mated spectrophotometrically ($\epsilon_{266}$ of methylaminopurine riboside = 15.9 × $10^3$),[48] 62.6 %. Estimated from phosphate analysis (Calcd. for $C_{11}H_{14}N_5O_{13}P_3Li_4\cdot4H_2O$, 12.97. Found:8.02), purity was 62.0 %. Paper chromatography: $R_f$ 0.57 (solvent A), 0.65 (solvent H). Since the main contaminant was LiCl which had no significant effect on the enzymic properties of actomyosin, no further purification was attempted.

6-Dimethylamino-9-$\beta$-D-ribofuranosylpurine 5'-Triphosphate (VIIIb).—143 mg. of ribosyldimethylaminopurine monophosphate,[17,49] 0.33 g. of orthophosphoric acid (85 %) and 2.9 g. of DCC was caused to react in 6 ml. of pyridine and 1.5 ml. of tri-$n$-butylamine. Recovery from charcoal was 82.3 %. Ion-exchange chromatography showed the following results. 0.003 $N$ HCl gave 11.5 % monophosphate ($TOD_{268}$ 100), 0.003 $N$ HCl + 0.05 $M$ LiCl gave 17.6 % diphosphate ($TOD_{268}$ 153) and 0.003 $N$ HCl + 0.15 $M$ LiCl gave 71.0 % triphosphate ($TOD_{268}$ 619). The triphosphate fraction was lyophilized and washed with anhydrous MeOH. The residual white powder weighed 22 mg. Purity calculated on a weight basis ($C_{12}H_{18}N_5O_{13}P_3Li_2$), as estimated spectrophotometrically ($\epsilon_{268}$ of ribosyldimethylamino-purine = 18.3 × $10^3$),[50] 44.8 %. Paper chromatography: $R_f$ 0.15 (solvent A). As the main contaminant was LiCl, no further purification was attempted.

Adenosine 5'-Sulfatopyrophosphate (IX).—200 mg. of ADPNa.$4H_2O$, which was purchased from Sigma Co., was dissolved in 12 ml. of aqueous solution containing 1.2 g. of sodium bicarbonate. It caused to react with 800 mg. of pyridine sulfurtrioxide[51] at 45° for 40 min.[18] Evolution of $CO_2$ along with liberation of pyridine was observed. The whole was poured into 500 ml. of ice water, adjusted to $p$H 6 with 1 $N$ formic acid and adsorbed on a column ( 5 × 7 cm.) of charcoal-celite (4:3), at a flow-rate of 4 ml. per min. The column was washed with water (500 ml.) thoroughly until

no turbidity was observed with $BaCl_2$. Elution was achieved by 500 ml. of 50 % EtOH–water solution containing 1 % ammonia. The effluent was adsorbed on a column of Dowex 1 × 2 resin (4 × 7 cm., $Cl^-$-form, 200–400 mesh), washed with 2 volumes of water and eluted with 3 l. of water along with 2 l. of 2 $N$ LiCl by a concave gradient elution technique. After the first peak of ADP ($TOD_{260}$ 2659), the peak of adenosine 5'-sulfatopyrophosphate (TOD 350) was observed. The second fraction was adsorbed on a charcoal-celite (4:3) column (2 × 5 cm.), washed until no $Cl^-$ was detected by aqueous $AgNO_3$ and finally eluted with 300 ml. of 50 % EtOH–water containing 1 % of ammonia. The effluents were concentrated to a small volume in a rotary evaporator at less than 25° and converted to the Li-salt by passing through a column (1 × 1 cm.) of Amberlite IR 120 resin. The effluent and the washings were combined, evaporated to a small volume and lyophilized. 52 mg. of the Li-salt was obtained. Purity estimated spectrophotometrically on a weight basis ($C_{10}H_{12}N_5O_{13}P_2S_1Li_3$), 15.6 %[52]; yield, 1.5 %. The structure of this material was tested by acidic hydrolysis. When it was hydrolyzed with 0.1 $N$ HCl at 37° for 18 min.,[53] ADP ($R_f$ 0.19, solvent S') and $SO_4$ ($R_f$ 0.28, $BaCl_2$-rhodizonate)[54] were detected on the paper chromatogram, accompanied with spots of base and of organic phosphate[55] ($R_f$ 0.57). Paper chromatography:

| Compound | Solvent S' | Solvent H' |
|---|---|---|
| AMP | 0.21 | 0.27 |
| ADP | .19 | .40 |
| IX | .34 | .37 |
| ATP | .15 | .45 |
| Inorganic sulfate | .28 | |
| Inorganic phosphate | .22 | |

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# The Effects of Charged Groups on the Chromophores of Lysozyme and of Amino Acids[1,2,3]

BY JOHN W. DONOVAN, MICHAEL LASKOWSKI, JR., AND HAROLD A. SCHERAGA

At acid $p$H, the difference spectrum of lysozyme, obtained when a solution at one $p$H is measured against another at a different $p$H, appears to be due to the effect of the charges of ionizable carboxyl groups on nearby indole chromophores of the molecule. The dependence of the difference spectrum on $p$H, temperature and ionic strength is presented. A comparison of the difference spectra of lysozyme with those of amino acids containing chromophores found in proteins indicates that, although the effect of the charged groups upon the chromophores appears to occur mainly as an inductive effect through covalent bonds in amino acids, a large part of the charge effect takes place through the solution in the case of lysozyme.

## Introduction

The general red shift of the spectra of chromophores present in protein molecules, as compared with the spectra of these same chromophores in smaller molecules, e.g., amino acids, has been known for some time.[4] Some part of the observed modifications of the spectra of a protein's chromophores

must be attributed to the influence of non-charged groups (including the amide bonds) in the protein, while another part must be caused by the presence of charged functional groups on the protein. The effects of charges on the spectra of a protein's chromophores can be observed when ionizable groups of the protein gain or lose protons as the $p$H of the solution containing the protein is changed.

Apparently almost any change in the environment of a chromophore will produce some change in its spectrum. A discussion of spectral perturbations with particular reference to proteins has been presented.[5] The effect of the solute on the chromo-

phores of proteins has been correlated with the refractive index of the solutions.[6-8] Solvent effects have been used to determine the number of chromophores which are in the interior of a protein molecule.[9] Changes in the configuration of protein molecules have been followed by means of the accompanying spectral changes.[6,9-13]

The spectral changes occurring in the experiments presented here for lysozyme have been interpreted as due to the effect of charges present in the molecule on the indole chromophores. These charge effects on the indole chromophore may be of use in deciding between proposed configurations of the lysozyme molecule, when its amino acid sequence and positions of disulfide bonds become available.

Changes in the spectrum of the indole chromophore have been observed when chymotrypsin undergoes autolysis.[14] Similar changes are observed when the $p$H of solutions of pepsin is changed.[15]

Because the effects of charges on the chromophores' spectra are small, the experiments have been carried out almost exclusively by the method of difference spectrophotometry.[16] The interpretation of the lysozyme experiments has been aided by results of similar experiments with amino acids containing these chromophores, and pertinent data for these molecules are also given here.

**Difference Spectra.**—For most of the compounds described here, a red shift is observed for the longer wave length ultraviolet absorption bands when the molecule becomes more negatively charged by dissociation of protons. If the molecule which the spectrum $\epsilon(\lambda)$, in Fig. 1, represents is modified so that the entire spectrum is shifted along the wave length axis by a constant amount, then the new spectrum will be $\epsilon(\lambda - \Delta\lambda)$, where $\Delta\lambda$ is the wave length shift ($\Delta\lambda > 0$). The difference in energy linearity between wave length and frequency will be ignored here. No intensity change is assumed, for simplicity. The "difference spectrum," $\Delta\epsilon(\lambda)$, is measured by reading the optical density of the

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(20) M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 571 (1960).

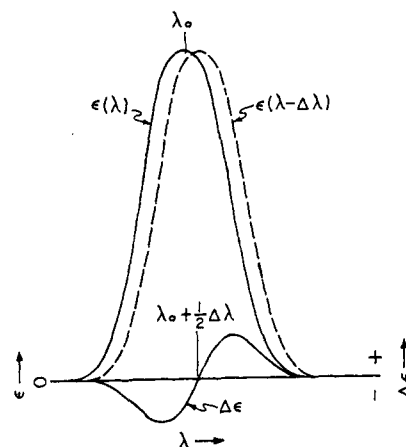(21) S. J. Leach and H. A. Scheraga, *ibid.*, **82**, 4790 (1960).

Fig. 1.—The origin of the difference spectrum, not to scale. For symbols, see text.

solution having the shifted spectrum against that having the normal spectrum. The shifted spectrum may be expanded in a Taylor series about some wave length, $\lambda_k$

$$\epsilon(\lambda_k - \Delta\lambda) = \epsilon(\lambda_k) + \sum_{n=1}^{\infty} \frac{(-\Delta\lambda)^n}{n!} \left(\frac{d^n\epsilon}{d\lambda^n}\right)_{\lambda_k} \quad (1)$$

If $\Delta\lambda$ is sufficiently small, and the spectrum sufficiently well behaved (*i.e.*, higher derivatives of $\epsilon$ with respect to $\lambda$ are small enough for the terms containing them to be neglected compared to the first order term), a good approximation to the difference spectrum is given by[22] (omitting the subscripts on $\lambda$)

$$\Delta\epsilon(\lambda) \equiv \epsilon(\lambda - \Delta\lambda) - \epsilon(\lambda) \simeq -\Delta\lambda[d\epsilon(\lambda)/d\lambda] \quad (2)$$

At this level of approximation, the difference spectrum is equal to the negative of the slope of the original spectrum, multiplied by the wave length shift. This approximation is particularly good at the maximum in the difference spectrum since $d^2\epsilon/d\lambda^2 = 0$ at this point.

The $p$H dependence of the difference spectrum can be treated as follows. The molecule which contains the chromophore has the absorption coefficient $\epsilon(\lambda)$ when it is in its most highly positively charged state (low $p$H). Upon dissociation of a proton, accompanied by a red shift in the spectrum, its absorption coefficient becomes $\epsilon(\lambda - \Delta\lambda)$. The total concentration, $C$, in moles per liter, of the chromophore molecules is the same for the reference solution as for the sample solution. The $p$H of the reference solution is chosen so that essentially all its chromophore molecules are non-dissociated molecules. The sample solution, at higher $p$H, however, will contain both dissociated and non-dissociated chromophore molecules, in a ratio determined by the degree of dissociation, $\alpha$. Assuming that both Beer's Law and the Law of additive optical densities hold, the optical density of the reference solution is given by

$$D(\lambda) = \epsilon(\lambda)C \quad (3)$$

and that of the sample solution by

$$D'(\lambda) = [\epsilon(\lambda)(1 - \alpha) + \epsilon(\lambda - \Delta\lambda)\alpha]C \quad (4)$$

a one cm. cell length being assumed. The dif-

(22) This has also been noted by Chervenka.[14]

ference spectrum, $\Delta D$, is then

$$\Delta D(\lambda) = D'(\lambda) - D(\lambda) = [\epsilon(\lambda - \Delta\lambda) - \epsilon(\lambda)]\alpha C \quad (5)$$

Combining equations 2 and 5

$$\Delta D(\lambda) = -\Delta\lambda(d\epsilon(\lambda)/d\lambda)\alpha C = \delta(\lambda,\Delta\lambda)\alpha C \quad (6)$$

Here, $\delta$ is the "molar difference absorption coefficient," and the minus sign appears again, because of the choice of reference solution. The values of $\delta$ for different dissociations in the same molecule will, in general, differ. The sign of $\delta$ for the longest wave length "band" in the difference spectrum will be positive for a red shift and negative for a blue shift.

If a small change in intensity of the spectrum occurs simultaneously with the wave length shift, the difference spectrum will still be recognizable by its approximation to the derivative of the original spectrum. If the change in intensity is large, it is not profitable to speak of the shift in terms of the derivative of the spectrum.

From equation 5, $\Delta D$ will show the same dependence on $pH$ as $\alpha$ does. Accordingly, the $pK$ of the dissociating group may be determined from a plot of $\Delta D$ vs. $pH$. The heat of dissociation of the group may be determined, using the van't Hoff relation, if $\Delta D$ vs. $pH$ plots are available at more than one temperature.[23]

**Spectrophotometric Errors.**—Since $\delta$ will ordinarily be small, high concentrations of absorber are necessary to obtain a difference spectrum with some accuracy. Errors may be introduced at high concentrations of absorber, since Beer's law may not hold. Beer's law (equation 3) can be written in an empirical, extended form

$$D(\lambda) = \epsilon(\lambda)C[1 - f(C,\lambda)] \quad (7)$$

where $1 \geqslant f(C,\lambda) \geqslant 0$, and represents the fractional apparent decrease in total concentration produced by stray light effects when the incident wave length is $\lambda$. If $f$ can be assumed to be the same for the dissociated and non-dissociated species (since their spectra are almost identical), then equation 6 becomes

$$\Delta D(\lambda) = \alpha\delta(\lambda,\Delta\lambda)C[1 - f(C,\lambda)] \quad (8)$$

Thus, if stray light effects only are considered, $\Delta D$, although no longer a linear function of the total concentration, remains a linear function of $\alpha$, and, accordingly, $pK$ values determined from the $pH$ dependence of $\Delta D$ will not be in error.

An observed shift in the peak of the difference spectrum with concentration can be ascribed to stray light. If $\delta$ in equation 8 is substituted from equation 6, and the partial derivative of equation 8 with respect to $\lambda$ is set equal to zero, the following condition for the maximum in $\Delta D$ is obtained

$$d^2\epsilon(\lambda)/d\lambda^2 - \frac{[\partial f(C,\lambda)/\partial\lambda][d\epsilon(\lambda)/d\lambda]}{1 - f(C,\lambda)} = 0 \quad (9)$$

If the stray light has a distribution such that $\partial f/\partial\lambda$ is zero (this will depend on the spectral sensitivity of the phototube as well as the actual wavelength distribution of intensity of stray light), then $d^2\epsilon/d\lambda^2$ will be zero for all $C$. Thus, the peak in $\Delta D$ will appear at the wave length of the point of inflection in $\epsilon(\lambda)$ at all concentra-

(23) J. Hermans, Jr., J. W. Donovan and H. A. Scheraga, *J. Biol. Chem.*, **235**, 91 (1960).

tions, regardless of how $f$ depends on $C$. If $\partial f/\partial\lambda$ is not zero, then the second term in equation 9 represents the departure of the peak from the point of inflection in $\epsilon(\lambda)$, which departure will increase with $f$.

## Experimental

**Materials.**—The egg white lysozyme was obtained from Armour and Co., and used as received. Lot 381–187 is characterized elsewhere.[24] The titration curve of Lot D638040 indicates the presence of five more titratable carboxyl groups than 381–187. The methylated lysozyme 381–187M5 is the same material as that for which a titration curve and amide and methoxyl analyses have been presented.[24] The following model compounds were used as received: L-phenylalanine, H. M. Chemical Co., C.P. Grade lot No. 10.87.7; Imidazole, Eastman Kodak 4733; L-Histidine, Nutritional Biochemicals Corp. No. 2204; α-Benzoyl-L-lysine, Mann Research Labs. No. 3128; L-Tryptophan, Nutritional Biochemicals Corp. No. 4442; Acetyl-L-Tryptophan, Acetyl-L-Tryptophan Ethyl Ester, Glycyl-L-Tryptophan Monohydrate, Mann Research Labs. Nos. 108258, 1612 and 2468, respectively. The glycyltryptophan was supplied with analysis: C 55.7%, H 6.0%, N 14.85%. Sp. Rot. +21.2° in 1 $N$ HCl. Carbonate-free KOH was prepared from reagent grade KOH by the method of Kolthoff.[25] All other chemicals used were reagent grade.

**Apparatus.**—The $pH$ meter, electrodes and calibration have been described previously.[24] A Beckman DU spectrophotometer with hydrogen lamp source and photomultiplier attachment was used. In general, the instrument was operated at maximum photomultiplier sensitivity and minimum slit width, except that when it was possible to reduce slit width below 0.02 mm., the sensitivity knob on the monochromator housing was turned clockwise instead, increasing electrical sensitivity as the optical sensitivity was decreased. Only when this sensitivity control was at its clockwise limit would the voltage on the photomultiplier be decreased. At wave lengths below about 290 m$\mu$ the sensitivity control was ordinarily at the counterclockwise limit. For controlled temperature experiments, water from a constant temperature bath was circulated through thermospacers; otherwise solutions were measured at room temperature (24 ± 2°, approx.). The readings at constant temperature were made at 1° ± 0.2, 25° ± 0.1 and 40° ± 0.3 (measured temperature of water in a sample cell). At 1°, a small cheesecloth bag of silica gel inserted in the cell compartment prevented condensation of moisture on the windows of the cells.

**Procedure.**—Aliquots of "$pH$ control" solutions were added to aliquots of a stock solution of the compound to be studied. Volume additivity was assumed. The "$pH$ control" solutions contained only KOH or HCl in KCl and were made up with the same ionic strength as the stock solution. No buffers, as such, were added, for fear of complicating the spectral changes observed, particularly with the protein solution. The most acid of these resulting solutions was used as reference solution and placed in the reference cell. The higher $pH$ solutions were successively placed in the sample cell for measurements. The same two 1 cm. silica cells were used for all the experiments. They were the two most nearly alike of a matched set of four. One was reserved for the reference solution, the other always held the sample. The positions and orientations of these cells in the cell holder were always the same.

## Results

**A. Model Compounds. 1. Histidine.**—The difference spectrum of histidine, Fig. 2, is very similar to that of imidazole and shows only a single peak, corresponding to the longer wave length portion of the first derivative of the spectrum. Since the observed spectrum apparently has a flat-topped peak,[26,27] this separation in wave

(24) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 2154 (1960).

(25) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1922).

(26) L. J. Saidel, *Arch. Biochem. Biophys.*, **54**, 184 (1955).

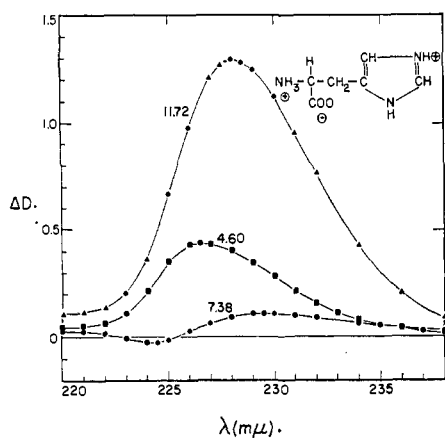(27) L. J. Saidel, A. R. Goldfarb and S. Waldman, *J. Biol. Chem.* **197**, 285 (1952).

Fig. 2.—The difference spectra of 1.6 × 10⁻³M solutions of histidine at 25° and 1.0 ionic strength. The common reference solution for all three curves has a pH of 0.24.
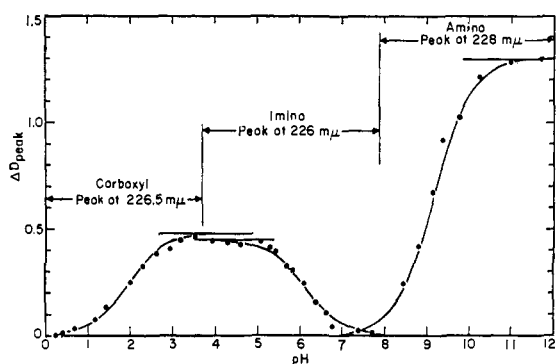


Fig. 3.—The pH dependence of the difference spectrum of histidine at 25°. The solutions are 1.6 × 10⁻³M, the ionic strength, 1.0, the reference pH, 0.24. The curves drawn on the figure are theoretical with pK's of 1.9, 6.2 and 9.2. The peak wave length given for each ionizable group applies only in the pH region indicated, i.e., when the reference solution has a pH at the lower boundary of, or within, this region.

length of the positive and negative portions of the derivative of the spectrum is not unexpected. The positive peak in the difference spectrum observed in the pH region of the carboxyl group ionization becomes negative as the imino group ionizes and then positive again as the amino group ionizes. This behavior is in agreement with the red and blue shifts of the edge of the absorption band observed by Saidel, et al.[26,27] The pH dependence of the difference spectrum is shown in Fig. 3, and a summary of the data is presented in Table I. The observed pK's of Fig. 3 are about 1.9, 6.2 and 9.2, which agree fairly well with those cited by Cohn and Edsall.[28]

2. **Phenylalanine.**—A portion of the difference spectrum of phenylalanine due to the ionization of the carboxyl group is shown in Fig. 4.

3. **Tryptophan and Derivatives.**—Difference spectra of tryptophan and glycyltryptophan at various pH's are shown in Figs. 5 and 6. The change in the spectrum of the indole chromophore

(28) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Co., New York, N. Y., 1943, p. 85.
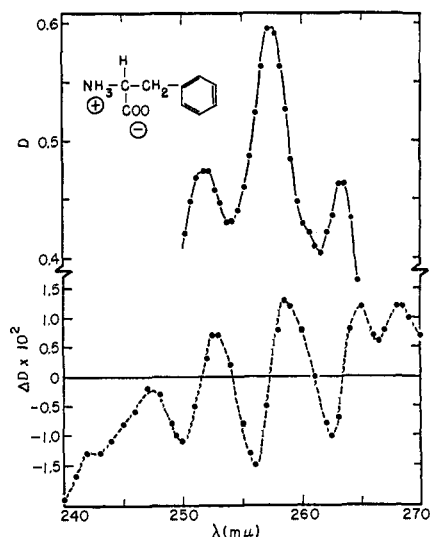


Fig. 4.—The spectrum (solid line) and the difference spectrum (dashed line) of 3.1 × 10⁻³M solutions of phenylalanine, at 0.15 ionic strength and 25°. Sample pH, 3.30; reference pH; 1.05; slit width, 0.03 mm.
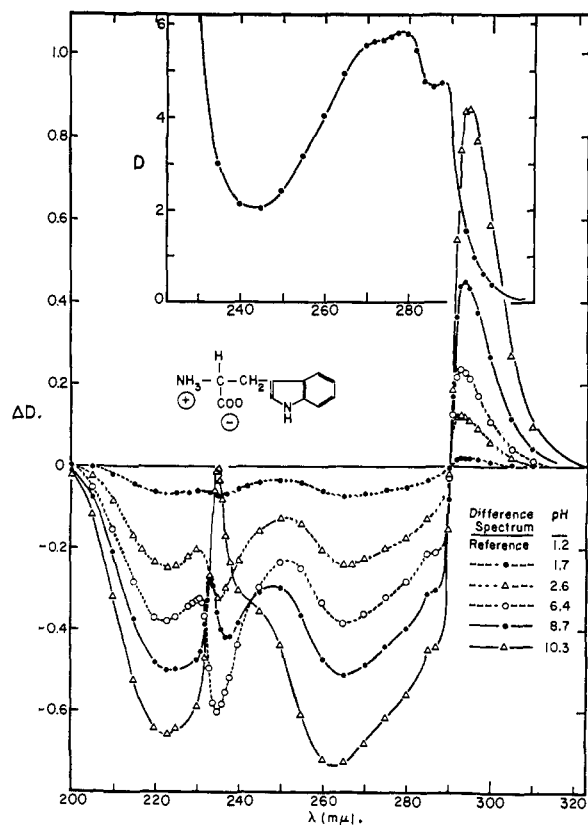


Fig. 5.—Difference spectra of tryptophan at 25°. The solutions are 1.0 × 10⁻³M, the ionic strength, 0.15. The spectrum of tryptophan at pH 7 is shown in the upper part of the figure, on the same wave length scale, and also at a concentration of 1.0 × 10⁻³M, for comparison.

near 295 mµ when a proton is dissociated from the molecule appears to be the same whether the proton is dissociated from the carboxyl or amino group, although δ is larger for the latter (Table I).

Table I

Summary of Some Data Pertaining to Difference Spectra of Model Compounds

| Compound | Chromophore | Ionizable group | $\lambda_{\text{peak}}$[a] (m$\mu$) | $\delta_{\text{peak}}$ | $\left(\dfrac{\delta}{\epsilon}\right)_{\text{peak}}$[b] | Peak half width (m$\mu$) | $\dfrac{\delta\alpha\text{-amino}}{\delta\alpha\text{-carboxyl}}$ |
|---|---|---|---|---|---|---|---|
| Tryptophan | Indole | Carboxyl | 293 | 240 | 0.053 | 9 ⎫ | |
| Tryptophan | Indole | Amino | 295 | 680 | .150 | 11 ⎭ | 2.83 |
| Acetyltryptophan | Indole | Carboxyl | 296 | 270 | .059 | 9 | |
| Glycyltryptophan | Indole | Carboxyl | 294 | 270 | .059 | 11 | |
| Glycyltryptophan | Indole | Amino | 294 | 68 | .015 | 11 | |
| Imidazole | Imidazole | Imino | 225 | 190 | ... | 6 | |
| Histidine | Imidazole | Carboxyl | 226.5 | 300 | .053 | 7 ⎫ | |
| Histidine | Imidazole | Amino | 228 | 825 | .145 | 8 ⎭ | 2.74 |
| Histidine | Imidazole | Imino | 226 | −285 | .050 | 7 | |
| Phenylalanine | Benzene | Carboxyl | 259[c] | 4.2 | .022 | 2.5 ⎫ | |
| Phenylalanine | Benzene | Amino | ~260[d] | ~14[d] | ~.07[d] | ... ⎭ | ~3 |
| O-methyltyrosine[e] | Phenol | Carboxyl | 284 | 55 | .039 | 7 ⎫ | |
| O-methyltyrosine[e] | Phenol | Amino | 285 | 178 | .127 | 7 ⎭ | 3.24 |
| $\alpha$-Benzoyllysine | Benzoyl | Carboxyl | 250 | 600 | ... | 20 | |
| $\alpha$-Benzoyllysine | Benzoyl | Amino | ~250[f] | <30 | ... | ... | |

[a] Concn. usually about 0.025%, in 0.15 $M$ KCl except histidine, 1 $M$ KCl. [b] This ratio is given for the peak values in each case. The positive peak in the difference spectrum will be at a longer wave length than the peak in the absorption spectrum for ionizations which have positive values of $\delta$. [c] This is the wave length of the largest positive peak in the difference spectrum. [d] Calcd. from the wave length shift given by Beaven and Holiday,[4] using eq. 2. [e] Data of Wetlaufer, et al.[30] [f] Expected position, if present.

Fromageot and Schnek[29] have stated that it would be possible to determine the $pK$ of the carboxyl
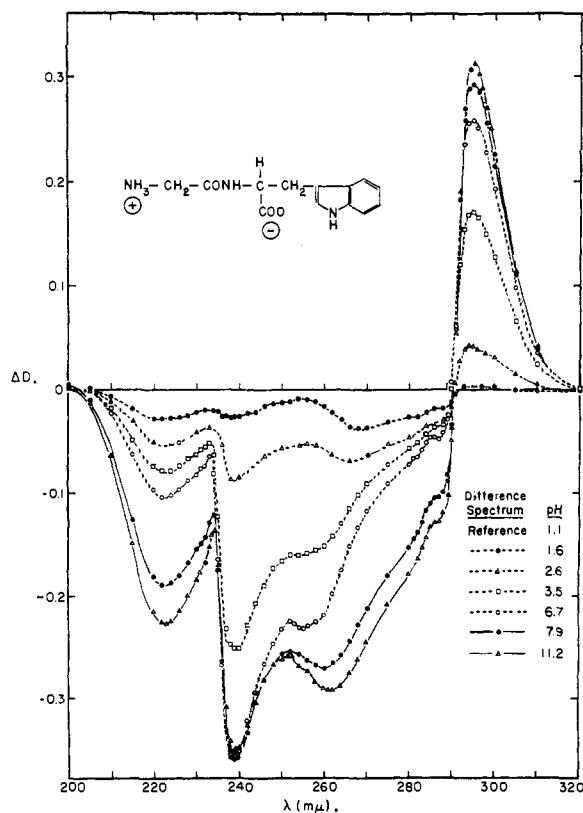


Fig. 6.—Difference spectra of glycyltryptophan at 25°. The solutions are 1.0 × 10⁻³$M$, the ionic strength, 0.15.

group in glycyltryptophan from the $p$H dependence of the spectrum near 250 m$\mu$.

(29) C. Fromageot and G. Schnek, Biochim. et Biophys. Acta, 6, 113 (1950).

(30) D. B. Wetlaufer, J. T. Edsall and B. R. Hollingworth, J. Biol. Chem., 233, 1421 (1958).

The slit widths used in obtaining these difference spectra were 0.03–0.05 mm. at wave lengths above 290 m$\mu$, so this portion of the spectrum is well resolved. Between 290 and 250 m$\mu$, the slits used were fairly large (0.05–1.0 mm.), and some details of the spectra are not revealed. When small slit widths are used in this region, positive peaks at about 284 and 274 m$\mu$ can be resolved in the difference spectrum.[6,23]

The wave length of the maximum $\Delta D$ increases with concentration for all compounds examined. This is presumed to be due to stray light effects. With glycyltryptophan, for 0.44 × 10⁻³ $M$, the maximum was observed at 291.5 m$\mu$; for 2.84 × 10⁻³ $M$, at 301 m$\mu$ (amino ionization). This increase became a non-linear function of concentration above 1.5 × 10⁻³ $M$.

The difference spectra of acetyltryptophan and acetyltryptophan ethyl ester are not shown, but pertinent data are given in Table I. As would be expected, the acetyl derivative shows no spectral change at high $p$H's. The acetyl ethyl ester, in which both the carboxyl and amino groups have been masked, does not show a difference spectrum, although after standing for some time in basic solution a difference spectrum is observed as saponification liberates an ionized carboxyl group.

For tryptophan, acetyltryptophan and glycyltryptophan, experiments carried out in several ways showed no dependence of the height of the 295 m$\mu$ peak on ionic strength. For example, an experiment in which two $p$H 6.8 solutions, 6.2 × 10⁻⁴ $M$ in tryptophan, having ionic strengths of 0.11 and 0.0054, were measured against one another showed no optical density difference within experimental error (about 0.002 in $\Delta D$) between 320 and 285 m$\mu$. Results of an experiment using glycyltryptophan are shown in Fig. 7.

4. $\alpha$-Benzoyllysine.—The spectrum and difference spectrum of this compound are shown in Fig. 8. The difference spectrum is attributable to the ionization of the $\alpha$-carboxyl group ($pK' = 3.8$).
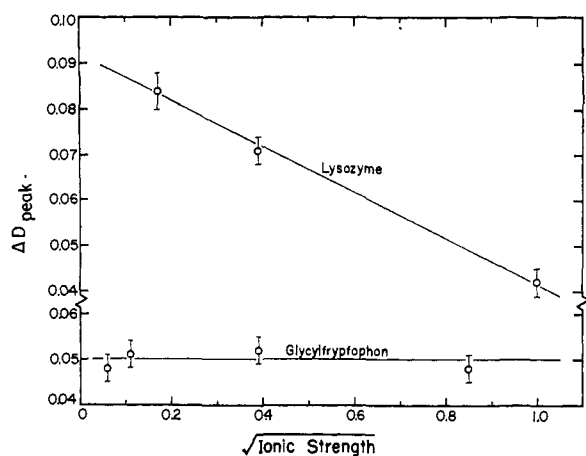
Fig. 7.—The ionic strength dependence of the difference spectra of glycyltryptophan and lysozyme at 25°. The $\Delta D$ values are given for the wave length at which the peak in $\Delta D$ occurs. For glycyltryptophan, the difference spectrum was obtained with solutions of $p$H 11 to 12 as samples, solutions of $p$H 6 as references, at $10^{-3}M$. The lysozyme solutions are 0.24% (1.6 × $10^{-4}M$) Armour Lot 381–187, $p$H 5.0 vs. $p$H 1.2 (see text).



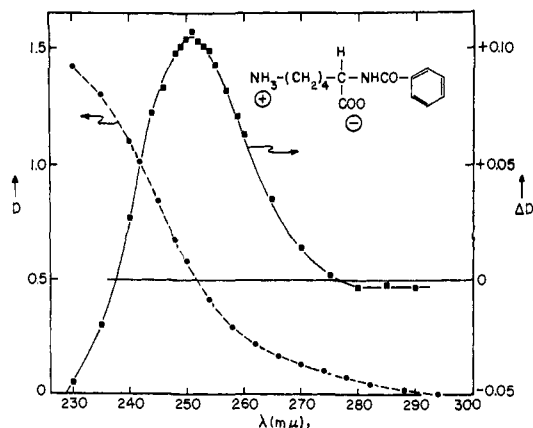Fig. 8.—The Spectrum and difference spectrum of α-benzoyllysine at 25°, obtained with 0.03 mm. slit. Spectrum, 2.4 × $10^{-4}M$, $p$H 7.4; difference spectrum, 1.8 × $10^{-4}M$; reference, $p$H 1.53, ionic strength 0.037; sample, $p$H 7.40, ionic strength about 0.002.

The ionization of the ε-amino group has no effect (within experimental error) on the benzoyl chromophore, since no change in $\Delta D$ is observed between $p$H 7 and $p$H 13. The data are summarized in Table I.

B.   **Lysozyme.**—The difference spectrum of lysozyme, Armour Lot D638040, is shown in Fig. 9. The difference spectrum of Lot 381–187 appears to be identical with this. The $p$H dependence of the 295 m$\mu$ peak at 1 and 25° is shown in Fig. 10. At this concentration of lysozyme, the optical density at 295 m$\mu$ was 3.0. An error of one part per thousand in the concentration of either the reference or sample solution will thus produce an error of 0.003 in $\Delta D$. For all the points shown in this figure, the entire difference spectrum between 320 and 270 m$\mu$ was obtained and inspected for irregularities before accepting the value of $\Delta D_{295}$.
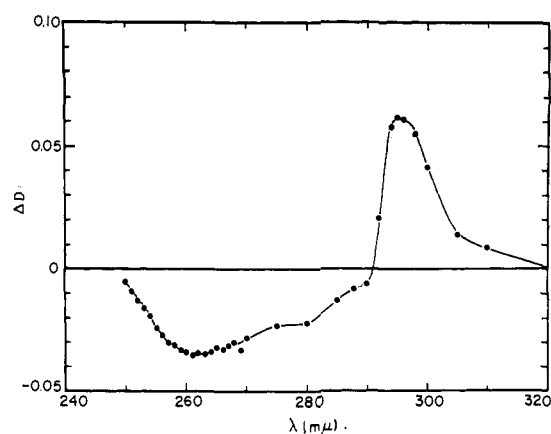
Fig. 9.—The difference spectrum of 0.23% solutions of Armour Lot D638040 lysozyme at 25° and ionic strength 0.15. Sample, $p$H 5.04; reference, $p$H 1.15.
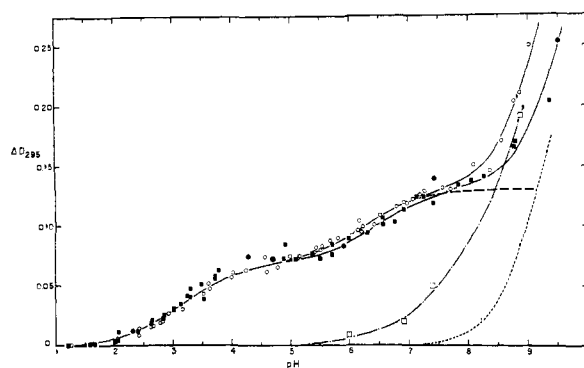


Fig. 10.—The $p$H dependence of the difference spectrum of 0.24% (1.6 × $10^{-4}M$) solutions of Armour Lot 381-187 lysozyme at 0.15 ionic strength. Reference $p$H's are approximately 1.2. Open circles, 25°. Filled squares, 1°. Open squares, the $p$H dependence of the tyrosine ionization in the methylated lysozyme, 381-187M5, determined as a difference spectrum under the same conditions, except that the $p$H 1.2 reference contained 381-187M5. Dashed curve without points is a calculated difference spectrum for tyrosine, with $pK_{int}$ 10.80 for the three groups, and 0.080 for $w$. The solid curves are calculated difference spectra for the following groups on the lysozyme 381-187 molecule: 1 group with $pK_{int}$ 4.20, $\Delta H$ = 0, $\Delta D$ = 0.070; 1 group with $pK_{int}$ 6.85, $\Delta H$ = 3 kcal., $\Delta D$ = 0.060; 3 groups with $pK_{int}$ 10.80, $\Delta H$ = 6 kcal., $\Delta D$ = 1.10. For these curves, the charge on the molecule at any $p$H has been calculated from the titration curve of 381-187, using the assumptions listed in the text.

A plot of $\Delta D$ against concentration, Fig. 11, indicates that $f$, in equation 8, is effectively not a function of C for these concentrations of lysozyme.

Figure 7 shows the effect of ionic strength on the height of the 295 m$\mu$ peak. The values presented on the figure were obtained at $p$H 5.0 from three $\Delta D$–$p$H curves, one of which is presented in Fig. 10. At the lowest ionic strength, the values obtained for $\Delta D_{295}$ had to be corrected for turbidity of solutions (shown by the failure of $\Delta D$ to become zero at 320 m$\mu$ and above—compare Fig. 9 and the insert in Fig. 5) and are probably not as accurate as the values at higher ionic strength. The ionic strength dependence above $p$H 6.5 was not
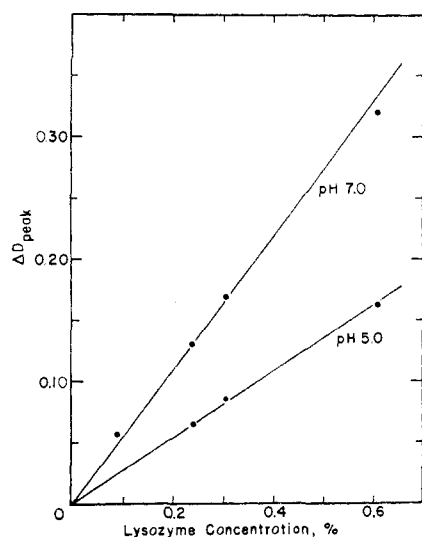
Fig. 11.—A plot of the relation of equation 8, for Armour Lot 381–187 lysozyme, at 25° and 0.15 ionic strength. The points shown for $p$H's 5.0 and 7.0 are obtained from $\Delta D$-$p$H curves of the type given in Fig. 10. At $p$H 5, the maximum $\Delta D$ was observed at 294.5, 295.0, 296.5 and 298.0 m$\mu$ for the (increasing) concentrations shown. The reference $p$H is approximately 1.

determined, because of the large turbidity at the lowest ionic strength above this $p$H.

## Discussion

**A. Model Compounds. General Comments.**— For the amino acids, $\Delta D$ is essentially independent of ionic strength. This indicates that the effect of the charge is transmitted to the chromophore predominantly along a path within the molecule (inclusive of "bound" solvent, which ions cannot enter). The path "within the molecule" must be nearly the same for the $\alpha$-carboxyl and $\alpha$-amino groups, since they are attached to the same ($\alpha$) carbon atom and the chromophore is always one $CH_2$ group removed from the $\alpha$ carbon atom. Thus it is not unexpected that the ratio of the effects of the ionization of these groups on the spectrum of a chromophore is constant, within experimental error, from one amino acid to another, irrespective of the chromophore. These ratios of the values of $\delta$ for the $\alpha$-amino and $\alpha$-carboxyl groups for most of the amino acids which contain chromophores are presented in Table I.

**1. Histidine.**—The data for histidine indicate that changes in the histidine spectrum caused by changes of charge on proteins appear very likely. However, because of the very high optical density of protein solutions at the wave lengths at which histidine difference spectra may be observed, it seems doubtful that they will be detected with present equipment unless the protein contains unusually small amounts of tyrosine, tryptophan and phenylalanine. It is interesting that for histidine, the ionization of the imidazoyl chromophore itself causes a smaller change in the spectrum than the ionization of one of the attached groups.

**2. Phenylalanine.**—The difference spectrum closely resembles a first derivative of the spectrum, with negative maxima at wave lengths where

the original spectrum has maximum positive slope and nodes at places of zero slope. The amount of shift of the central vibrational band, calculated from the observed difference spectrum using equation 2, is approximately 1.5 Å. A shift of 5 Å. has been observed for this band by Beaven and Holiday,[4] when protons are dissociated from both groups on the molecule. The shorter wave length band also shows a red shift with increasing $p$-H[26,27,29]

**3. Tryptophan and Derivatives.**—The data summarized in Table I call for little comment. The values of $\delta$ for the $\alpha$-carboxyl group are larger when an $\alpha$-amino group is not present to decrease the effect of the carboxyl group, presumably either by "withdrawing some negative charge" from the $\alpha$-carbon atom or by modifying the structure of the "bound" solvent.

**4. $\alpha$-Benzoyllysine.**—The failure of the $\epsilon$-amino group to affect the spectrum of the benzoyl chromophore must be due to its distance from the chromophore. There are insufficient data available in this case to determine whether the charge effect could transfer could take place through the solvent.

**Estimation of $\delta$ for Proteins.**—A comparison of the data for tryptophan and glycyltryptophan shows that, upon insertion of a saturated carbon atom ($-CH_2-$) and an amide group ($-CONH-$) between the charged amino group and the chromophore, the effect of the charge on the chromophore is reduced roughly ten-fold. If the assumption (consistent with $\Delta D$ being independent of ionic strength for these small molecules) is made that the effect of the charge is transferred through the covalent bonded structure only, then the amide group and the $CH_2$ group probably contribute about equally to the ten-fold decrease in the effect of the amino group on the chromophore of glycyltryptophan. Derick[31] has observed a three-fold reduction in the effect of the polar substituent upon the ionization of the carboxyl group in chloroaliphatic acids by the interposition of a single $CH_2$ group in the carbon chain separating the carboxyl group from the chlorine atom. Taft,[32] using data on the rates of hydrolysis of esters of carboxylic acids, has calculated that the $CH_2$ group reduces inductive effects by a factor of 2.8. Saidel[26] has observed that the effect of the ionization of a carboxyl group on the spectrum of the amide group at 205 m$\mu$ is reduced by a factor of 2.7 by an intervening $CH_2$ group. (Saidel has been able to determine the $pK'$ of the amino and carboxyl groups of glycylglycine from the effects of these groups on the spectrum of the peptide amide linkage.)

If this approximate three-fold reduction of an inductive effect holds true for glycyltryptophan, then the amide group also must reduce the inductive effect by a factor of approximately three. With these assumptions, predictions may be made about the positions in a protein molecule which charge-carrying groups can occupy and still noticeably affect the spectrum of its chromophores,

(31) C. G. Derick, J. Am. Chem. Soc., 33, 1181 (1911).
(32) R. W. Taft, Jr., Chapter 13 in "Steric Effects in Organic Chemistry," M. S. Newman, ed., John Wiley and Sons, Inc., New York, N. Y., 1956, p. 592.

if the charge effect transfer takes place only through covalent bonds by an inductive mechanism. Predictions for the indole chromophore are summarized in Table II.

TABLE II

ESTIMATED VALUES OF $\delta$ AT 295 m$\mu$ FOR THE INDOLE CHROMOPHORE IN A PROTEIN

| Position of residue with respect to tryptophan residue | Residue with ionizable group | | |
|---|---|---|---|
| | Aspartic acid | Glutamic acid | Lysine |
| Adjacent | 10 | 3 | 0.8 |
| Once removed | 1 | 0.3 | 0.08 |

**B. Lysozyme. Origin of the Difference Spectrum.**—A comparison of the difference spectrum of lysozyme (Fig. 9) with that of tryptophan or glycyltryptophan (Fig. 5 and 6) shows that, below $p$H 7, the difference spectrum appears to be due entirely to the indole chromophore. Neither the peaks at 280 and 287 m$\mu$, characteristic of the phenolic chromophore,[20,30,33] nor the peaks near 260 m$\mu$, characteristic of the benzene chromophore, are observed.

**The Phenolic Ionization in Lysozyme.**—In Fig. 10, the sharp rise in $\Delta D_{295}$ above $p$H 8 is produced by the phenolic ionization. The difference spectrum of a methylated derivative (381–187M5), which shows only the phenolic ionization and not the indole difference spectrum, is also shown in this figure (open squares). Since essentially all the carboxyl groups in this derivative have been esterified,[24] this molecule, at $p$H 8, should have nine more positive charges on it than the native molecule. This should lead to an acid shift of the $p$H of the phenolic ionization, if the same electrostatic factor can be assumed, of 0.63 $p$H units. Near $p$H 9, this is roughly correct, but at more acid $p$H's, a larger shift is observed.

A theoretical curve (dashed line without points) for the ionization of the three phenolic groups (assumed identical) has been calculated for lot 381–187, using the assumptions: (1) The total $\Delta D_{295}$ due to phenolic ionization is 6900 per mole of lysozyme, this being the normal value for the ionization of three tyrosyl residues. (2) The isoelectric point of lot 381–187 is 11.1, the same as for lot 003L1.[34] (The 1.5 fewer titratable acid groups in 381–187 should not change the isoelectric point very much.) (3) The $pK_{\text{int}}$ of the phenolics is 10.8.[29,34] (4) The electrostatic factor, $w$, is 0.080.[34] (5) No chloride ion is bound by the molecule between $p$H 11.1 and 7. This has been shown by Carr.[35] When this calculated curve for phenolic ionization is subtracted from the $\Delta D$–$p$H curve, there remains a constant $\Delta D$ of 0.13 above $p$H 8 (dashed line). This corresponds to an optical density change of 800 per mole of lysozyme which is due to the indole chromophores.

Tanford and Wagner[34] have observed that the degree of ionization of the three phenolic groups in lysozyme, calculated from spectral changes at 288, 290 and 295 m$\mu$, varies with wave length. In

addition, they observed a total change in optical density at 295 m$\mu$ of 7500 per mole of lysozyme, 600 per mole larger than the expected 6900 per mole. Just as would be predicted from the shape of the difference spectrum of the indole chromophore, which has a peak in $\Delta D$ at 295 m$\mu$, the degree of ionization of the phenolic groups calculated by them at lower $p$H's is greatest at 295 m$\mu$. Thus, a correction of the observed spectral changes for the effect of the indole change appears to reconcile the phenolic ionization data obtained at different wave lengths and gives a normal value for the optical density change due to the ionization of the phenolic groups.

Because of these inconsistencies, Tanford and Wagner hesitated to calculate a value of the heat of ionization of the phenolic groups from the temperature dependence of the observed spectral changes. From the small portion of the tyrosine ionization observed in Fig. 10, a value of 6–7 kcal. may be estimated for these groups. It seems easiest to reconcile this apparently normal $\Delta H$ with the abnormal $pK$'s of these groups by assuming that local electrostatic effects or steric effects[34] are the cause of the abnormality, rather than hydrogen bonding effects[34] which should lead to large values of $\Delta H$ for large abnormalities in $pK$,[36] as observed here.

**The $p$H Dependence of the Difference Spectrum of Lysozyme.**—When the tyrosine ionization is subtracted from the $\Delta D$–$p$H curve shown in Fig. 10, there remains a curve which appears to be the titration curve of two types of ionizable groups, one with apparent $pK$ 3.15, the other with apparent $pK$ 6.20. These curves are "flatter" than theoretical curves for single groups, but this is expected because of the simultaneous ionization of other groups (assuming that the curves are due to two groups only), which presumably do not affect the spectra of the chromophores. These values of $pK$, determined from the points of half-ionization of the "two" groups on the $\Delta D$–$p$H curve, are similar to those obtained from titration curves[37] and from kinetic data.[24] It is possible that a smaller contribution of several groups in the acid range could cause the lower portion of this curve, but the higher portion is most likely due to one group.

It is unlikely that this $\Delta D$ is due to electrostatic effects resulting from a configuration change or aggregation of the molecules, since the data of Jirgensons[38] and Yang and Foster[39] show that the optical rotation and viscosity are constant throughout this $p$H region. This $\Delta D$ is probably not due to the transfer into the solvent of a tryptophan chromophore which had been "buried" in the molecule, since Herskovits and Laskowski[40] have shown that a constant number (most probably, all) of the tryptophan chromophores in lysozyme are "exposed" to the perturbing solvents (ethylene

(33) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga. *Biochim. et Biophys. Acta,* **19,** 581 (1956).

(34) C. Tanford and M. L. Wagner, *J. Am. Chem. Soc.,* **76,** 3331 (1954).

(35) C. W. Carr, *Arch. Biochem. Biophys.,* **46,** 417 (1953).

(36) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.,* **76,** 6305 (1954).

(37) S. Beychok and R. C. Warner, *ibid.,* **81,** 1892 (1959).

(38) B. Jirgensons, *Arch. Biochem. Biophys.,* **74,** 70 (1958).

(39) J. T. Yang and J. F. Foster, *J. Am. Chem. Soc.,* **77,** 2374 (1955).

(40) T. T. Herskovits and M. Laskowski, Jr., unpublished experiments. 1960.

glycol, glycerol, polyethylene glycol) over the $pH$ range 1.5 to 7.0 at 25°.

The observed $\Delta D$ must then actually be caused by the ionization of acid groups. These groups must be carboxyls, since the methylated derivative does not show this indole difference spectrum, but only the phenolic ionization shown in Fig. 10. Since a normal $\beta$- or $\gamma$-carboxyl group with intrinsic $pK$ 4.6 would have an apparent $pK$ of 4.1 because of electrostatic interactions, these carboxyl groups appear to be quite abnormal. However, if the charge effect transfer does not take place through covalent bonds only (see below), the lower $pK$ carboxyl "group" could be the (normal) $\alpha$-carboxyl group.

**The Temperature Dependence of the Difference Spectrum.**—Within the experimental error of about ± 1 kcal., the curves drawn in Fig. 10 give a zero $\Delta H$ of ionization for the lower $pK$ "group," and about 3 kcal. for the higher $pK$ "group." However, no attempt has been made to adjust the values of $\Delta D$ observed at one temperature to the values of $\Delta D$ at the other, that is, the difference absorption coefficient is assumed to be constant with temperature. Although the values of $\delta$ observed for tryptophan do not appear to be a function of temperature,[23] it is still possible that the values of $\delta$ for the indole chromophores in proteins may be a function of temperature. If the estimates of the enthalpies of ionization of these groups are correct, then the abnormality of these groups is probably due to local electrostatic effects.

**The Number of Ionizable Groups which Affect the Spectrum of the Indole Chromophore in Lysozyme.**—The applicability of the assumption of inductive charge effect transfer can be tested for lysozyme by calculating, with the aid of Table II,

the number of ionizable groups necessary to cause the observed $\Delta D_{295}$ of 400 per mole at $pH$ 5 for one "group." Forty carboxyl groups adjacent to tryptophan residues is the minimum number required. The partial sequence of lysozyme presented by Jollès[41] indicates that, of the nine free carboxyl groups in this lot of lysozyme, seven are not adjacent to tryptophan residues. It appears that inductive effects alone are insufficient to explain the magnitude of the observed effect.

The dependence of $\Delta D_{295}$ for lysozyme on ionic strength (Fig. 7) seems to indicate that at least a large portion of the charge effect transfer is occurring through the solvent, in contrast to model compounds.

The simplest explanation of these effects for lysozyme, which could account for the order of magnitude of $\Delta D$, is that the charge effect takes place through the solvent at small distances between the charged groups (probably few) and the indole chromophores (probably few) producing the difference spectrum. Not *all* the carboxyl groups influence one or more of the indole chromophores, since then: (1) The $\Delta D$–$pH$ curve would look very much like the acid portion of the titration curve of the protein. In fact, the titration curve shows no "step" at about $pH$ 5, as observed in Fig. 10. (2) The $\Delta D$–$pH$ curve for lysozyme lot D638040, which appears to have five more titratable carboxyl groups than lot 381–187, would give a larger $\Delta D$ at any $pH$. In fact, the $\Delta D$ values observed for the complete ionization of each "group" in lot D638040 are the same, within experimental error, as the $\Delta D$ values observed for lot 381–187.

(41) P. Jollès, private communication. See also J. Jollès and P. Jollès, 1° Symp. Internazionale sul Lisozima di Fleming, Milano, April 3–5, 1959.

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# Rauwolfia Alkaloids. XXXVI.[1] Non-hypotensive Sedatives Derived from Methyl Reserpate

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Alkyl ethers of methyl reserpate have been prepared by reactions of the hydroxy compound with diazoalkanes in the presence of fluoroboric acid. Since the products were found to possess potent sedative activity, other methods for their preparation were sought. It was found that alcoholysis reactions with methyl reserpate $p$-bromobenzenesulfonate proceed with inversion to produce ethers of methyl 18-epireserpate, which have comparable sedative activity. The stereochemistry of the epi-ethers was elucidated by relating each series to common intermediates in which all asymmetric centers other than $C_{18}$ are preserved.

Interest in reserpine, the primary sedative and hypotensive principle of the genus Rauwolfia, has stimulated efforts in this Laboratory to separate these two types of physiological action by suitable modification of the structure of the molecule.[2] Isolation of the second type of activity was realized in the preparation of methyl reserpate, 18-(3,5-dimethoxy-4-ethoxyformyloxybenzoate),[2a] a com-

(1) A preliminary account of this investigation was published in *Experientia*, **17**, 14 (1961).

(2) (a) R. A. Lucas, M. E. Kuehne, M. J. Ceglowski, R. L. Dziemian and H. B. MacPhillamy, *J. Am. Chem. Soc.*, **81**, 1928 (1959); (b) R. A. Lucas, R. J. Kiesel and M. J. Ceglowski, *ibid.*, **82**, 493 (1960).

pound which has a hypotensive effect comparable to that of reserpine, but which has greatly diminished sedative properties. Since reserpine itself has an ester group at the 18-position, considerable emphasis in past investigations has been placed on variation of the ester function itself. It seemed desirable, therefore, to extend the study to determine the effects of other substituent types on pharmacological activity. This paper reports the preparation of a number of ethers derived from methyl reserpate, which are potent sedatives with rapid onset of action, but which are essentially devoid of hypotensive effect.