The Spectrophotometric Titration of the Sulfhydryl and Phenolic Groups of Aldolase*

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The ionization of the phenolic and sulfhydryl groups of rabbit muscle aldolase can be followed as a function of *p*H by measurements of ultraviolet difference spectra in the region 230–320 mμ. In the denaturing solvent 4 M urea, the sulfhydryl and phenolic titration curves are reversible, and the *pK* values of these groups are normal. In aqueous solution large numbers of both these types of groups are found to be "buried" in the interior of the native molecule. They ionize only when the conformation of the molecule is disrupted. The titration curves are abnormal, irreversible, and time-dependent. From the disappearance of spectral perturbation characteristic of these "buried" groups, the amount of conformational change can be calculated as a function of *p*H. The spectrophotometric titration of the sulfhydryl groups can be used as a straightforward method for determining the total number of these groups in a protein, if a suitable denaturing solvent is available. Only one difference spectrum is required for such a determination.

If the absorption spectrum of the ionized form of a molecule is different from that of the un-ionized form, then the change in the absorption as a function of *p*H can be used to determine the titration curve of the ionizing group of the molecule. This titration is normally carried out by means of a difference method in which either the ionized or un-ionized form is used as a reference solution in determining the absorption of the solution being titrated. The titration of the phenolic chromophore in proteins has been regularly done spectrophotometrically since the time of the first studies made in this way (Stenström and Reinhard, 1925; Crammer and Neuberger, 1943).

The spectrophotometric titration of sulfhydryl groups in proteins has not been extensively studied, although the extinction coefficient of the ionized sulfhydryl group at 235 mμ is nearly twice that of the ionized phenolic group at 295 mμ. In fact, the only spectrophotometric titration of sulfhydryl groups in a protein that the author knows of was performed by Benesch and Benesch (1959), who treated gelatin with a reagent that introduced sulfhydryl groups into the molecule. The change in the spectrum near 235 mμ due to the ionization of the sulfhydryl group could be easily observed, since gelatin contains essentially no tyrosine or tryptophan, both of which absorb strongly in this wavelength region.

The two principal difficulties which have discouraged the titration of sulfhydryl groups in proteins have been: (1) The generally high absorbance in the 235-mμ region caused by other chromophores in protein molecules, notably tyrosine and tryptophan, makes the change in absorbance caused by the sulfhydryl ionization a relatively small change in proteins with reasonable tyrosine and tryptophan content. (2) The change in the absorbance produced by the ionization of the phenolic chromophores in the 235-mμ region is approximately five times as large as the change produced by this chromophore in the 295-mμ region. Particularly for "buried" sulfhydryl groups, this change in absorption with *p*H of the phenolic groups occurs simultaneously with that of the sulfhydryl groups.

A difficulty which does not appear to have been recognized, but which appears in the present study (see below), is the change in absorbance in the 235-mμ region caused by the change in the perturbation of other chromophores (notably tyrosine and tryptophan) as the conformation of the molecule undergoes change in the *p*H region of the titration.

EXPERIMENTAL

The preparation and storage of aldolase has been described (Taylor *et al.*, 1948; Stellwagen and Shachman, 1962). The enzyme was dialyzed against deionized distilled water before use. Solutions so dialyzed were used within a few days, although dialyzed solutions stored at 4° for as long as three weeks showed no significant differences in the present experiments.

Spectrophotometric measurements were made using a Cary Model 11 spectrophotometer with a thermostated sample-cell holder at 25° ± 0.5°. Silica 1-cm cells were used for all measurements. For the perturbation difference spectra some measurements were made in 1-cm "tandem" cells (Herskovits and Laskowski, 1962). If these were not used, solvent baselines were determined separately and subtracted. For both spectra and difference spectra, special care was taken with solvent baselines and reference-reference baselines. At high *p*H, small corrections (a few per cent of the total difference absorption) were necessary for the absorption of KOH and urea in the 230-mμ region. In general, dilution corrections were not considered necessary for the titrations, since these were carried out by adding 10 M KOH to the sample solution. The reference solution was at neutral *p*H. Deionized water was used in making up all solutions, and the determination of the difference spectrum was completed within at most 5 minutes after the adjustment of the *p*H of the sample solution.

The concentration of aldolase was determined spectrophotometrically. The "optical factor" was determined by evaporating to dryness (24 hours in a 110° oven in air) a solution of 25 mg of aldolase which had been dialyzed in the cold against frequent changes of distilled water for one week. The spectrum of an aliquot was carefully determined. The "optical factor" for a *p*H 2 solution (no added salt) was found to be 0.832 ± 0.004 optical density units per mg (peak at 277 mμ) and for the neutral solution (*p*H 5–7) 0.938 ± 0.004 optical density units per mg (peak at 280 mμ). (Limits of error are estimated.) Previously

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published data (Baranowski and Niederland, 1949; Szabolcsi and Biszku, 1961) give the latter value as 0.91. The molecular weight was assumed to be 142,000 (Stellwagen and Schachman, 1962).

Reagent grade urea was twice recrystallized from ethanol by the method of Steinhardt (1938). The L-tryptophan was Mann assayed lot B2224. *N*-Acetyl-L-tyrosine was prepared from Mann assayed *N*-acetyl-L-tyrosine ethyl ester lot A5762 by exposure to alkali and subsequent neutralization. L-Tyrosine and L-cysteine were obtained from Nutritional Biochemicals Corp. Mercaptoethanol was Eastman White Label grade. These were used without further purification. All other chemicals were reagent grade.

Results and Discussion

Perturbation of the Sulfhydryl and Phenolic Chromophores.—The sulfhydryl chromophore is like the phenolic chromophore in that the absorption of the unperturbed un-ionized form is negligible in the wavelength region in which the ionized form has its absorption maximum (Noda *et al.*, 1953; Benesch and Benesch, 1955). This is important for the present study, since the "fabric" of a protein can complicate the interpretation of spectra considerably by perturbing the spectra of chromophores which are "buried" within it. Since sulfhydryl groups, like phenolic groups, are un-ionized at physiological *p*H, they are easily "buried" in the same way phenolic groups are. Hence the resulting perturbation of the un-ionized groups could make the interpretation of the absorption data much more complicated. This is commonly found to be true for the phenolic groups, since the perturbation of the un-ionized form causes its spectrum to shift into the 295-mμ region. In such cases, the phenolic difference peak appears at wavelengths longer than 295 mμ. However, the spectrum of the un-ionized sulfhydryl group is sufficiently far removed from that of the ionized form that any reasonable amount of perturbation of the spectra of the "buried" groups cannot significantly affect any results obtained by neglecting the absorption of the un-ionized chromophores.

The "Buried" Chromophores in Aldolase.—When a neutral *p*H solution of aldolase is used as reference and the phenolic ionization is followed spectrophotometrically, it is found that the peak in the difference spectrum does not appear at 295 mμ, but at 298 mμ. Furthermore, the maximum molar difference extinction coefficient observed at high *p*H, which is found to be independent of time, is $82.0 \pm 0.5 \times 10^3$. If the usual extinction coefficient for phenolic ionization, 2.33×10^3 per mole (Beaven and Holiday, 1952), is used to calculate the number of tyrosine residues per mole of aldolase from this extinction coefficient, 35 tyrosine residues per mole are obtained. If, instead, an acid *p*H solution of aldolase is used as the reference, then the difference peak of the phenolics appears at 295 mμ, with a molar difference extinction coefficient of $98.8 \pm 0.5 \times 10^3$. This value corresponds to 42.4 ± 0.3 tyrosine residues per mole of aldolase, and is in agreement with the amino acid analysis of Velick and Ronzoni (1948). Thus, the neutral *p*H aldolase solution is not the proper reference for measurements intended to determine the total number of phenolic groups. When the acid solution is measured against the neutral solution as reference, the "acid difference spectrum" of aldolase is obtained. This spectrum has been presented by Drechsler *et al.* (1959) and Stellwagen and Schachman (1962), and is also reproduced in Figure 1 over a more extended wavelength range. Since the acid solution contains disorganized aldolase (Stellwagen and Schachman, 1962), in which all the chromophores appear to be in contact with the solvent (see below), this difference spectrum represents the perturbation of some of the chromophores which are partly or completely "buried" in the fabric of the protein molecule in its native conformation in neutral solution. Although there may be a small contribution to the difference spectrum by electrostatic charge effects on the chromophores in the acid *p*H solution, the amount of

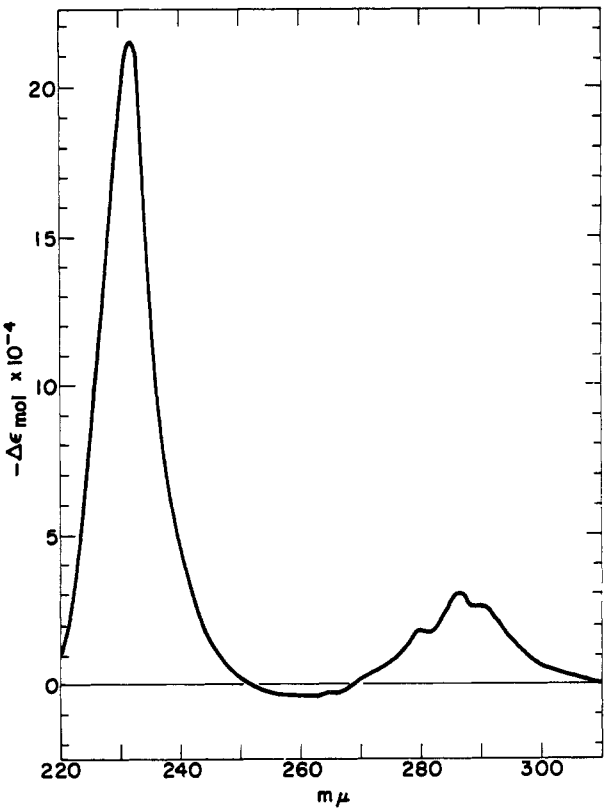

FIG. 1.—The acid difference spectrum of aldolase. The spectrum was determined at concentrations of $2.0–4.0 \times 10^{-6}$ M. The sample *p*H was 3.4, the reference *p*H was 6.5.

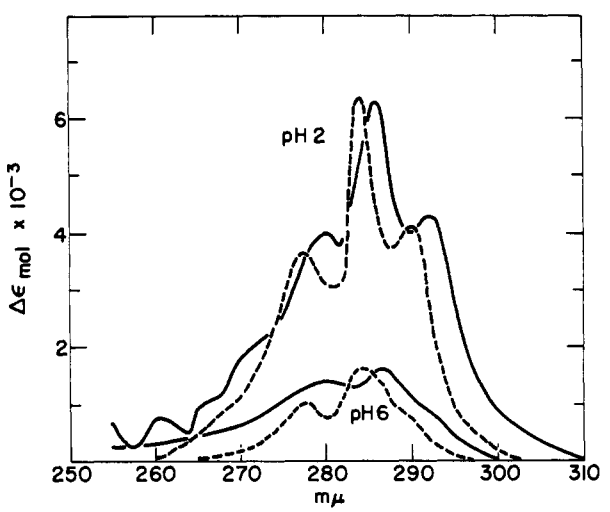

FIG. 2.—Perturbation difference spectra of aldolase produced by 20% ethylene glycol. These spectra (solid lines) are compared with perturbation difference spectra (dashed lines) calculated for 10 moles of tryptophan plus 42 moles of tyrosine (at *p*H 2) and for 1 mole of tryptophan plus 14 moles of tyrosine (at *p*H 6). The perturbation difference spectra for aldolase were determined at a concentration of 1.6×10^{-5} M.

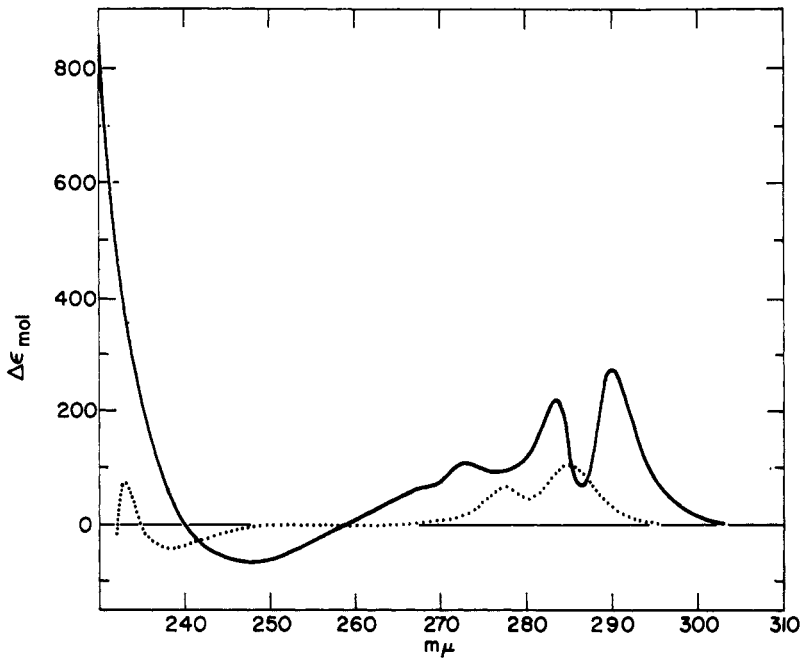

FIG. 3.—Perturbation difference spectra of tyrosine and tryptophan caused by 20% ethylene glycol. Solid curve, tryptophan; dotted curve, tyrosine. These spectra were determined at a concentration of 8.4×10^{-4} M for tyrosine, 2.6×10^{-4} M for tryptophan, in neutral solution.

such contribution is so small as to be nearly negligible (Donovan *et al.*, 1961). The numbers and types of these chromophores "buried" in the native molecule can be determined by using the solvent perturbation spectrum technique of Herskovits and Laskowski (1960, 1962). Figure 2 gives the perturbation spectrum of neutral and acid *p*H solutions of aldolase produced by 20% ethylene glycol. The corresponding spectra for tyrosine and tryptophan are given in Figure 3.

The *p*H 2 perturbation difference spectrum of aldolase appears to be fitted fairly well by the combination of 10 tryptophan chromophores and 42 tyrosine chromophores (phenylalanine chromophores are present in the spectrum, but no attempt has been made to include them in the calculations, since their absorption is relatively small). This fit of the acid perturbation difference spectrum indicates that all these chromophores are in contact with the solvent at *p*H 2. There is a red shift for the chromophores attached to the protein backbone, as is common with chromophores in proteins (Beaven and Holiday, 1952), but the use of the free amino acids rather than their acyl esters as model compounds probably accounts for most of the wavelength discrepancy observed in Figure 2.

The perturbation difference spectrum obtained at *p*H 6 appears to be fitted best by about 14 tyrosine chromophores and 1 tryptophan chromophore. (For both the *p*H 6 and the *p*H 2 perturbation difference spectra there are unexplained discrepancies in fitting near 280 mμ.) If this fitting of the *p*H 6 curve is correct, then in the native molecule only 1 of the 10 or 11 tryptophan chromophores is exposed to solvent. This result is in agreement with that of Velick and Ronzoni (1948), who found that the chemical reactivity of the tryptophan groups was unusually low in the native molecule. If 14 of the 42 tyrosine groups are exposed to solvent, then 28 phenolic groups must be "buried." It will be shown below that three-fourths of the sulfhydryl groups also appear to be "buried" in the native molecule.

The Perturbing Power of the Protein Fabric.—It is of interest to compare the perturbing power of the protein fabric with that of the solvent ethylene glycol.

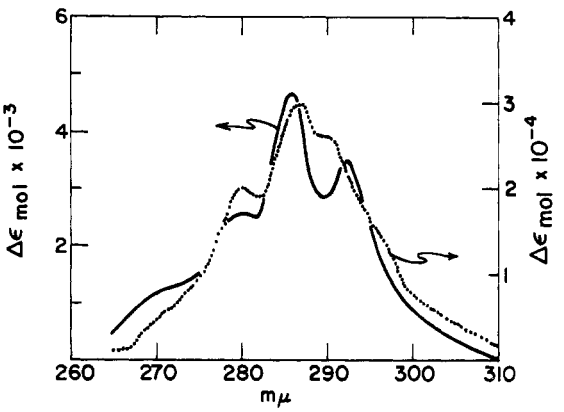

FIG. 4.—Comparison of the perturbation difference spectrum of the "buried" tyrosine and tryptophan chromophores caused by the protein fabric with the perturbation difference spectra of these chromophores caused by 20% ethylene glycol. The solid curve is the perturbation difference spectrum of the "buried" chromophores, when these chromophores are perturbed by 20% ethylene glycol, and is equal to the difference between the two solid curves of Figure 2. The dotted curve is the perturbation difference spectrum of these same chromophores when perturbed by the protein fabric (i.e., when they are "buried" in the native molecule). This dotted curve is actually the acid difference spectrum (Stellwagen and Schachman, 1962).

The perturbation spectrum of the "buried" chromophores caused by the protein fabric (the acid difference spectrum) is remarkably similar in shape to that of the *p*H 2 perturbation difference spectrum due to ethylene glycol. A comparison of the perturbation extinction coefficients can be made as follows: Since the perturbation difference spectrum at *p*H 2 is that of all the chromophores, and the perturbation difference spectrum at *p*H 6 is that of the "unburied" chromophores, the difference between these two perturbation difference spectra gives the perturbation difference spectrum of the "buried" chromophores caused by 20% ethylene glycol. This is compared with the acid difference spectrum in Figure 4. The protein fabric is a stronger

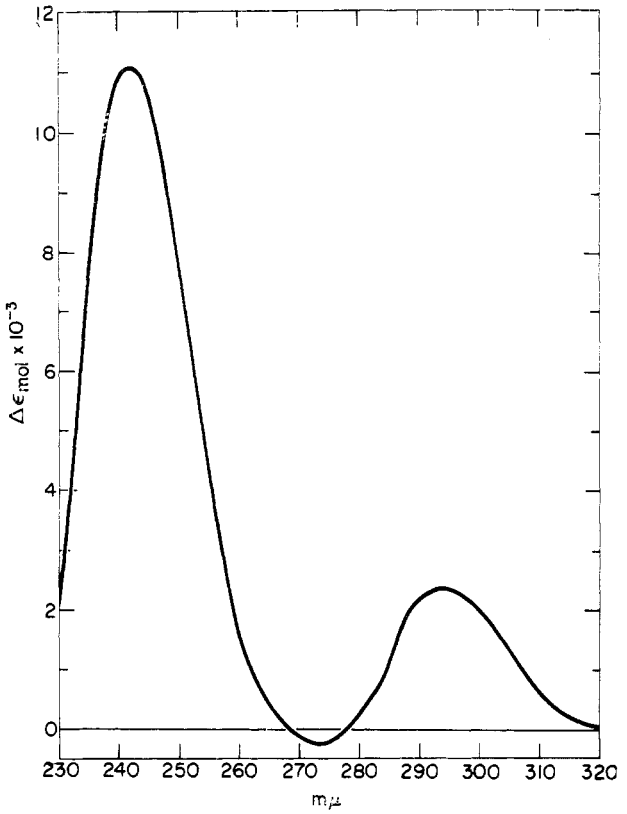

FIG. 5.—The difference spectrum of acetyltyrosine. The sample *p*H is 11.0; the reference *p*H is 7.5. Determined at a concentration of 9.4×10^{-5} M.

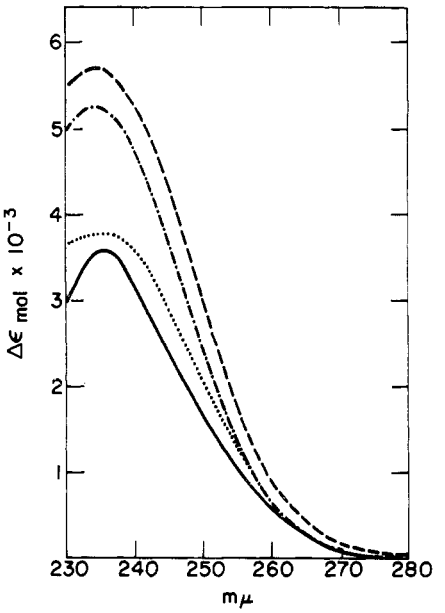

FIG. 6.—Difference spectra for the ionization of the sulfhydryl group. The *p*H of the reference solutions was between 6 and 7. – – – – Mercaptoethanol in water, *p*H 12.2; – · – · – in 4 M urea, *p*H 12.9, 1.5×10^{-4} M; · · · · cysteine in 4 M urea, *p*H 12.3, 1.0×10^{-4} M; ——— aldolase in 4 M urea, see text for *p*H, 1.6×10^{-6} M.

perturbant than 20% ethylene glycol by a factor of 6, approximately.

The short wavelength acid difference spectrum (Fig. 1) and perturbation difference spectrum of tyrosine and tryptophan (Fig. 3) merit a comparison. As shown above, the longer-wavelength region of the acid difference spectrum can be approximated by multiplying the 20% ethylene glycol perturbation difference spectrum of the proper combination of tyrosine and tryptophan chromophores by a factor of 6. The shorter-wavelength region cannot be so approximated, even when the approximately 2-mμ blue shift of the model compounds is taken into consideration. This may be because of the contribution of chromophores (phenylalanine, histidine, sulfhydryl, peptide) which do not contribute significantly at longer wavelengths, and also because the unfolding of the helical regions of the molecule may produce an additional effect near 230 mμ (Imahori and Tanaka, 1959; Glazer and Smith, 1960; Rosenheck and Doty, 1961).

Spectrophotometric Titration Curves.—Just as the aldolase molecule undergoes a conformational change in acid solution giving rise to the acid difference spectrum, it undergoes a similar conformational change in alkali, as indicated by optical rotatory data (see below). It will be presumed here that in alkaline solution the difference spectrum produced by the conformational change is identical to that observed in acid solution, and that the amount of conformational change at any *p*H can be represented by a suitable fraction, *F*, of the acid difference spectrum. With this assumption, the difference spectrum of aldolase at alkaline *p*H vs. a neutral *p*H aldolase solution as reference can be considered to be made up of suitable proportions of three components: (1) the difference spectrum for the ionization of the phenolic group (Fig. 5); (2) the difference spectrum for the ionization of the sulfhydryl group (Fig. 6); and (3) the acid difference spectrum (Fig. 1). At any *p*H a minimum of three equations must be solved to determine the number of ionized phenolic groups, ionized sulfhydryl groups, and fraction of conformational change from the observed alkaline difference spectrum. These equations require eight extinction coefficients at three wavelengths (sulfhydryl ion does not absorb at 295 mμ). Before titrating aldolase in aqueous solution and attempting to apply these equations, a simplification was made in order to check on some of the extinction coefficients. This simplification consisted in performing the spectrophotometric titration in 4 M urea, in which aldolase is completely disorganized (Stellwagen and Schachman, 1962). Thus, all the terms in *F* drop out of the equations, and only two equations are required.

Titration in 4 M Urea.—The number of ionized phenolic groups can be obtained directly from the absorption change at 295 mμ. At high *p*H the total molar extinction at 295 mμ is 98.8×10^3, which corresponds to 42.4 phenolic groups per mole of aldolase. To determine the number of sulfhydryl groups ionized it is necessary to correct for the phenolic absorption in the region of sulfhydryl ion absorption. The difference extinction coefficients for the phenolic group were obtained in two ways, which gave good agreement: (1) Above *p*H 10 all the sulfhydryl groups are ionized, while the phenolic groups are still ionizing. Thus, the differences between the difference spectra are due only to the ionization of phenolic groups. Several difference spectra at these higher *p*H values in 4 M urea gave the ratios $\Delta\epsilon_{243}/\Delta\epsilon_{295} = 4.73 \pm 0.02$ and $\Delta\epsilon_{235}/\Delta\epsilon_{295} = 2.85 \pm 0.04$ for the difference spectra of the phenolic groups *only*. (2) The difference spectrum of acetyltyrosine was determined in aqueous solution (Fig. 5). The same ratios determined from this difference spectrum were, respectively, $\Delta\epsilon_{242}/\Delta\epsilon_{294} = 4.77 \pm 0.02$ and $\Delta\epsilon_{234}/\Delta\epsilon_{294} = 2.9 \pm 0.1$ (there is a 1-mμ blue shift). The ratio $\Delta\epsilon_{242}/\Delta\epsilon_{294}$, obtained from the extinctions at the two peaks in the difference spectrum for acetyltyrosine, is considered reliable. However, the ratio $\Delta\epsilon_{234}/\Delta\epsilon_{294}$ is determined from an extinction on the steep

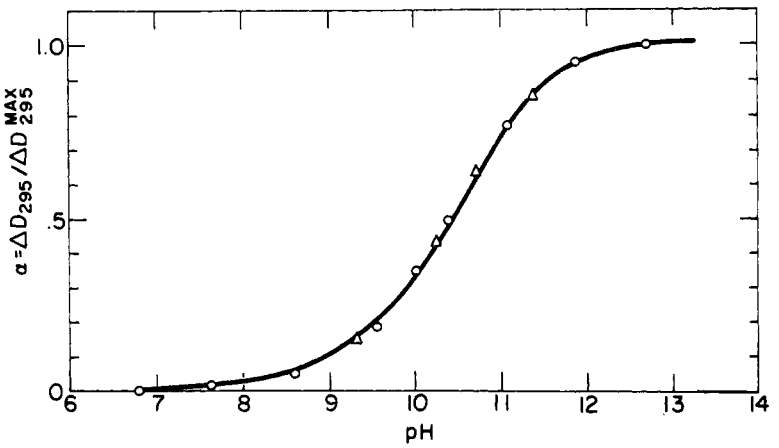

FIG. 7.—Spectrophotometric titration curve of the phenolic groups of aldolase in 4 M urea at 25°. The degree of ionization, α, as determined at 295 mμ, is plotted vs. pH. Forward titration, ⊙; reverse titration from pH 12.7, △. Determined at an aldolase concentration of 1.11×10^{-5} M.

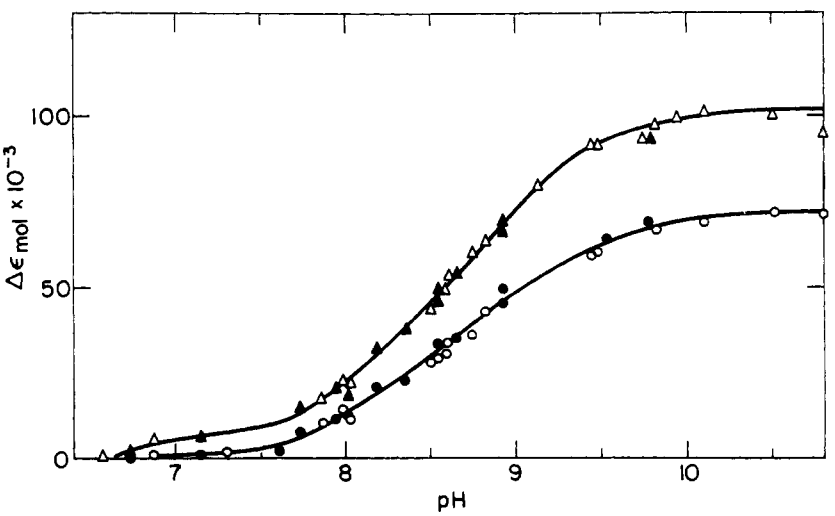

FIG. 8.—Spectrophotometric titration curves of the sulfhydryl groups of aldolase in 4 M urea at 25°. The molar difference extinction coefficient at two wavelengths is plotted vs. pH. 235 mμ: forward titration, △; reverse from pH 9.8, ▲. 243 mμ: forward titration, ○; reverse from pH 9.8, ●. Determined at aldolase concentrations of $1.5–4.0 \times 10^{-6}$ M.

portion of the absorption curve. Because of the large error in this ratio which would be caused by only a small error in the correction for the blue shift, this ratio for acetyltyrosine is not considered reliable, even though the agreement with the other ratio is reasonable. Even for the calculations for aqueous solutions the ratio determined in urea was used. The extinction coefficients derived from the ratios discussed above are listed in Table I. When the coefficients listed there for 4 M urea are used for the calculations in that solvent, the titration curves so obtained are given in Figures 7 and 8.

Figure 7 shows the titration curve for the phenolic groups in 4 M urea. The curve is reversible, and the apparent pK of these groups in urea is 10.42. The pK of phenol in 4 M urea has been determined to be 10.29 (Donovan *et al.*, 1959). In 4 M urea, then, the phenolic groups all appear to be exposed to the solvent, and ionize both normally and reversibly.

Figure 8 shows the titration curves calculated for the sulfhydryl ionization in 4 M urea, at the two wavelengths 235 and 243 mμ. At lower pH values the 235-mμ titration curve is not in agreement with the 243-mμ curve. This appears to be due to the difference spectrum for the ionization of histidine, which probably has a maximum near 230 mμ, and is negligible near 243 mμ (Donovan *et al.*, 1961). When a correction is made for the absorption change occurring up to pH about 7.5, the 235-mμ curve appears to be in agreement with the 243-mμ curve. Within experimental error, the pH of half-ionization is the same for both curves, 8.66 ± 0.10. This value of apparent pK seems low. Mercaptoethanol was determined to have a pK of 9.5 in 4 M urea. Its pK in water has been reported to be 9.5

TABLE I
MOLAR DIFFERENCE EXTINCTION COEFFICIENTS

Compound	Solvent	$\Delta\epsilon_{235}$	$10^{-3} \times \Delta\epsilon_{243}$	$\Delta\epsilon_{295}$
	Phenolic Group			
Acetyltyrosine	Water	6.8[a]	11.10	2.33[b]
Aldolase	4 M urea	6.65	11.02	2.33[b]
	Sulfhydryl Group			
Mercaptoethanol	Water	5.8	4.9	
Mercaptoethanol	4 M urea	5.3	4.2	
Cysteine	4 M urea	3.8	3.3	
Aldolase[c]	4 M urea	3.6	2.7	
Aldolase[c]	Water	4.8	3.8	

[a] See text. [b] Beaven and Holiday (1952). [c] Assuming 27 sulfhydryl groups per molecule.

(Edsall and Wyman, 1958). The sulfhydryl groups in thiolated gelatin in aqueous solution have a pK of 9.8 (Benesch and Benesch, 1959). The microscopic pk values for the cysteine sulfhydryl group are 8.53 (in the presence of the positively charged amino group) and 10.03 (with the uncharged amino group) (Benesch and Benesch, 1955).

The reversibility of the titration curve was checked by bringing samples to pH 9.8 for 1 minute and then quickly reducing the pH and measuring the difference spectrum. Above pH 9.5, oxidation of sulfhydryl groups appears to be rather rapid (a few per cent decrease in sulfhydryl extinction in about 10 minutes) so spectra were obtained immediately after the sample was brought to high pH. Often the measurement of the pH was postponed until after the spectrum was measured, on the assumption that oxidation of the sulfhydryl groups would not change the pH as much as it would the spectrum. Since the titration curves of both the sulfhydryl and phenolic groups are reversible, and the pK of the phenolic groups is normal, it is concluded that the pK of 8.7 for the sulfhydryl groups is a normal pK for these groups in 4 M urea.

The Difference Extinction Coefficient of the Ionized Sulfhydryl Group in Aldolase.—Amino acid analysis (Velick and Ronzoni, 1948) and p-mercuribenzoate titration (Swenson and Boyer, 1957; Stellwagen and Schachman, 1962) indicate that 27 sulfhydryl groups are present in aldolase. If this number is correct, the extinction coefficients of the ionized aldolase sulfhydryl groups in 4 M urea at 235 and 243 mμ are as shown in Table I. These values agree reasonably well with the extinction coefficients of cysteine in 4 M urea, as determined in this laboratory. The extinction coefficients for sulfhydryl compounds in urea appear to be smaller than in water (Table I and Fig. 6). The difference spectra of the ionized sulfhydryl group in cysteine and in mercaptoethanol are presented in Figure 6. The difference absorption curve for the aldolase sulfhydryl group in 4 M urea was obtained at pH 8.5, where the contribution of the phenolic ionization was negligible, since the pK values of these groups are separated by 1.7 pH units. The shape of this absorption curve was retained, but its height was adjusted to agree with the calculated extinction coefficients listed in Table I. It was not possible to determine the spectrum of the aldolase sulfhydryl group in aqueous solution in a similar way. Because of the "burying" of the sulfhydryl groups in the native molecule, their "pK" approaches that of the phenolic groups (see below). Consequently, at no pH in aqueous solution is the spectrum of the sulfhydryl groups free from superimposed phenolic spectrum.

Titration in Water.—Since salt tends to cause aggregation of the protein, with resultant turbidity (Stellwagen and Schachman, 1962), no salt was added to the solutions for the titration carried out in water. As discussed above, there are three equations in three different wavelengths necessary to completely specify the variables. The three wavelengths chosen were: 235 mμ, approximately the peak in the sulfhydryl difference spectrum, and 243 and 295 mμ, both peaks in the difference spectrum of the phenolic groups. A minimal set of equations is thus:

$$\Delta\epsilon_{295} = \Delta\epsilon^{F}_{295}\cdot F + \Delta\epsilon^{T}_{295}\cdot T$$

$$\Delta\epsilon_{243} = \Delta\epsilon^{F}_{243}\cdot F + \Delta\epsilon^{T}_{243}\cdot T + \Delta\epsilon^{S}_{243}\cdot S$$

$$\Delta\epsilon_{235} = \Delta\epsilon^{F}_{235}\cdot F + \Delta\epsilon^{T}_{235}\cdot T + \Delta\epsilon^{S}_{235}\cdot S$$

Here, F is the fraction of the conformational change, T is the number of phenolic groups ionized per molecule of aldolase, and S is the number of sulfhydryl groups ionized per molecule of aldolase. It is evident that the limits on these parameters are as follows: $0 \leq F \leq 1$, $0 \leq T \leq 42$, $0 \leq S \leq 27$. The difference extinction coefficients for the conformational change were taken directly from the acid difference spectrum, without adjustment. The values for the phenolic difference extinction coefficients were taken from Table I. The difference extinction coefficients for the ionized sulfhydryl group were determined in the following manner. Several difference spectra at high pH (where $F = 1$, $T = 42$, and $S = 27$) were determined, and the observed difference extinction coefficients at the three chosen wavelengths were entered into the equations above. The values of the difference extinction coefficients for the sulfhydryl group were calculated for the several difference spectra, and the results were averaged. These difference extinction coefficients are listed also in Table I. This completed the evaluation of the necessary extinction coefficients. The three equations used for the calculation of the titration curves in water were then:

$$10^{-3}\,\Delta\epsilon_{295} = -15.0\cdot F + 2.33\cdot T$$

$$10^{-3}\,\Delta\epsilon_{243} = -24.0\cdot F + 11.10\cdot T + 3.80\cdot S$$

$$10^{-3}\,\Delta\epsilon_{235} = -125\cdot F + 6.65\cdot T + 4.80\cdot S$$

The titration curves of the phenolic and sulfhydryl groups and the fraction of conformational change as a

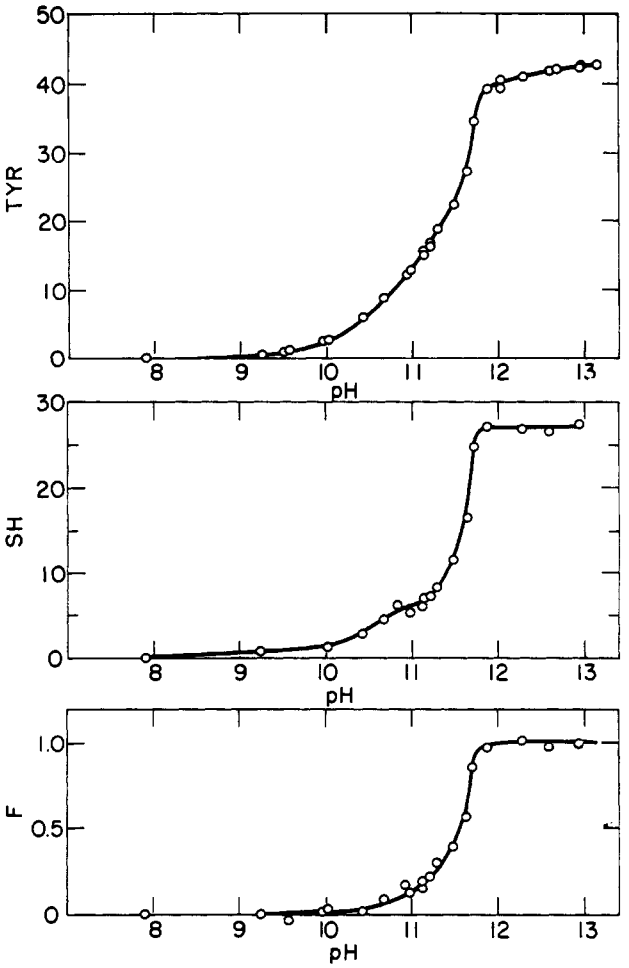

FIG. 9.—The spectrophotometric titration curves of the phenolic and sulfhydryl groups of aldolase in aqueous solution, and the conformational change as a function of pH. The ordinates give, top to bottom, the number of phenolic groups and the number of sulfhydryl groups ionized per molecule of aldolase as a function of pH, and the fraction of conformation change, F, as a function of pH. Determined at 25°, aldolase concentration 3.6×10^{-6} M.

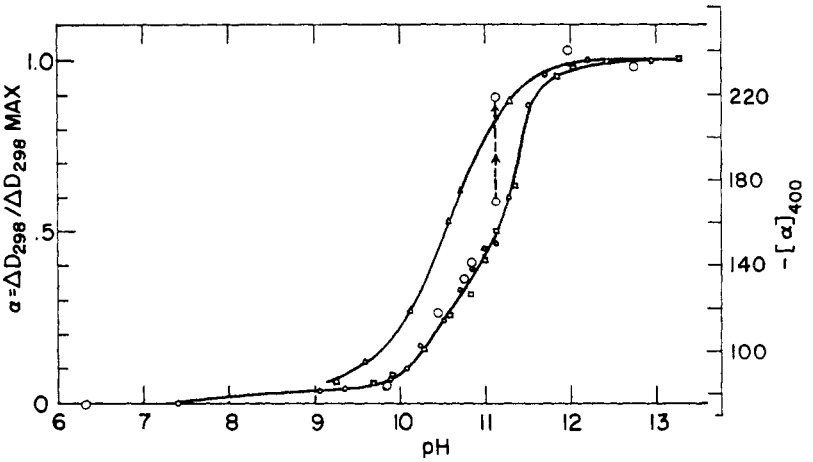

FIG. 10.—The reverse titration curve of the phenolic groups of aldolase in aqueous solution, at 25°. The apparent degree of ionization, α, as determined at 298 mμ, is plotted as a function of pH. The specific rotation, $[\alpha]$, determined at 400 mμ, is also presented as a function of pH. For details, see text. Large circles, $[\alpha]_{400}$ vs. pH, determined at a concentration of 3.5×10^{-5} M. The vertical dashed line connects the initial and final values of specific rotation from a kinetic experiment at pH 11.13. Small circles and squares, forward titration curves determined at 2.0×10^{-6} and 6.0×10^{-6} M, respectively. Small triangles, reverse titration determined at 2.0×10^{-6} M.

function of pH resulting from the application of these equations to the difference spectra determined in the alkaline pH range are given in Figure 9. These curves are not reversible.

The time dependence of the ionization of the phenolic groups in aldolase reported by Hass and Lewis (1963) was observed in the pH range 10.8–11.7. Since the spectral measurements were made quickly, the titration curves shown are not equilibrium curves in this pH range. Measurements at later times would probably not give so steep a titration curve, and would not give so good an indication of the abnormality of the ionizable groups in the native molecule.

The titration curves shown in Figure 9 are obviously not normal titration curves. Although no pK values can be assigned to the ionization of these groups, the mid-points of the ionization occur at relatively high pH values: 11.5 for the sulfhydryl groups and 11.4 for the phenolic groups. The essential identity of these two mid-points strongly suggests that the anomalous behavior is due to the groups' being "buried" in the molecule and ionizing as they are released, when the conformational change occurs (see below). However, there are indications that the pK values of the sulfhydryl groups would be lower than those of the phenolic groups if these groups could be titrated in this solvent in an "unburied" condition. At pH values above 11.8 (after the conformational change is completed), the number of phenolic groups titrated is still increasing with pH (compare the same pH region in Figure 7), while the number of sulfhydryl groups titrated remains constant with pH. In the "unburied" state, in this solvent, it seems that the intrinsic pK of the sulfhydryl groups would be of the order of one pK unit lower than that of the phenolic groups.

The shape of the titration curves at lower pH values is interesting. Although speculation about these regions is somewhat hazardous, in view of the limited data presented on conformation of the molecule, it appears that there is a small number of sulfhydryl groups with a different behavior from the larger portion of the sulfhydryl groups. If the conformational change occurring up to pH 11.0 can be neglected (total change in F is 0.1), the lower portion of the sulfhydryl titration curve can be construed as the titration of about 6 relatively "unburied" sulfhydryl groups with an apparent pK 10.5. This is approximately the number of sulfhydryl groups which Swenson and Boyer (1957) have found to be relatively rapidly reactive with p-mercuribenzoate in the native conformation of the molecule.

It is tempting to try to fit the lower portion of the phenolic titration curve with the 14 phenolic groups which perturbation difference spectra indicate are accessible to solvent in the native molecule. However, the degree of conformational change, if any, caused by 20% ethylene glycol has not been determined, so the number may be misleading. In addition, there is not any sharp break in the titration curve for the phenolic groups, as there is for the sulfhydryl groups. Actually, there may be a distribution of phenolic groups of various degrees of accessibility to solvent.

Observations on Reversibility.—The reversal of the titration of the sulfhydryl groups was not attempted in aqueous solution. Attempts to reverse the titration curve of the phenolic groups gave reversal curves of the type shown in Figure 10. The reversal curve is time dependent, approaching (but apparently not reaching) the forward curve with time. The reverse curve follows a more "normal" course than the forward curve, in that the mid-point is closer to the pK of phenol in aqueous solution. Because of the time dependence, the mid-point of the curve cannot be interpreted as an apparent pK. It is interesting that, at lower pH values, the reverse curve does not give negative values of α, the apparent degree of ionization. If the reverse curve were characteristic of a random-coiled molecule with chromophores accessible to the solvent, then the total change in extinction for the reverse curve would be *greater* than that for the forward curve, since the extinction coefficients for some of the un-ionized phenolic groups will not be increased by perturbation by the protein fabric, as they would be if they were reincorporated into the native conformation. Thus the failure to attain a negative α (α could theoretically attain the value of -0.2) must mean that upon reversal of the titration curve the molecule assumes a conformation remarkably like the native molecule, as far as the environment of the phenolic and indole chromophores is concerned. These chromophores must again be "buried" in a hydrophobic region of the molecule.

The reassociation of the three disorganized subunits (Stellwagen and Schachman, 1962) does not appear to

be the cause of the irreversibility of the titration curve. The time dependence of the renaturation has been observed to be first order (Deal and Van Holde, 1963; Stellwagen and Schachman, 1962) as well as the time dependence of the recovery of the acid difference spectrum on reconstitution (Donovan, unpublished results). This suggests a time-dependent conformational change in the molecule first formed from the reassembled subunits. In this conformational rearrangement, the phenolic and sulfhydryl groups which become "buried" presumably are "buried" in their nonionized form. It seems likely that at higher *p*H values, the phenolic groups may be ready to be reincorporated into hydrophobic regions, while the sulfhydryl groups still may largely be in the ionized form, since their *pK* is probably lower than that of the phenolic groups. As the *pH* is decreased, the number of molecules with sulfhydryl groups in the nonionized form will increase, and thus the rate of conformational change will increase. Figure 10 indicates that by *p*H 9, all the phenolic groups that were "buried" in the native molecule are now "reburied," and it may be presumed that at least most of the sulfhydryl groups have been returned to their former condition.

Although the titration curve of the phenolic groups cannot be reversed from *p*H 11.7, as indicated in Figure 10, it seemed likely that it might be reversible from lower *p*H values, particularly from *p*H values below 11.0. In fact, the titration curve, when reversed from *p*H 10.7, did not coincide with the forward curve. In this case, α actually became negative below *p*H 9.0, assuming a constant value of -0.03 between *p*H 9 and 8.

To support the conclusions from difference spectra that the aldolase molecule undergoes a conformational change in alkaline solution, the change in speicfic rotation as a function of *p*H, measured under the same conditions as for the titration curves, is also presented in Figure 10. The large increase in levorotation observed is characteristic of a denaturing transition. An exact correlation of the change in specific rotation with the parameter *F* should not be expected, however. The difference spectra give a measure of solvent penetration at *particular sites* within the molecule, while the specific rotation gives a measure of both helical content and exposure of the peptide chromophores to solvent *throughout the entire molecule.*

A Spectrophotometric Method for the Determination of the Number of Sulfhydryl Groups in a Protein.—If a suitable denaturing solvent for a protein is available, then the determination of the difference spectrum at *one* *p*H, suitably chosen, suffices to determine the number of ionizable sulfhydryl groups present in the protein. It is important that no conformational changes affecting the difference spectrum take place in this solvent over the *p*H range of the sulfhydryl ionization. Consider Figure 8. At *p*H values above 10 all the sulfhydryl groups are ionized. Any difference spectrum at *p*H values above 10 will contain the difference spectrum of all the sulfhydryl groups present in the protein, together with a difference spectrum of the number of phenolic groups which have ionized at that *p*H (e.g., in 4 M urea at *p*H 10.3, about one-half the phenolics will contribute to the difference spectrum). The solution of two simultaneous equations gives directly the number of sulfhydryl groups. For aldolase in 4 M urea, the following two equations (in which the difference extinction coefficients are taken from Table I) were used:

$$10^{-3}\,\Delta\epsilon_{295} = 2.33 \cdot T$$

$$10^{-3}\,\Delta\epsilon_{243} = 11.02 \cdot T + 2.70 \cdot S$$

The use of an equation at 235 mμ will require a correction for the absorption of ionized histidine, if the *p*H of the reference solution is below about 7.5. Slight modification in the difference extinction coefficients for the phenolic and sulfhydryl groups may become necessary, as data on different molecules (both model compounds and proteins) become available.

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References

Baranowski, T., and Niederland, T. R. (1949), *J. Biol. Chem. 180*, 543.

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Benesch, R., and Benesch, R. E. (1959), *in* Sulfur in Proteins, Chapt. I.2, Benesch, R., Benesch, R. E., Boyer, Paul D., Klotz, Irving M., Middlebrook, W. Robert, Szent-Györgyi, Andrew G., and Schwarz, David R., eds., New York, Academic, p. 15.

Benesch, R. E., and Benesch, R. (1955), *J. Am. Chem. Soc. 77*, 5877.

Crammer, J. L., and Neuberger, A. (1943), *Biochem. J. 37*, 302.

Deal, W. C., and Van Holde, K. E. (1963), *Biochemistry 2*, 246.

Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1959), *J. Mol. Biol. 1*, 293.

Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1961), *J. Am. Chem. Soc. 83*, 2686.

Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, Academic, Chaps. 8 and 9.

Glazer, A. N., and Smith, E. (1960), *J. Biol. Chem., 235*, PC 43.

Hass, L. F., and Lewis, M. S. (1963), Abstracts of the American Chemical Society Meeting, January, 1963, p. 34A.

Herskovits, T. T., and Laskowski, M., Jr. (1960), *J. Biol. Chem. 235*, PC 56.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.

Imahori, K., and Tanaka, J. (1959), *J. Mol. Biol. 1*, 359.

Noda, L. H., Kuby, S. A., and Lardy, H. A. (1953), *J. Am. Chem. Soc. 75*, 913.

Rosenheck, K., and Doty, P. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1775.

Steinhardt, J. (1938), *J. Biol. Chem. 123*, 543.

Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.

Stenström, W., and Reinhard, M. (1925), *J. Phys. Chem., 29*, 1477.

Swenson, A. D., and Boyer, P. D. (1957), *J. Am. Chem. Soc. 79*, 2174.

Szabolcsi, G., and Biszku, E. (1961), *Biochim. Biophys. Acta 48*, 335.

Taylor, J. F., Green, A. A., and Cori, G. T. (1948), *J. Biol. Chem. 173*, 591.

Velick, S. F., and Ronzoni, E. (1948), *J. Biol. Chem. 173*, 627.