance at 612 nm of each sample was determined as explained in the Methods section and plotted as a function of pH. As seen in Fig. 3 the spectral properties of BDC^+

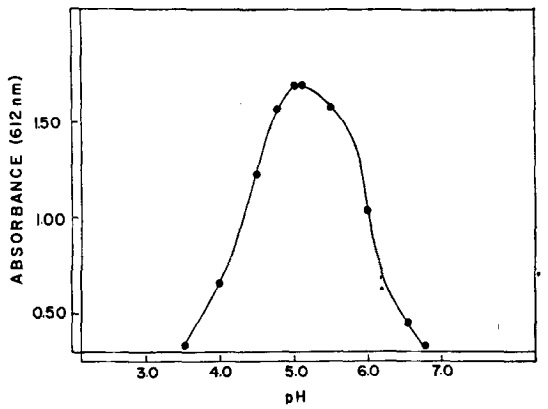

FIG. 3. The pH dependence of the absorbance of BDC^+ in 0.04 M sodium acetate buffer, containing 4 M guanidine hydrochloride. A sample of BDC-OH (6.61 mg) was dissolved in 10 ml of reagent-grade acetone; 100-μl aliquots of this stock solution were transferred to 10-ml volumetric flasks and diluted to volume with solutions of 0.04 M sodium acetate buffer, containing 4.0 M guanidine hydrochloride, ranging in pH from 3.5 to 6.8. The absorbance of each solution was determined at 612 nm using the appropriate blank in each case.

are highly pH dependent; however, in the range pH 5.0–5.2 one observes a ΔA_{612} of the order of only 0.5% of the total absorption at this wavelength.

K_1 and K_2 for the three species present in a given solution were determined by obtaining a computer fit of Eq. 5 to the experimentally determined pH profile of the BDC^+ cation.

$$[BDC^+] = \frac{BDC_{total}}{(1 + 1/K_1[H^+] + K_2[H^+])}, \tag{5}$$

where

$$K_1 = \frac{[BDC^+]}{[BDC\text{-}OH]\,[H^+]} \tag{6}$$

and

$$K_2 = \frac{[BDCH^{++}]}{[BDC^+]\,[H^+]} \tag{7}$$

Equation 8 describes the relationship of the BDC_{total} concentration to the three species present.

$$(BDC_{total}] = [BDC\text{-}OH] + [BDC^+] + [BDCH^{++}] \tag{8}$$

Substitution of Eq. 6 and 7 into Eq. 8 yields Eq. 5.

The values for $K_{a,1}$ and $K_{a,2}$ were determined from Eq. (9).

$$K_a = 1/K. \tag{9}$$

Table 2 contains the values determined for K_1, K_2, pK_{a_1}, pK_{a_2}, K_{a_1} K_{a_2} resulting from the computer fit of Eq. 5 to the experimentally determined pH profiles in 0.1 M sodium acetate and in 0.1 M sodium acetate buffer, containing 4 M guanidine hydrochloride.

TABLE 2

EQUILIBRIUM CONSTANTS, ACID DISSOCIATION CONSTANTS AND pK_a FOR THE THREE SPECIES COMPOSING BDC_{total}

	Solvent system	
	0.1 M Sodium acetate, 4 M guanidine hydrochloride	0.1 M Sodium acetate
K_1	$3.58 \times 10^5\ M^{-1}$	$1.32 \times 10^4\ M^{-1}$
$K_{a,1}$	2.79×10^{-6} M	7.58×10^{-5} M
$pK_{a,1}$	5.55	4.12
K_2	$5.06 \times 10^4\ M^{-1}$	$2.99 \times 10^5\ M^{-1}$
$K_{a,2}$	1.98×10^{-5} M	3.34×10^{-6} M
$pK_{a,2}$	4.70	5.48

In 0.1 M sodium acetate buffer, pH 5.10, containing 4 M guanidine hydrochloride, the pK_a for the equilibrium of $BDC^+ \leftrightharpoons BDCH^{++}$ is 4.70 and the pK_a for the equilibrium of $BDC\text{-}OH \leftrightharpoons BDC^+$ is 5.55. At any pH the amount of BDC^+ present will depend upon these equilibria. Figure 4 illustrates the percentage of BDC_{total} that each of the three species represents as a function of pH. The pH profile for formation of BDC^+ can be

understood as a function of the two competing equilibria. As can be seen, above pH 7.5, BDC-OH is the only species present in amounts greater than 0.1 % of BDC_{total}. As the pH is lowered, BDC-OH is converted to BDC^+; further decreases in the pH begin to yield significant amounts of $BDCH^{++}$. At pH 5.10 the two competing equilibria result in the maximal formation of BDC^+. At pH 2.0 the only species present in significant amounts is $BDCH^{++}$.

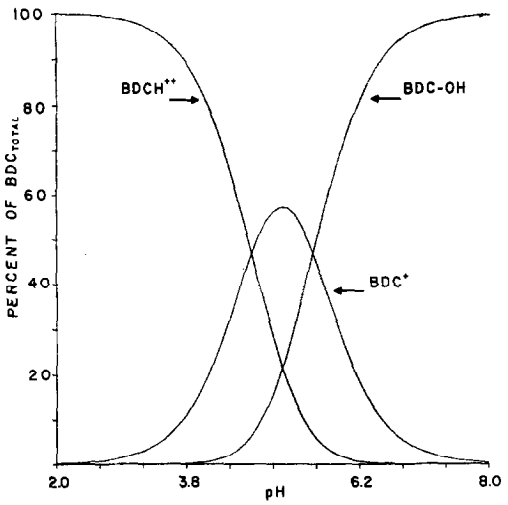

FIG. 4. The percentage of BDC_{total} represented by BDC-OH, BDC^+, and $BDCH^{++}$ as a function of pH in 0.1 M sodium acetate containing 4 M guanidine hydrochloride. The percentage of BDC_{total} for each of the three species at any given pH was calculated from the equilibrium constants (Table 2) assuming that these are the only species present in this pH range.

In 0.1 M sodium acetate buffer, the pK_a for the equilibrium $BDC^+ \rightleftharpoons BDCH^{++}$ is 5.48 and the pK_a for the equilibrium BDC-OH $\rightleftharpoons$ BDC^+ is 4.12. Figure 5 represents the percentage of BDC_{total} that each of these three species represents as a function of pH in acetate buffer.

The pK_a of the BDC-OH $\rightleftharpoons$ BDC^+ equilibrium is lowered by 1.43 pK_a units in the sodium acetate buffer as compared to the sodium acetate–guanidine hydrochloride buffer system. By contrast the pK_a of the $BDC^+ \rightleftharpoons BDCH^{++}$ equilibrium is raised by 0.78 pK_a units in the sodium acetate buffer system. These two shifts in pK_a have the net result of producing a lower maximal amount of BDC^+ in the sodium acetate buffer system and shifting the pH optimum from 5.10 in sodium acetate–guanidine hydrochloride to 4.80 in sodium acetate alone. This lower maximal concentration of BDC^+ accounts for the decrease in $\varepsilon_{M,app}$ as the guanidine hydrochloride content of a given buffer is reduced from 6 M to zero; see Table 1. Both of the shifts in pK_a can be attributed to the guanidine hydrochloride itself and not a general salt effect, since the apparent molar absorption coefficient for BDC^+ in 0.1 M sodium acetate buffer, pH 5.1, containing 4 M sodium chloride, is identical with that determined in sodium acetate alone.

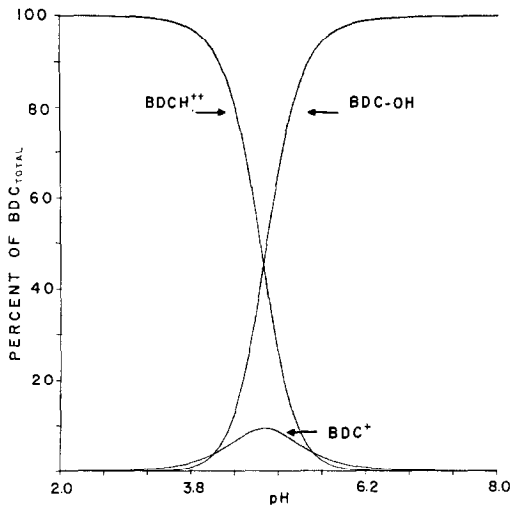

FIG. 5. The percentage of BDC_{total} represented by BDC-OH, BDC^+, and $BDCH^{++}$ as a function of pH in 0.1 M sodium acetate. The percentage of BDC_{total} for each of the three species at any given pH was calculated from the equilibrium constants (Table 2) assuming that these are the only species present in this pH range.

From the k_{obs} of the interconversion of BDC-OH and BDC^+, k_1 and k_{-1} can be calculated from Eq. 2 and 3. Equation 2 was derived with the following assumptions:

(1) The interconversion of BDC^+ and $BDCH^{++}$ is very fast compared to the interconversion of BDC-OH and BDC^+.

(2) $[BDC\text{-}OH]_0 = BDC_{total}$, $[BDC^+]_0 = 0$, $[BDCH^{++}] = 0$.

(3) $[BDC\text{-}OH]_t = BDC_{total} - [BDC^+]_t - K_2\,[H^+]\,[BDC^+]_t$,

where K_2 is the equilibrium constant at pH 5.10.

$[\ ]_0$ = concentration at zero time, $[\ ]_t$ refers to concentration at any given time.

Table 3 contains the values for $t_{1/2}$, k_1 and k_{-1} in both buffer systems as determined at pH 5.10. From the rate constants, k_1 and k_{-1}, it is apparent that k_{-1} is most affected

TABLE 3

KINETIC CONSTANTS FOR THE BDC-OH TO BDC^+ EQUILIBRIUM

	0.1 M Sodium acetate, pH 5.1, 4 M Guanidine hydrochloride	0.1 M Sodium acetate, pH 5.1
$t_{1/2}$	1.28 min	0.35 min
k_1	3.92×10^4 min^{-1} M^{-1}	1.95×10^4 min^{-1} M^{-1}
k_{-1}	0.11 min^{-1}	1.49 min^{-1}
k_{obs}	0.55 min^{-1}	2.0 min^{-1}

by the composition of the buffer. When the buffer is changed from one containing no guanidine hydrochloride to one containing 4 M guanidine hydrochloride, the forward

rate constant, k_1, is doubled while the reverse rate constant, k_{-1}, decreases 14-fold. Thus, the large change in the equilibrium constant K_1, upon addition of 4 M guanidine hydrochloride, is largely a reflection of the decreased rate of the conversion of BDC^+ back to BDC-OH.

This directs attention to the simple fact that in the absence of guanidine hydrochloride, the maximum concentration of BDC^+ formed is only approximately 10% of BDC_{total}. This indicates that less BDC^+ is present than noted by molar excess values

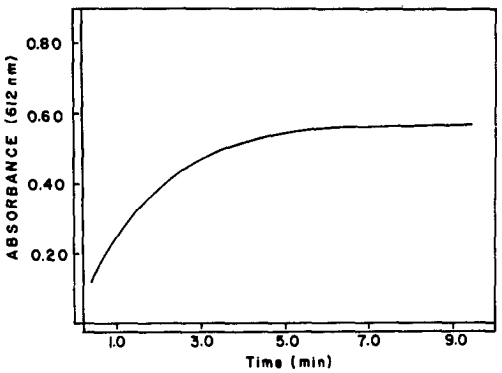

FIG. 6. Time course for the establishment of equilibria among BDC-OH, BDC^+, $BDCH^{++}$, in 0.1 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. BDC-OH (5.95 mg) was dissolved in 10 ml of reagent-grade acetone. Ten microlitres were added to 3 ml of 0.1 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. The increase in absorbance of this solution was followed at 612 nm as a function of time on a recording spectrophotometer.

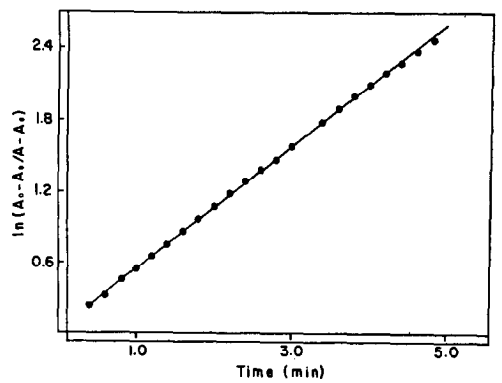

FIG. 7. Determination of the half-life for the establishment of equilibria in 0.1 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. The data obtained for the time course of the establishment of equilibria as shown in Fig. 5 were plotted utilizing the following equation: $\ln(A_0\text{-}A_e/A\text{-}A_e) = k_{obs}t$. The half-life was determined by division of 0.693 by k_{obs} (slope of the line).

reported in chemical modification experiments. In a preceding report (2) demonstrating the inactivation of malate dehydrogenase with BDC^+, a 20-fold molar excess of BDC_{total} was used. In terms of BDC^+, this concentration was actually only a constant 2-fold molar excess over enzyme, and thus the reagent is even more effective than one would suspect.

In order to determine the time required to ensure quantitative reaction of BDC^+ with the sulfhydryl residue, it was necessary to determine the time required to establish the equilibrium between BDC-OH and BDC^+. Figure 6 illustrates the time course for the formation of BDC^+ from BDC-OH and the establishment of the equilibrium in 0.1 M sodium acetate buffer, pH 5.10, containing 4 M guanidine hydrochloride. The $t_{1/2}$ for this equilibrium was determined from Fig. 7, which is a plot utilizing Eq. 4 of the experimental data given in Fig. 6. The $t_{1/2}$ for the disappearance of BDC-OH is 1.28 min. Since the establishment of equilibrium will be 99.9% complete after 10 half-lifes (13

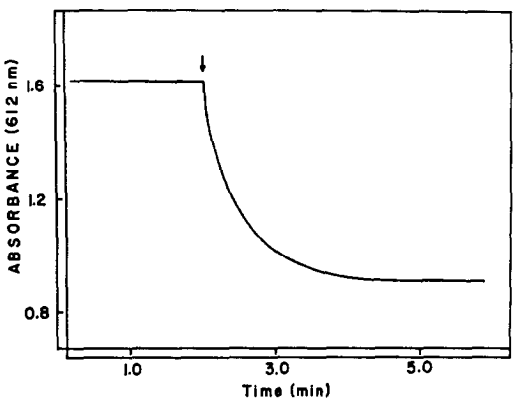

FIG. 8. Time course for the reaction of BDC^+ and protein sulfhydryl in 0.1 M sodium acetate, pH 5.10, containing 4 M guanidine hydrochloride. At the time (indicated by arrow) the protein solution was introduced and the decrease in absorbance of 612 nm followed as a function of time. See 'Experimental Procedures' for details.

min) it can be safely assumed that the absorbance read after 20 min will represent equilibrium values. The establishment of the same equilibrium in sodium acetate buffer demonstrates a half-life of 0.35 min, so 4 min would ensure that equilibrium had been established.

Figure 8 illustrates the time course for the reaction of protein sulfhydryls with BDC^+ in 0.1 M sodium acetate, pH 5.1, containing 4 M guanidine hydrochloride.[5] In this experiment the sulfhydryl concentration is 50% of the BDC_{total} concentration. As seen in the figure, 4 min after introduction of sulfhydryl-containing protein the reaction is 99.9% complete. In the preceding report (*1*) describing the analytical technique for sulfhydryl residues, we have suggested a 20-min incubation period to follow dilution of the protein, BDC-OH solution. Therefore, this incubation period is sufficient to ensure the complete reaction of all protein sulfhydryls and the establishment of the equilibrium value for BDC^+.

The reaction of BDC^+ with simple thiols over a wide range of sulfhydryl concentrations is represented in Fig. 9. As can be seen, the reaction is linear only when the sulfhydryl concentration is between 15 and 90% of the BDC_{total} concentration. The

[5] Although BDC^+ has been indicated as the species which reacts with sulfhydryls, it is recognized that the $BDCH^{++}$ ion may, in fact, be the reactive species, due to the greater positive charge at the central carbon. This possibility will have no effect on the technique for sulfhydryl determination or the kinetic analysis discussed in this paper.

nonlinearity at low sulfhydryl concentrations could be the result of an impurity in the system, which reacts with sulfhydryl groups. The actual sulfhydryl concentration would be reduced by the amount of impurity which had reacted with free sulfhydryl groups This would have the net effect of having more BDC^+ than would theoretically be expected. Attempts to determine if an impurity in the system is a cause of nonlinearity have not been successful. Repeated recrystallization of 4,4'-*bis*-dimethylaminobenzophenone, the starting material in the synthesis, and of BDC-OH itself have not removed the observed nonlinearity. Recrystallization of the guanidine hydrochloride has, likewise, resulted in no change.

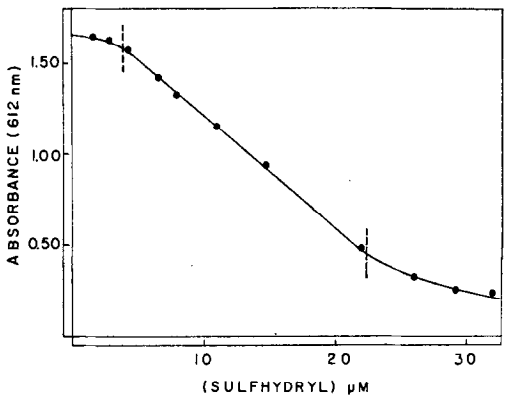

FIG. 9. Determination of the sulfhydryl content of 2-mercaptoethanol. A stock solution of 2-mercaptoethanol (1.42 mM) was prepared. Aliquots of 10–225 μl were added to a series of 10-ml volumetric flasks containing 100 μl of a stock solution of BDC-OH in acetone (6.5 mg/10 ml). Each flask was diluted to volume with 0.04 M sodium acetate buffer, pH 5.1, containing 4.0 M guanidine hydrochloride thoroughly mixed, and their absorbances at 612 nm determined. The dotted line delineates the linear portion of this graph and denotes that portion used for determination of sulfhydryl content. Sulfhydryl content was determined by dividing the slope of the linear portion of this graph by the apparent molar absorption coefficient for BDC^+ under these conditions.

An equilibrium between BDC^+ and BDC-SR at pH 5.10 seems to be the most probable cause of nonlinearity. If such an equilibrium exists, the concentration of BDC^+ present at all sulfhydryl concentrations would be greater than expected. At nearly equal concentrations of BDC^+ and sulfhydryl, a finite amount of BDC^+ would exist in solution. Both of these predictions agree with observed data. As further support of this explanation it has been noted that addition of a large (100 M) excess of sulfhydryl does result in the complete consumption of BDC^+.

It is evident that whether the nonlinearity is due to an impurity or an equilibrium between BDC^+ and BDC-SR, accurate, reliable values for sulfhydryl concentrations can be determined at sulfhydryl concentrations lower than 90% or higher than 15% of the BDC_{total}. For that range of sulfhydryl group concentrations where linearity is observed, accurate determinations are possible if the slope method as described previously (*1*) is used for their calculation. This procedure, in effect, cancels any deviation imported to the system by either the presence of a small amount of impurity or an equilibrium reaction between BDC^+ and BDC-SR.

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