Studies on Myosin–Azomercurial Complexes*

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ABSTRACT: The acidity constants of the azomercurial phenolic group in an adduct of 0.1 mole of 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate/10^5 g of myosin was determined in various solvents. The pK_a of 3 mg of adduct/ml of 0.1 M KCl was 9.16, while after denaturation in 8 M urea or 5 M guanidine, it was considerably lower, close to that of the azomercurial linked to β-mercaptoethylamine. The acidity constants were concentration dependent and were affected by the species of cation and species of anion as well as by the total salt concentration. The cations could be ranked in the series $Li^+ < Na^+ < K^+ < Rb^+$ and the anions could be ranked in the series $Cl^- < Br^- < ClO_4^- < I^-$ according to the pK_a of the adduct in solutions of their salts. Prior reaction of myosin with <3.0 moles of *p*-mercuribenzoate (PMB)/10^5 g of myosin, amounts which stimulate the ATPase activity, does not alter the acidity constants appreciably. Reaction with >4.0 moles of PMB/10^5 g of myosin results in larger shifts of pK_a.

The pK_a shifts could result from changes in hydrogen bonding of the phenolic group, stacking interactions with aromatic amino acids, or changes in the structure of the water in the immediate environment of the protein. The azomercurial is a useful probe for changes in protein conformation.

Myosin is thought to undergo subtle conformational changes in various environments which are reflected in its ATPase[1] activity and its ability to interact with actin (Rainford *et al.*, 1964; Sekine and Kielley, 1964). The changes in ATPase activity or ability to interact with actin are not accompanied by significant changes in the optical rotatory dispersion of myosin at salt concentrations less than about 2 M, even though myosin is a protein with a high helical content (Warren *et al.*, 1966). In an attempt to learn more about such changes, studies of myosin–azomercurial complexes were undertaken and they are reported in this communication.

Myosin ATPase activity is influenced by the ionic composition of the medium. Calcium ions have long been known to increase the enzyme activity (Bailey, 1942; Banga and Szent-Györgyi, 1943), an enhancement which is prevented by magnesium ions. Curiously, EDTA also activates myosin ATPase activity (Friess, 1954; Kielley *et al.*, 1956), an effect which requires the presence of potassium ions, or better still, ammonium ions. EDTA stimulation may result from the removal of the last traces of magnesium ions from the myosin (Ebashi *et al.*, 1960). Potassium ions alone activate enzyme activity (Mommaerts and Green, 1954), but in high concentrations, all monovalent cations and anions inhibit the ATPase activity, and the ions can be arranged in a lyotropic series according to their inhibiting effect (Warren *et al.*, 1966).

The sulfhydryl groups of myosin play an important role in its activity as an ATPase and in its ability to interact with actin. Although Bailey and Perry (1947) reported that the ATPase activity and the affinity for actin disappeared *pari passu* when several reagents which react with sulfhydryl groups were added to myosin, subsequent studies disclosed the sulfhydryl groups to be heterogeneous. Gilmour and Gellert (1961) were able to distinguish three types of sulfhydryl groups (rapidly reacting, slowly reacting, and very slowly reacting) on the basis of the rate of mercaptide formation with *p*-mercuribenzoate (PMB). Treatment of myosin with iodoacetamide (Barany and Barany, 1959) or with dithioglycolic acid dimethyl ester (Gaetjens *et al.*, 1964) inhibits the ATPase activity without a comparable effect upon the ability to bind actin. Stepwise reaction of the myosin sulfhydryl groups with increasing amounts of phenylmercuriacetate (Greville and Needham, 1955) or PMB (Kielley and Bradley, 1956) results in progressive activation of the nucleoside triphosphatase activity using ATP or CTP as substrate until about 3–4 SH groups/10^5 g of protein react. Further additions of phenylmercuriacetate or PMB result in progressive inhibition of enzyme activity. When ITP or GTP are used as substrates, no activation is observed.

Klotz and Ayers (1957) found that the acidity constant of an ionizable group on an azomercurial dye was much lower when the dye was attached to bovine

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[1] Abbreviations used: ATPase, adenosine triphosphatase; PMB, *p*-mercuribenzoate; CTP, ITP, and GTP, cytidine, inosine, and guanosine triphosphates; HAPM, 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate.

TABLE I: Acidity Constants of Some HAPM[a] Adducts.[b]

Solvent	pK_a of HAPM Adduct with		
	β-Mercaptoethylamine	β-Lactoglobulin[d]	Skeletal Myosin[d]
In 0.1 M KCl	8.04	9.04	9.16
In 0.1 M KCl + 8 M urea[c]	8.52 (8.02)	9.20 (8.71)	9.09 (8.59)
In 0.1 M KCl + 5 M guanidine-HCl	8.09	8.62	8.45

[a] HAPM is 4-(*p*-hydroxybenzeneazo)phenylmercuriacetate. [b] Refer to text for experimental details. Values are means of three to eight titrations. [c] Values in parentheses are "corrected" for the presence of 8 M urea (Donovan *et al.*, 1959). [d] Protein concentration, 3 mg/ml. The adduct contained 0.1 mole of HAPM/10^5 g of protein.

plasma albumin than when the dye was linked to cysteine. Upon denaturation of the protein by 8 M urea, the pK_a of the protein–azomercurial complex was nearly the same as the pK_a of the cysteine–azomercurial adduct. Thus, the pK_a of the azomercurial–protein complex varied with the conformation of the protein. Using a similar azomercurial dye, Dowben *et al.* (1965) studied actin–azomercurial complexes and found pK_a changes accompanying the G–F transformation of actin as well as changes upon denaturation. Although a precise interpretation of the pK_a changes cannot be made with certainty, a study of myosin–azomercurial complexes provides some information about the structural changes in various environments.

Experimental Procedure

Myosin was prepared from rabbit psoas muscle as described by Mommaerts (1958), except that at no time was the ionic strength of the medium permitted to fall below 0.025. The last traces of actomyosin were removed after the addition of ATP to a solution at ionic strength 0.25 (Weber, 1956). The preparation gave a single, symmetric peak upon sedimentation in the ultracentrifuge, gave a sharp single band upon electrophoresis in 12 M urea–acrylamide gel (Small *et al.*, 1961), and did not show a viscosity drop upon the addition of ATP. The thiol content of the myosin preparations was determined by the method of Boyer (1954) using a reaction time of 30 min at 20°; only preparations with >7.2 SH groups/10^5 g of protein were used.

4-(*p*-Hydroxybenzeneazo)phenylmercuriacetate (HAPM) was prepared as described previously (Zak *et al.*, 1965). The dye forms an extraordinarily insoluble chloride which does not react readily with thiols. Myosin was precipitated by lowering the salt concentration to 0.025 M and the precipitate was washed with 0.025 M ammonium acetate (pH 7.2) until the wash was free of chloride. The myosin was then dissolved in 0.5 M ammonium acetate (pH 7.2) and 0.1 mole of HAPM/10^5 g of myosin as an 0.2% solution in 2% potassium carbonate was added slowly. The mixture was stirred overnight in the cold room and then centrifuged to remove traces of unreacted dye. The myosin–azomercurial adduct was precipitated by lowering the salt concentration to 0.025 M and redissolved in the required solvent. HAPM adducts were also prepared using myosin which had been first reacted with *p*-mercuribenzoate.

The amount of dye bound was determined by adding four volumes of 0.2 M glycine buffer (pH 10.6) to an aliquot and measuring the absorbance at 434 mμ, using a molar absorptivity of 2.73×10^4. Total protein was determined by the method of Lowry *et al.* (1951).

Myosin ATPase activity was assayed in an incubation medium containing 0.15 M KCl, 10 mM $CaCl_2$, 30 mM Tris, and 2.0 mM ATP (pH 7.4) at 25° for 5 min. The inorganic phosphate liberated was measured by the method of Horwitt (1952). The activity of the native myosin preparations was 0.20–0.24 μmole of P_i/min per mg of protein. After reaction of the myosin with 2.5 moles of PMB/10^5 g of protein, the ATPase activity was 0.46–0.63 μmole of P_i/min per mg of protein; and after reaction with 5.0 moles of PMB/10^5 g of protein, the ATPase activity was <0.09 μmole of P_i/min per mg of protein.

The acidity constants (pK_a) for the conjugate acid of the azomercurial adducts were calculated from plots of absorbance at 434 mμ *vs.* pH (Klotz and Ayers, 1957), from measurement obtained with a Beckman Model B spectrophotometer and a Leeds and Northrup Model 7664 pH meter modified by the Instruments Development Products Co., Chicago, to permit simultaneous determinations of absorbance and pH.

Viscosity was measured in Ostwald–Fenske viscometer tubes having an outflow time for water of about 75 sec. Ultracentrifuge sedimentations were carried out in a Spinco Model E analytical ultracentrifuge using cells with Kel-F centerpieces.

Optical rotatory dispersion studies were carried out with a Cary Model 60 spectropolarimeter. The helicity was evaluated by fitting the data obtained between 600 and 290 mμ to a Moffitt–Yang (1956) equation and by calculating the mean residue rotation at the 233-mμ trough. The azomercurial is optically inactive and protein–azomercurial adducts do not show a Cotton

effect near the absorption maximum of the azomercurial.

ATP was purchased from Pabst Laboratories, *p*-mercuribenzoate from the California Corp. for Biochemical Research, Tris from Sigma Chemical Co., bovine plasma albumin from Pentex, Inc., and all other reagents were reagent grade commercial preparations.

Results

Acidity Constants of the Azomercurial Adducts. The acidity constants (pK_a values) of HAPM linked to β-mercaptoethylamine, β-lactoglobulin, or myosin in several solvents are listed in Table I. The pK_a of the phenolic OH group of HAPM in the soluble mercaptide formed with β-mercaptoethylamine was 8.04 in 0.1 M KCl. The pK_a of this adduct was not appreciably changed if the solvent also contained 5 M guanidine hydrochloride or 8 M urea, using values corrected for the anomalous dissociation in the latter case (Donovan *et al.*, 1959).

Protein–HAPM adducts have higher pK_a values than adducts with low molecular weight thiols. The pK_a of the β-lactoglobulin adduct in 0.1 M KCl was 9.04 and that of the myosin adduct was 9.16. In 5 M guanidine hydrochloride or 8 M urea, the pK_a's of the protein–HAPM adducts approximated the pK_a's of the β-mercaptoethylamine adduct. Typical titration curves are shown in Figure 1. The protein–HAPM adducts gave asymmetric titration curves which may indicate a conformational change at high pH above 10.0–10.2.

Physical Properties of the Myosin Adduct. The values for the reduced viscosity and sedimentation constants obtained using solutions of 1.5 mg of protein/ml of 0.20 M KCl–0.05 M phosphate buffer (pH 7.2) are listed in Table II. The values of $-b_0(\lambda_0 = 212\ m\mu)$, $-a_0$, and $-[R']_{233}$ calculated from the optical rotatory dispersion data are also listed in Table II. The values for the myosin–HAPM adduct are judged not to be significantly different from those for myosin alone. By these physical criteria, the myosin–HAPM adduct resembles myosin itself.

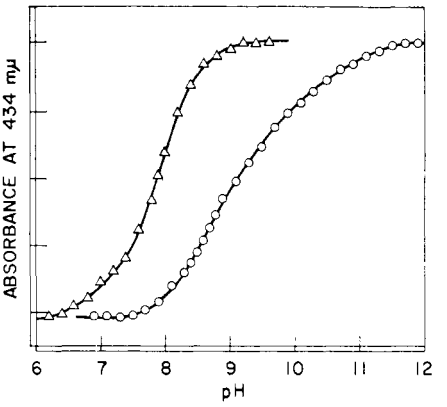

FIGURE 1: Spectrophotometric titration of the phenolic groups of HAPM adducts: (Δ- - -Δ) to β-mercaptoethylamine in water and (O- - -O) to myosin, 3.0 mg/ml of 0.5 M KCl.

TABLE II: Effect of Mercaptide Formation on Some Properties of Myosin.

	Myosin	Myosin–HAPM[a]
η_{red}[b] (dl/g)	2.32	2.27
$s_{20,w}$[b] (S)	6.18	6.34
$-a_0$	218	160
$-b_0$ (λ_0 = 212 mμ)	418	412
% helix (100% = 700; 0% = −100)	64	64
$-[R']_{233}$	7,910	7,260
% helix (100% = 12,700; 0% = 1,800)	56	52

[a] Adduct of 0.1 mole of HAPM/10^5 g of protein. [b] Determined at a protein concentration of 1.5 mg/ml in 0.20 M KCl–0.05 M phosphate buffer (pH 7.2).

Effect of Salt Concentration and Protein Concentration. In general, the pK_a was higher in solvents containing a higher salt concentration. Thus, the pK_a of myosin–HAPM adducts was 9.06 in 0.5 M KCl and 9.42 in 1.4 M KCl (Figure 1). The pK_a of cysteine–HAPM adducts was 7.95 in water, 8.10 in 0.5 M KCl, 8.15 in 1.0 M KCl, and 8.19 in 1.4 M KCl. Similar values were obtained using other salts.

In general, the pK_a for a given myosin–HAPM adduct was higher at greater protein concentrations. Thus, the pK_a's for myosin–HAPM in 0.5 M KCl were 8.99, 9.05, and 9.16 for protein concentrations of 0.6, 1.5, and 3.0 mg/ml, respectively. The addition of bovine plasma albumin to a final concentration of 2% did not change the acidity constants (Table III).

Effect of Prior Reaction of Some Sulfhydryl Groups with PMB. Prior reaction of myosin with 3.0 moles of PMB/10^5 g of protein, or less, before reaction with HAPM, concentrations which result in activation of the myosin ATPase activity, resulted in very small increases in the acidity constants of the subsequently formed azomercurial complexes at all ionic strengths compared to myosin–HAPM adducts (Figure 2). If more than 4.0 moles of PMB/10^5 g of protein was added before forming the HAPM adduct, the pK_a's were markedly elevated. Thus, the pK_a's were 9.06, 9.10, and 9.36 for adducts formed with myosin, with 2.5 moles of PMB/10^5 g of protein, and with 5.0 moles of PMB/10^5 g of protein, respectively.

Effect of Various Cations and Anions on the Acidity Constants. The pK_a of myosin–HAPM adducts at a given protein concentration and at a given ionic strength depended upon the principal cation and principal anion (Table IV). The cations could be arranged

TABLE III: Effect of Salt Concentration and Protein Concentration on the Acidity Constants of Myosin–HAPM Adducts.[a]

Solvent (M)	Myosin Concn (mg/ml)	pK_a
KCl (0.5)	0.6	8.98, 8.99, 9.00
KCl (0.5)	1.5	9.05, 0.96, 9.06
KCl (0.5)	3.0	9.15, 9.15, 9.16, 9.16
KCl (1.4)	0.6	9.28, 9.36, 9.38, 9.41
KCl (1.4)	3.0	9.41, 9.52
KCl (0.5) + PP (0.01)[b]	3.0	9.12, 9.18, 9.21
KCl (0.5) + BPA (2%)[b]	1.5	9.08, 9.10, 9.11
KCl (0.5) + BPA (2%)[b]	0.6	8.93

[a] Adducts of 0.1 mole of HAPM/10^5 g of myosin. [b] BPA, bovine plasma albumin; PP, pyridoxal phosphate.

in the following series according to increasing values of the acidity constants of the azomercurial: $Li^+ < Na^+ < K^+ < Rb^+$. The anions could be arranged in the following series according to increasing values of the acidity constants: $Cl^- < Br^- < ClO_4^- < I^-$. When dissolved in 2.5 M LiBr, a solvent known to disrupt protein structure markedly, the myosin–HAPM adduct had an acidity constant of about 8.30, a value close to those of several protein–azomercurial complexes denatured by 8 M urea or 5 M guanidine hydrochloride.

TABLE IV: Acidity Constants of Myosin–HAPM Adducts in Solutions of Various Salts.[a]

Solvent (M)	Myosin Concn (mg/ml)	pK_a
LiCl (0.5)	0.6	8.80, 8.83, 8.84
LiCl (0.5)	3.0	8.95, 8.95, 8.96
NaCl (0.5)	3.0	8.94, 8.96
KCl (0.5)	3.0	9.15, 9.15, 9.16, 9.16
RbCl (0.5)	3.0	9.23, 9.25
LiBr (0.5)	3.0	8.89, 8.96
LiBr (2.5)	3.0	8.30, 8.26, 8.42
KBr (0.5)	3.0	9.32
KI (0.5)	3.0	9.60, 9.64
$NaClO_4$ (0.5)	3.0	9.46

[a] Adducts of 0.1 mole of HAPM/10^5 g of myosin.

Discussion

The high pK_a of the phenolic OH group of the azomercurial–myosin adduct could result from a number of circumstances. It resembles the anomalous acidity constants of the phenolic groups of certain tyrosine residues in proteins. Spectrophotometric titration of myosin has shown that some of the tyrosine residues have abnormal acidity constants (Stracher, 1960). In part, the high pK_a of phenolic groups could result from its location in an environment of dense negative charge. The shift upward of pK_a with increasing salt concentration speaks against a simple electrostatic effect. The phenolic groups on the tyrosine residues have been thought to take part in intramolecular hydrogen bonds resulting in anomalous acidity constants. While it is possible that the phenolic group of the azomercurial also participates in an intramolecular hydrogen bond, the same reservations that are applicable to this interpretation of tyrosine interactions are also applicable here (Williams and Foster, 1959).

TABLE V: Intrinsic pK and w Calculated for Myosin–HAPM Adducts in Various Salt Solutions.

Solvent (M)	Protein Concn (mg/ml)	pK_{int}	$w_{obsd}/5.7 \times 10^5$ g
KCl (0.5)	0.6	7.7	6.6×10^{-3}
KCl (0.5)	3.0	8.3	3.9×10^{-3}
KCl (1.4)	3.0	8.3	4.3×10^{-3}
NaCl (0.5)	3.0	8.3	3.4×10^{-3}
LiCl (0.5)	3.0	8.4	2.9×10^{-3}
KBr (0.5)	3.0	9.0	1.3×10^{-3}
KI (0.5)	3.0	9.4	0.9×10^{-3}
$NaClO_4$ (0.5)	3.0	9.7	0.8×10^{-3}

The intrinsic pK of the phenolic group of HAPM can be evaluated from the relation $pK_{int} = pH - \log \alpha/(1 - \alpha) + 0.868w\bar{Z}$, where α is the degree of ionization measured spectrophotometrically at any given pH, $\bar{Z}$ is the average charge at that pH, and w is an empirical electrostatic interaction factor. The data for the myosin–HAPM adduct (Table V) in 0.5 and 1.4 M KCl are plotted as [pH $-$ log $\alpha/(1 - \alpha)$] *vs.* $\bar{Z}$ in Figure 3. The average charge for myosin was calculated from the titration data of Mihalyi (1950) in 0.6 M KCl. The data fall on a straight line at pH values below 10, but deviate from linearity at pH values above 10–10.5. The deviations at high pH correspond to the asymmetry of the titration curves at high pH and may reflect a conformational change in the myosin. Lowey (1965) found a constant $-b_0$ value from optical rotatory dispersion measurements of myosin between pH 6 and 10, and lower values of $-b_0$ at pH values above 10. Duke *et al.* (1966) found

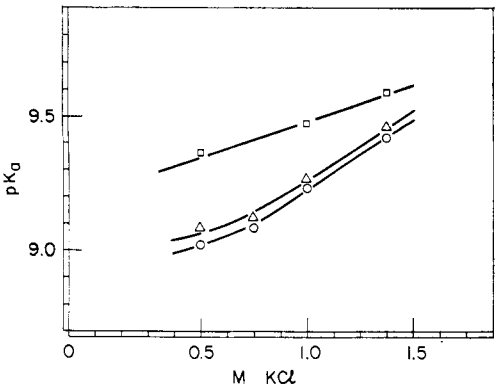

FIGURE 2: Acidity constants of several myosin–azomercurial adducts in varying ionic strength KCl solutions: (O- - - -O) myosin and 0.1 mole of HAPM/10^5 g of protein; (Δ- - - -Δ) myosin and 2.5 moles of PMB/10^5 g of protein, then +0.1 mole of HAPM/10^5 g of protein; and (□- - - -□) myosin and 5.0 moles of PMB/10^5 g of protein, then +0.1 mole of HAPM/10^5 g of protein. Protein concentration, 1.5 mg/ml.

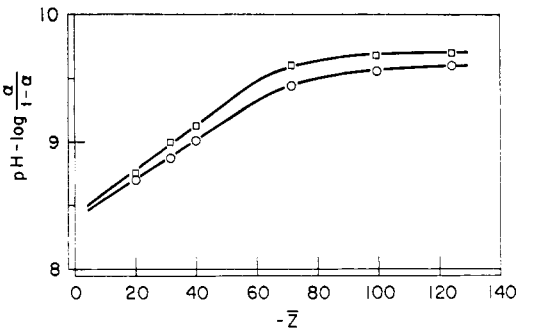

FIGURE 3: Data from the titration curves of myosin–HAPM adducts plotted according to eq 1. (O- - - -O) In 0.5 M KCl. (□- - - -□) In 1.4 M KCl. Protein concentration, 3.0 mg/ml.

increased fluorescence of myosin-8-anilino-1-naphthalenesulfonate mixtures above pH 9, additional evidence of a conformational change in this pH range. The pK_{int} in KCl is in the same neighborhood as the pK_a of the β-mercaptoethylamine–HAPM adduct.

The shift in pK_a of an azomercurial dye attached to proteins compared to low molecular weight thiol adducts was interpreted by Klotz (Klotz and Ayers, 1957; Klotz, 1960) in terms of an envelope of organized water surrounding the protein molecules, for which Klotz has coined the descriptive term *hydrotactoids*. The ionization of titratable groups embedded in this layer of water would be hindered because of the disruptive effect of ionic charges on the structure of the lattice. Such an envelope of structured water could arise from clusters of hydrogen-bonded water molecules stabilized by the protein (Némethy and Scheraga, 1962) or clathrate formation around protein side chains (Klotz, 1960).

While the causes of the pK_a shifts are not understood with certainty, the azomercurial appears to act as a valuable probe for changes of protein conformation. Thus, large changes are observed upon denaturation with 8 M urea or 5 M guanidine hydrochloride, and small increases in the acidity constants of the myosin–HAPM adducts with increasing salt concentration in the solvent. The latter could be explained by conformational changes which increased an intramolecular hydrogen bond of the phenolic group on the dye, by planar stacking interactions with aromatic amino acid residues in the protein, or by increased binding of water by the protein. In support of the latter view, Clark and Schachman[2] have evaluated bound water of several proteins by measuring changes in the solvent phase after sedimentation in the ultracentrifuge and found increased amounts of adsorbed water on several proteins with increased ionic strength of the solvent.

The concentration dependence of the acidity constant of the myosin–HAPM adduct undoubtedly reflects a concentration-dependent association of myosin which is also reflected in other physical measurements such as the marked concentration dependence of viscosity of myosin solutions. It is of interest that the pK_a of the azomercurial was not affected by the presence of 2% bovine plasma albumin. This could be interpreted as indicating a lack of association between bovine plasma albumin and myosin, or an association which does not alter the conformation of the myosin as judged by this probe.

The difference in effects of various cations and anions can also be interpreted in these terms. The various cations could be ranked according to the pK_a of the myosin–HAPM adduct in an 0.5 M solution of the salt giving a series from high to low pK_a that corresponds to the series of Warren *et al.* (1966). These authors ranked cations according to increasing effectiveness in inhibiting the ATPase activity of myosin, and the activity of several other enzymes. The anions could be similarly ranked, but in this case the order of anions in the series is exactly reversed from the series of Warren *et al.* Salts in solutions of this concentration are known to have effects upon the structure of solvent water which depend upon the species of cation and anion. Furthermore, ions can interact directly with proteins and such interactions vary with the species of ion and may result in differences in conformational changes. In 2.5 M LiBr, a solvent known to produce profound conformational changes in myosin and other proteins, the pK_a of the myosin–HAPM adduct was in the range of the denatured myosin–HAPM adduct. The addition of 10 mM pyrophosphate did not shift the pK_a of the azomercurial.

Prior reaction of myosin with 3.0 moles of PMB/10^5 g of myosin or less, amounts that stimulate the ATPase activity, resulted in azomercurial adducts which had

[2] J. B. Clark and H. K. Schachman, unpublished results.

pK_a's not very different from myosin itself. On the other hand, reaction of myosin with large amounts of PMB, more than 4.0 moles/10^5 g of protein, inhibits the ATPase activity (Rainford *et al.*, 1964), and produces profound conformational changes as judged, for example, by changes in fluorescence of a complex with 8-anilino-1-naphthalenesulfonate (Duke *et al.*, 1966), and by a decrease in helicity in optical rotatory dispersion studies. Azomercurial adducts formed from the latter type of PMB derivatives did have shifted pK_a's, but curiously, the shift was in the direction of higher values.

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