A Spectrophotometric and Spectrofluorometric Study of Intramolecular Interactions of Phenolic Groups in Ovomucoid*

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ABSTRACT: The phenolic groups of ovomucoid all ionize at abnormally high pH values, having pK''s of 12.0. Their absorption is hyperchromic and shifted to longer wavelengths than that of normal phenolic groups. A large decrease in absorption ($\Delta\epsilon_{286}/\epsilon_{277.5} = -0.29$) is accompanied by a shift of the absorption spectrum to shorter wavelength when ovomucoid solutions are made acid. A simultaneous increase of 300% in the fluorescence from these chromophores is observed. Both these changes are reversible, essentially independent of ionic strength, and have the same pH dependence as the ionization of a single acidic group of pK = 2.8. Esterification of ovomucoid has the same effect on its absorption and fluorescence as titration to low pH. Perturbation experiments with D_2O indicate that the phenolic chromophores interacting with acidic groups are at least partially exposed to water solvent, and that this exposure does not change upon titration to low pH.

Measurements of intrinsic viscosity indicate that ovomucoid undergoes a limited conformational change at acid pH. This conformational change does not affect the optical rotation at 350 mμ. These observations suggest that protonation of one or more carboxylate anions having the abnormally low pK' of 2.8 is accompanied by a limited conformational change in which intramolecular interactions of phenolic groups are altered.

Ovomucoid, a trypsin inhibitor from egg white, is one of the better characterized glycoproteins (Melamed, 1966) and possesses a number of interesting properties in addition to its ability to inhibit trypsin. It has a relatively high resistance to heat denaturation at acid pH, which may be related to its high cystine content, every 11th residue, on the average, being one end of a disulfide bridge. Despite this high degree of cross-linking, physical measurements indicate that the molecule has a larger hydrodynamic volume than expected for a compact protein of its molecular weight (approximately 30,000). Its hydrodynamic behavior may be due, in large part, to the polysaccharide side chains which make up about 30% by weight of the ovomucoid molecule. However, little is known about the conformation of these side chains, although a considerable amount of structural information has been obtained (Montgomery and Wu, 1963; Monsigny and Montreuil, 1966).

Ovomucoid is one of a small class of proteins which contain no tryptophan, and one of a yet smaller class of proteins which have extremely low quantum yields of fluorescence from the phenolic chromophores of their tyrosine residues. Early in this study, it was discovered that the ionization properties of all its phenolic groups are quite abnormal. These peculiar physical properties of ovomucoid, taken together with its important role as a trypsin inhibitor, led to this study of the relation between the optical properties of ovomucoid, its conformation, and the dissociation behavior of its ionizable groups.

Experimental Section[1]

Spectrophotometric measurements were obtained using a Cary Model 15 recording spectrophotometer, and in one instance, using the Turner Model 210 spectrofluorometer–spectrophotometer (Turner, 1964). Spectrofluorometric measurements were ordinarily obtained using a Baird-Atomic Model SF-1 (8-mμ band width), but the corrected spectra of Figure 6 were obtained with the Turner spectrofluorometer (10-mμ band width). Optical rotation measurements at 350 mμ were obtained using the Cary Model 60 spectropolarimeter; otherwise measurements were obtained using the Perkin-Elmer Model 141, at the 578-, 546-, 436-, 365-, and 313-mμ mercury lines.

For all the spectra, difference spectra, perturbation difference spectra, and fluorescence data reported, complete spectra were always obtained over the wavelength range of interest, to ensure against artifacts arising from turbidity or mismatch. Experiments were carried out at 22° except where noted. Both difference spectra and perturbation difference spectra were obtained in 1-cm path-length cells with ovomucoid

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[1] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

solutions of maximum absorbancy less than 2 (and ordinarily less than 1) absorbancy units. Solutions for fluorescence measurements did not exceed 0.2 absorbancy unit. Spectrophotometric titrations were carried out as described elsewhere (Donovan, 1965). Samples for D_2O perturbation spectra were prepared by placing 0.100 ml of buffered water solutions of ovomucoid in each of two spectrophotometer cells, and adding 1.000 ml of water or D_2O (Bio-Rad, 99.8%). The perturbation difference spectra were recorded using the 0.1 absorbancy unit slidewire, with the D_2O solution used as reference. Occasional corrections for turbidity were made by drawing a straight base line under the difference spectrum from 300 mμ to approximately 270 mμ. Final buffer concentrations used in the perturbation experiments were 0.1 M glycine-HCl, 0.05 M citrate, and 0.01 M acetate, in addition to 0.1 M HCl and unbuffered solutions. The pH of the D_2O solution did not differ from that of the water solution by more than 0.07 pH unit toroughout the pH range in which the acid difference spectrum is observed. Chromophore exposure to solvent was calculated relative to that of tyrosine.

Viscosity measurements were obtained at 25° using a Cannon–Übbelohde no. 50 dilution viscometer having a flow time for water of approximately 230 sec. The intrinsic viscosity was calculated from the intrinsic kinematic viscosity by the method of Tanford (1955), using 0.685 for the $\bar{v}$ of ovomucoid (Fredericq and Deutsch, 1949).

The concentration of ovomucoid was determined spectrophotometrically, using the optical factor 0.410 $D_{277.5\,m\mu}$ = 1 mg/ml (1-cm path). The optical factor was determined both by evaporating a Millipore-filtered water solution of ovomucoid of known absorbancy to dryness at room temperature *in vacuo* over P_2O_5 and by measuring the absorbancy of solutions prepared from ovomucoid of known moisture content (air drying at 105°). The first procedure gave an optical factor of 0.411, the second, 0.409. This is in agreement with the molar extinction coefficient reported by Chatterjee and Montgomery (1962).

The ovomucoid used in these experiments, prepared by the Lineweaver and Murray (1947) procedure, was obtained from the Worthington Biochemical Corp. Upon starch gel electrophoresis, it was observed to contain significant quantities of lysozyme, ovoinhibitor, and flavoprotein, and was purified by Mr. J. G. Davis of this laboratory, by adsorption to and elution from carboxymethylcellulose, followed by adsorption to and removal from DEAE-cellulose. Although the purified ovomucoid showed no traces of the original contaminants on starch gel, it showed a small amount of fluorescence near 345 mμ, characteristic of tryptophan (probably from traces of lysozyme remaining in this preparation). The unpurified ovomucoid had an acid difference spectrum with $-\Delta D_{max}/D_{max}$ of 0.20; the purified material, 0.29. The purified material contained 13.3% nitrogen on a dry weight basis. Fredericq and Deutsch (1949) report 13.2% nitrogen. Amino acid hydrolysates at 20, 40, and 70 hr (110°, 6 N HCl) showed 2.14, 2.08, and 2.06 moles of tyrosine, respectively, per 10,000 g, or approximately 6.0 moles of tyrosine/29,000 g. Stevens and Feeney (1963) report 1.90 moles of tyrosine/10,000 g for a 22-hr hydrolysate, or 5.5 moles of tyrosine/29,000 g (13.3% nitrogen). The complete amino acid analysis, purification procedure, and other characterizations of the present preparation will be reported elsewhere (J. G. Davis and J. W. Donovan, manuscript in preparation). A molecular weight value of 29,000 was assumed for calculations.

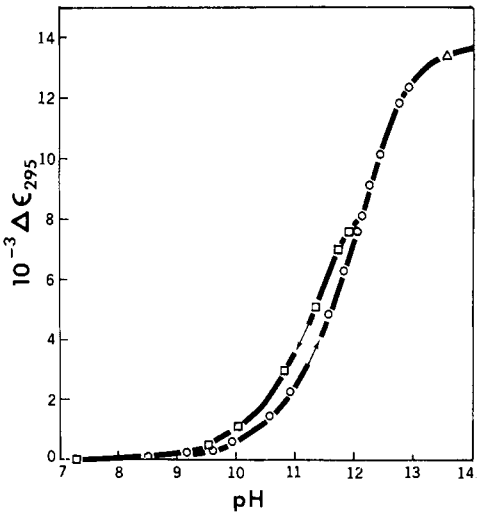

FIGURE 1: Spectrophotometric titration of the phenolic groups of ovomucoid. (○) Forward titration in 0.25 M KCl. (△) Obtained 15 sec after addition of KOH. (□) Reverse titration to lower pH from pH 12.1. Ovomucoid concentration, 3×10^{-5} M.

Results

Titration of Phenolic Groups in Ovomucoid. The spectrophotometric titration of the phenolic groups of ovomucoid is shown in Figure 1. The total absorption change observed is in agreement with the tyrosine content obtained from amino acid analysis, when the difference extinction coefficient for tyrosine ionization at 295 mμ of 2.33×10^3/mole is employed (Beaven and Holiday, 1952). It is evident that the phenolic ionization is markedly abnormal, the point of half-ionization appearing at pH 12.0, approximately 2 pH units greater than the normal apparent pK for these groups (about 10.0 in a protein with the isoelectric point of ovomucoid). Above pH 12.5, increases in absorption of ovomucoid solutions were observed upon standing. This additional absorption was not due to slow ionization of phenolic groups, since differences between absorption spectra measured at different times showed a gradual increase in absorption with decreasing wavelength, quite unlike the difference spectrum for ionization of phenolic groups. These time-dependent spectra did not pass through the isosbestic points of phenolic ionization near 270 mμ.

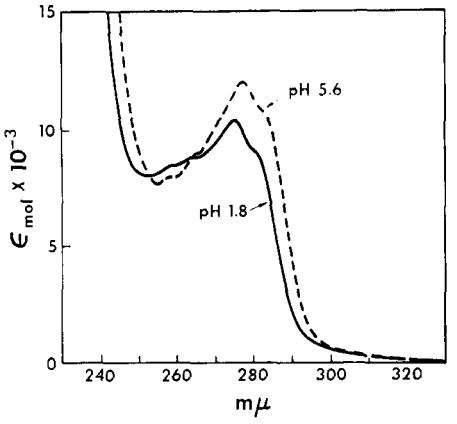

FIGURE 2: Spectra of ovomucoid at neutral and acid pH.

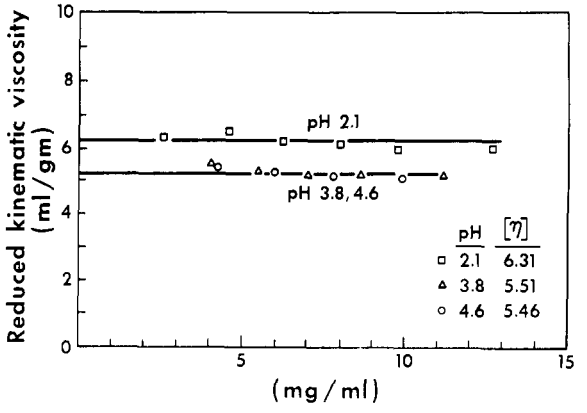

FIGURE 4: Concentration dependence of the reduced kinematic viscosity of ovomucoid in 0.25 M KCl.

Accordingly, at higher pH, spectra were taken very soon after adjustment of pH. The point at highest pH in Figure 1 was read directly at 295 mμ immediately after addition of KOH, and before an absorption scan was made. Because of this time dependence, the reversibility of the phenolic ionization was determined only from pH 12. Amino acid analysis of an ovomucoid sample exposed to high pH showed reduced lysine and half-cystine content, but a normal tyrosine content.

Absorption Change in Acid. When a neutral solution of ovomucoid is made acid, its spectrum undergoes a blue shift with a relatively large decrease in intensity (Figure 2). The absorption maximum shifts from approximately 277.5 to 275.0 mμ. The acid difference spectrum shows peaks at 278 and 286 mμ. The pH dependence of the acid difference spectrum, observed at the wavelength of maximum change in absorption, 286 mμ, is shown in Figure 3. The decrease in absorption is reversible, apparently independent of ionic strength, and appears to follow, to a close approximation, the sigmoid curve calculated for the ionization of a single group of pK = 2.8. Essentially the same data as presented in this figure have independently been obtained by Drs. T. Warrington and M. Laskowski, Jr. (private communication). The insert at upper right of Figure 3 demonstrates that the change in the spectrum is independent of ovomucoid concentration over more than a tenfold range.

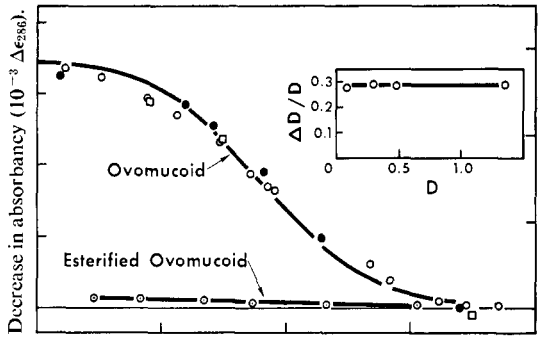

FIGURE 3: pH dependence of the acid difference spectrum of ovomucoid and the methyl ester of ovomucoid. Open circles: titration to low pH; squares: reversal from pH 1.2, both in 0.25 M KCl. Filled circles: titration to low pH in the absence of salt. The line drawn through the points is a theoretical titration curve of a single group with pK = 2.80. The inset shows that the acid difference spectrum at pH 1 obeys Beer's law.

To show that the decrease in absorption of ovomucoid at acid pH is in fact a change in absorption, and not due to an increase in fluorescence (see below), the absorption spectra of neutral and acid solutions of ovomucoid were measured using the Turner Model 210 as a spectrophotometer. The absorbing solutions are placed after the first monochromator, and before the second. This arrangement nearly completely eliminates "stray" light due to sample fluorescence. Within experimental error, the spectra obtained in this way were the same as those obtained with the Cary Model 15. Thus, although fluorescence of ovomucoid increases with decreasing pH, the observed absorption decrease is correct, within the accuracy of the measurements. The change in fluorescence emission with pH did not affect the measurement of absorption for two reasons. (1) The geometry of the Cary Model 15 spectrophotometer is not favorable for detecting fluorescence from samples placed in 1-cm cells in the center of the sample compartment (Mihalyi, 1965). (2) The fluorescence emission is quite weak compared to the intensity of the transmitted light.

Intrinsic Viscosity Change in Acid. Since blue shifts of protein spectra commonly result from denaturation of the protein, the intrinsic viscosity of ovomucoid was determined at neutral and acid pH. A 15% increase in intrinsic viscosity of ovomucoid is observed when the pH is lowered to pH 1 (Figure 4). However, the relatively small value of the intrinsic viscosity at pH 1, together with the absence of concentration dependence of the

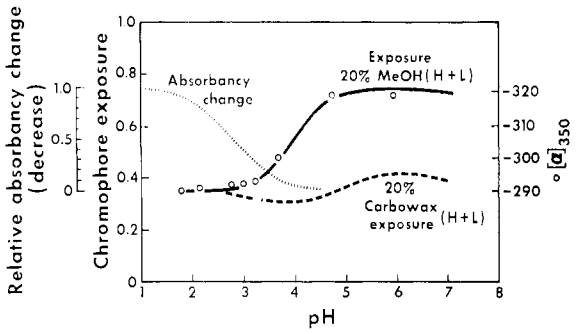

FIGURE 5: Comparison of the pH dependence of the acid difference spectrum of ovomucoid with its optical rotation at 350 mμ (circles) and with its solvent perturbation difference spectra in methanol (solid line) and in polyethylene glycol (dashed line) (Herskovits and Laskowski, 1962). Chromophore exposure is expressed relative to thioglycolic acid reduced ovomucoid in 8 M urea.

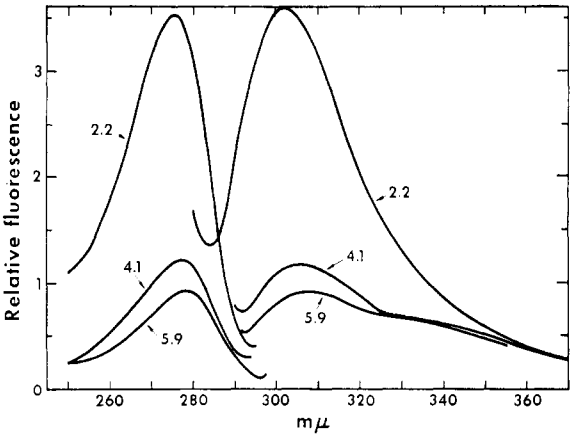

FIGURE 6: Corrected fluorescence excitation and emission spectra of ovomucoid at pH 5.9, 4.1, and 2.2. Excitation spectra were scanned with the emission monochromator set at the fluorescence emission maximum, and *vice versa;* ovomucoid (1×10^{-5} M) in 0.25 M KCl.

reduced viscosity at this pH, indicates that the amount of conformational change occurring is not large.

Optical Rotation Change at Acid pH. Although the viscosity measurements indicate a conformational change between pH 3.8 and 2.1, optical rotation measurements at 350 mμ do not show this change. In Figure 5, the pH dependence of the optical rotation at 350 mμ is shown together with the change in absorption and the change in the degree of exposure of the phenolic chromophores to two perturbants reported by Herskovits and Laskowski (1962). The optical rotation follows the change in exposure of phenolic chromophores to perturbants such as ethylene glycol or methanol, in confirmation of the observations of Herskovits and Laskowski. The acid difference spectrum is not accompanied by a change in optical rotation.

Fluorescence Change in Acid. Corrected fluorescence excitation and emission spectra of ovomucoid at three pH values are shown in Figure 6. The excitation spectra are very similar to the absorption spectra of ovomucoid and have maxima at the same wavelengths (about 278 mμ at neutral pH, and 275 mμ at acid pH). There is a small increase in fluorescence emission when the pH is changed from 5.9 to 4.1. A threefold increase in fluorescence emission is observed when the pH is reduced to 2.2.

The pH dependence of the fluorescence emission of ovomucoid is shown in Figure 7. Little change in fluorescence is observed in the pH range in which Herskovits and Laskowski report change in exposure of phenolic chromophores.

Quantum Yield of Fluorescence of Ovomucoid. When the fluorescence of solutions of ovomucoid and of tyrosine having the same maximum absorption (about 0.1 absorbancy unit) were compared at neutral pH, the fluorescence observed from ovomucoid was only 0.029 that observed for tyrosine. If the quantum yield for tyrosine is taken as 0.21 (Teale and Weber, 1957), then the quantum yield for ovomucoid is calculated to be 0.006. This is smaller than the value reported by Teale (1960). The results presented in Figure 7 indicate that the quantum yield for ovomucoid at acid pH is 0.025.

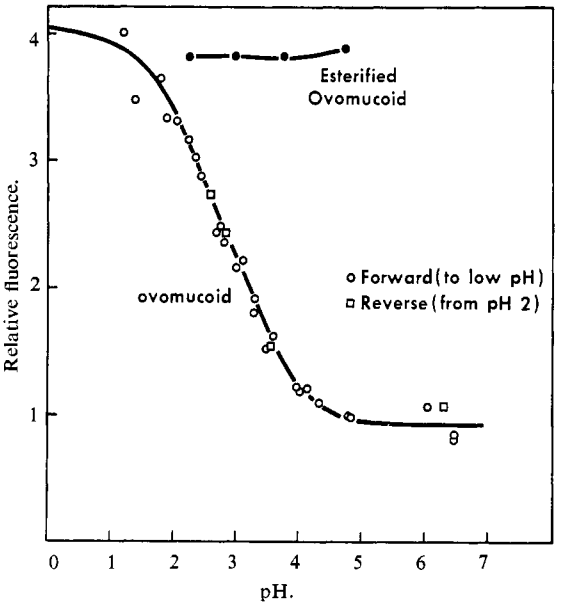

FIGURE 7. pH dependence of the fluorescence emission of ovomucoid and esterified ovomucoid. Excitation at 278 mμ. Fluorescence is shown as the ratio to the fluorescence at neutral pH; ovomucoid (3.3×10^{-5} M) in 0.25 M KCl.

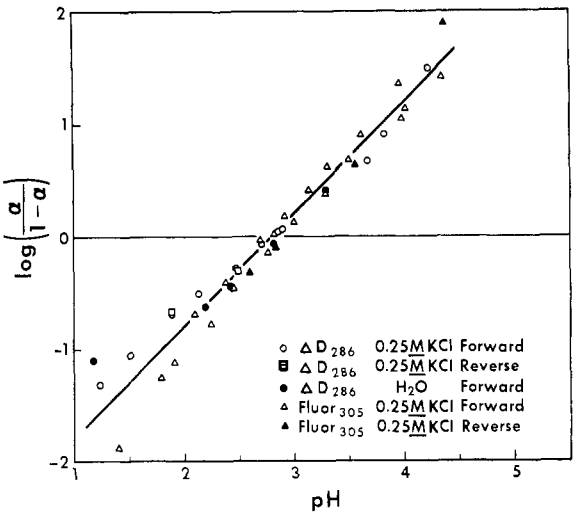

FIGURE 8: Hill plots of the change in absorption and emission as a function of pH. The line drawn through the points has a slope (Hill coefficient n) of unity.

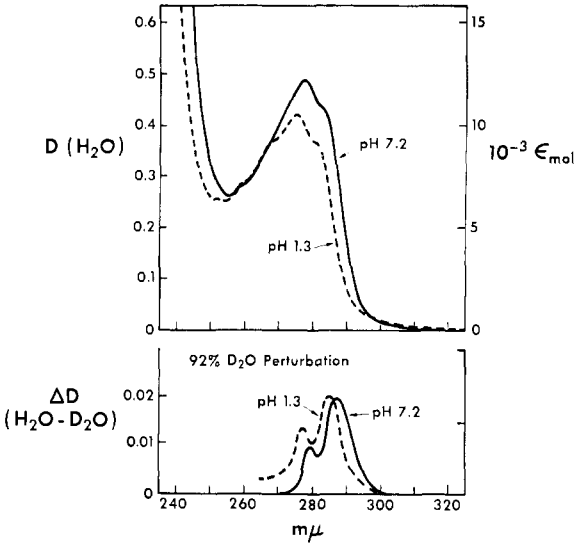

FIGURE 9: Comparison of the spectra (upper) and D_2O perturbation difference spectra (lower) of ovomucoid at neutral and acid pH. The left ordinates give the absorbancies and absorbancy changes as measured in 1-cm path cells.

Absorption and Fluorescence Changes Compared. The change in absorption and the change in fluorescence as a function of pH are presented together in the form of Hill plots in Figure 8. Here $\alpha/(1-\alpha) = (D - D_{min})/(D_{max} - D)$ for the absorption data, and $\alpha/(1-\alpha) = (F_{max} - F)/(F - F_{min})$ for the fluorescence data, since the absorption decreases and the fluorescence increases with decreasing pH. The fluorescence and absorption changes have the same dependence upon pH (apart from sign). This pH dependence can be characterized as the ionization of one or more noncooperative (Hill coefficient (n) = 1) groups with an apparent pK of 2.8.

Absorption and Fluorescence of Esterified Ovomucoid. Since it appeared that these changes in absorption and fluorescence of ovomucoid were related to the dissociation of protons from acidic groups, perhaps carboxyl groups, an ester derivative of ovomucoid was prepared by the method of Fraenkel-Conrat (1951). The derivative obtained after esterification for 3 days at room temperature in acidified methanol had an acid difference spectrum approximately one-half that of the native material. Spectrophotometric titration of the phenolic groups with alkali showed that these were as abnormal as those of the native material. Since alkali causes rapid hydrolysis of these methyl esters, this titration behavior can only be taken as an indication that the conformation of the protein was neither severely changed by the esterification procedure, nor readily reverted to the native conformation. Further evidence for little conformation change was obtained after esterification for 7 days. Values of the optical rotatory parameters obtained at neutral pH for this ester and for native ovomucoid, respectively, were a_0 −380 and −410°, b_0 −75 and −70°. (Tomimatsu and Gaffield (1965) have reported an a_0 of −524° and a b_0 of −86° for an unpurified commercial preparation of ovomucoid.) The ester derivative had an absorption maximum at 276 mμ, and showed no acid difference spectrum (Figure 3). Its fluorescence spectrum was pH independent, and essentially identical with that observed for native ovomucoid at pH 2 (Figure 7). These experiments indicate that the ester derivative of ovomucoid has a conformation similar to that of ovomucoid at low pH. Esterification appears to be equivalent to protonation of acidic groups.

Solvent Perturbation of Phenolic Chromophores of Ovomucoid by D_2O. When the perturbation of the exposed phenolic chromophores in ovomucoid is carried out using D_2O as a perturbant, the perturbation difference spectrum is observed to shift to shorter wavelength with decreasing pH, as the absorption spectrum does (Figure 9). Table I lists the exposure observed with D_2O as perturbant in the acid pH range. There appears to be a constant exposure to D_2O (and therefore, presumably also to H_2O) in this pH range.

The chromophore exposure to solvent given in Table I is calculated relative to the perturbation observed for the *amino acid* tyrosine. Herskovits and Laskowski (1962) calculated exposure relative to thioglycolic acid reduced ovomucoid in 8 M urea (Figure 5). Exposures calculated on different bases are not always comparable. Herskovits and Laskowski show that the reduced ovomucoid in 8 M urea is perturbed by methanol nearly 30% *more* than are the chromophores in a water solution of amino acids containing the same amount of the same chromophores as ovomucoid. The difference spectral constants ($\Delta\epsilon/\epsilon$) obtained using 20% methanol were 0.054 for thioglycolic acid reduced ovomucoid in 8 M urea, and 0.042 for the model mix-

TABLE I: Perturbation of the Phenolic Chromophores of Ovomucoid by 92% D_2O.

pH	λ_{max}[a] Absorption (ϵ)	λ_{max}[a] Perturbation ($\Delta\epsilon$)	$-\left(\frac{\Delta\epsilon_{max}}{\epsilon_{max}}\right)$[b]	Rel Exposure[c] Peak Height[d]	Rel Exposure[c] Area[e]	Half-Width of Difference Peak (mμ)
			Ovomucoid			
1.33	275.0	284.5	0.036	0.62	0.62	7.1
2.60	276.0	287.0	0.037	0.64	0.60	7.6
3.23	277.0	287.5	0.037	0.64	0.59	7.9
3.67	277.0	287.5	0.033	0.57	0.54	8.8
4.78	277.5	287.5	0.034	0.59	0.60	8.5
7.18	277.5	287.5	0.036	0.62	0.68	8.8
			Tyrosine			
1.24	274.5	283.5	0.056	0.97	1.00	6.7
6.20	274.5	284.0	0.058	1.00	0.98	6.7

[a] To nearest 0.5 mμ. [b] ϵ_{max} in H_2O at each pH. D_2O perturbation is a blue shift. [c] Compared to tyrosine at pH 6.2. [d] Difference peak at 284–287 mμ. [e] Area under difference spectrum between 275 and 300 mμ.

ture of amino acids (Herskovits and Laskowski, 1962). The ratio of these two constants allows the calculation of the exposure of the phenolic chromophores of ovomucoid, as determined from perturbation by methanol, relative to tyrosine. At pH 7, the exposure of the chromophores of ovomucoid to methanol, relative to tyrosine, calculated from their data, is 0.58. Within experimental error, this exposure to methanol perturbant in their experiments is the same as that obtained in the present experiments using D_2O as perturbant, and calculated relative to tyrosine (Table I). The perturbation by ethylene glycol reported by Herskovits and Laskowski, when similarly corrected, yields an exposure of 0.56.

Discussion

Magnitude of the Changes in Absorption and Fluorescence. Both the changes in absorption and fluoresence of ovomucoid observed upon the addition of acid are quite large. It appears unlikely that these changes are due to environmental effects on only *one* of the phenolic chromophores. If only the fluorescence changes are considered, it might be possible for one of the phenolic groups to be strongly quenched by proximity to an abnormal carboxyl group or the C-terminal α-carboxyl group. Förster coupling might then result in quenching of all phenolic groups in the near neighborhood. However, the observed maximum molar *change in absorption* at acid pH, −3400, is larger than the molar *absorption* of two phenolic residues (about 1500 each at acid pH). These considerations make it appear that most, or all, of the phenolic groups in ovomucoid participate in interactions leading to changes in absorption and fluorescence.

The Absorption Change in Acid Solution. Since the intrinsic viscosity of ovomucoid increases only 15% in acid solution and remains nearly concentration independent (Figure 4), the "acid difference spectrum" does not result from a gross denaturation of the molecule. The optical rotation at 350 mμ is unchanged between pH 3.2 and 1.8, in which range the absorption of phenolic chromophores undergoes a large decrease (Figure 5). Thus, while the acid difference spectrum can be termed a blue shift, it cannot be termed a denaturation blue shift, in which all chromophores buried in the hydrophobic interior of a protein become exposed to the surrounding water solvent. This distinction is especially clear-cut for ovomucoid, because solvent perturbation experiments with D_2O indicate a constant exposure of the phenolic chromophores to solvent throughout the pH range in which the acid difference spectrum is observed.

The pH dependence of the absorption change indicates that protonation of one or more acidic groups with an apparent p*K* of 2.8 removes most of the hyperchromicity and red shift of the absorption spectrum of the phenolic chromophores. The absorption observed at pH 1 agrees reasonably well with that calculated for six normal phenolic groups plus nine buried disulfide chromophores (15% increase in absorption assumed for buried chromophores). Esterification of the acidic groups also reduces the hyperchromicity, as indicated by the shift in the absorption maximum to 276.0 mμ upon esterification, and the lack of dependence of the spectrum of the ester upon pH (Figure 3). These interrelationships suggest a direct effect of carboxylate anions on the phenolic chromophores of the tyrosine residues, giving rise both to hyperchromicity of the phenolic groups, and to abnormalities in the ionization behavior of both the acidic and the phenolic groups.

Identity of the Acidic Groups. Apart from the observed p*K* value of 2.8 and the loss of the acid difference spectrum on esterification, there is no evidence that the acidic groups interacting with the phenolic chromophores are actually abnormal carboxyl groups. They

have been assumed to be carboxyl groups since no other acidic groups have been demonstrated in ovomucoid, and sulfate groups attached to the polysaccharide side chains, the other likely possibility, would have much lower values of pK. The value of the observed pK is the same as that of the carboxyl group of sialic acid, which has a pK of 2.6 or 2.8 (Bettelheim, 1963). However, several analyses of ovomucoid (Chatterjee and Montgomery, 1962; Stevens and Feeney, 1963; Melamed, 1967) show less than 1 residue of sialic acid/30,000 g.

Recently, Melamed (1967) investigated the electrophoretic properties of ovomucoid, both before and after removal of sialic acid with neuraminidase. He suggests that the presence of a single carboxyl group, not the carboxyl group of sialic acid, but having an abnormally low pK, could account for anomalous electrophoretic properties of ovomucoid observed near pH 4.6. The unknown number of carboxyl groups revealed by the present experiments may be the *single* abnormal carboxyl group hypothesized by Melamed. If so, then the abnormalities observed in the ionization of all six of the phenolic groups in ovomucoid cannot be produced by direct interactions of these phenolic groups with the single carboxyl group, since this would be impossible on steric grounds. However, a conformational change of ovomucoid, controlled by the protonation of the single carboxyl group, would be a likely explanation for the results presented here.

Estimate of the Free Energy of Interaction. The free energies of interaction of the carboxylate ion(s) and the phenolic groups can be estimated from a comparison of the observed values of apparent pK (pK') with the values of pK' expected for the ionization of normal groups. Since $\Delta F^\circ = -2.3RT \log K$, then $\Delta(\Delta F^\circ)$, the free energy of interaction, will be related to ΔpK, the difference between the observed and expected values of pK' by $\Delta(\Delta F^\circ) = 2.3RT\,\Delta pK$. Observed p$K'$ values for the carboxyl and phenolic groups are 2.8 and 12.0, respectively (Figures 1 and 3). Estimated normal pK' values in ovomucoid (isoelectric point approximately 4.4; Bier *et al.*, 1953) are 4.6 and 10.0, respectively. Thus, $\Delta(\Delta F^\circ)$ carboxyl $= -2.5$ kcal and $\Delta(\Delta F^\circ)$ phenolic $= 2.8$ kcal. The difference in sign results because interaction facilitates ionization of the carboxyl group, and hinders ionization of the phenolic group. This calculation for the carboxyl group assumes a side-chain carboxyl group, not an α-carboxyl group.

The interaction energies calculated above suggest a one-to-one interaction of carboxyl and phenolic groups. The bulk of the experimental evidence presented suggests that this is *not* a one-to-one interaction. Furthermore, the interaction between carboxyl and phenolic groups appears to be indirect, and thus not a hydrogen-bonding interaction of the type discussed by Laskowski and Scheraga (1954). Disruption of a small hydrophobic portion of the ovomucoid structure could account for the observed strength of the interaction, and might, in addition, account for the absorption changes. (Yanari and Bovey (1960) and Wetlaufer (1962) have reviewed the causes of wavelength shifts of phenolic chromophores.)

Number of Abnormal Phenolic Groups. The spectrophotometric titration of the phenolic groups (Figure 1) indicates that all of these groups are abnormal, with an average apparent pK of 12.0. However, both the solvent perturbation studies of Herskovits and Laskowski (1962) at neutral pH and the D_2O solvent perturbation studies (Table I) indicate that about 40% of the six phenolic groups are buried. These experiments do not indicate whether all of the phenolic groups are partially buried, or whether there is a sharp division into buried and exposed groups. The argument which follows leads to the conclusion that those phenolic groups which undergo changes in absorption in acid are at least partially exposed to solvent.

As the pH of an ovomucoid solution is lowered, the position of the peak in the D_2O perturbation spectrum shifts to shorter wavelength. The shift of the peak in the perturbation spectrum has approximately the same pH dependence as the shift of the absorption maximum of ovomucoid (Table I and Figure 9). The absolute value of the perturbation difference spectrum decreases with pH, but in the same ratio as the decrease in the absorption spectrum with pH, so that $\Delta\epsilon/\epsilon$ remains constant. The same relative exposure of chromophores is obtained, whether the calculations are based on peak heights or areas. Since the maxima of *both* the spectrum and the perturbation spectrum undergo corresponding shifts to shorter wavelength with decreasing pH, the phenolic groups affected by the protonation of one or more carboxylate anions at acid pH are the same phenolic groups which are perturbed by D_2O in the solvent perturbation experiments. Thus, these phenolic groups are those exposed to the solvent. The reversibility of the titration curve of the acid difference spectrum indicates that the carboxyl groups are also exposed to the solvent. Exposure to water solvent does not appear to be consistent with strong hydrogen bonding between phenolic groups and carboxyl groups (Schellman, 1955; Klotz and Franzen, 1962).

In Table I, the half-width (width at half-height) of the D_2O perturbation spectrum is given as a function of pH. At neutral pH, the peak width is broader than at acid pH. The broad peak width is consistent with a perturbation difference spectrum which is the sum of the perturbation difference spectra of phenolic groups of different degrees of abnormality. As the abnormality is removed by lowering the pH, the perturbation difference spectrum becomes sharper as it shifts to a shorter wavelength, approaching, but not reaching, the half-width and wavelength of the perturbation difference spectrum of the amino acid tyrosine. Assuming the abnormal phenolic groups are red shifted an average of 3.0 mμ, as indicated by Table I, the maximum molar change in extinction resulting from normalization of these groups can be estimated. The magnitude of a difference spectrum resulting from a simple shift of the spectrum is given by the approximate relation (Chervenka, 1959; Donovan *et al.*, 1961) $\Delta\epsilon = -(d\epsilon/d\lambda)\Delta\lambda$, where $d\epsilon/d\lambda$ is the slope of the absorption

spectrum, and where $\Delta\lambda$, the wavelength shift, is taken as positive for a red shift. Since the slope of the long-wavelength end of the ultraviolet absorption spectrum of acetyltyrosine is -200 ± 10 $\epsilon/m\mu$, and the wavelength shift estimated above for ovomucoid is 3.0 $m\mu$, normalization of one of these abnormal phenolic groups in ovomucoid would produce a maximum molar change is extinction of -600. The observed maximum $\Delta\epsilon$ of -3400 (Figure 3), even after correction for the 14% decrease in maximum absorption which accompanies the blue shift in the spectrum of ovomucoid, suggests that all six phenolic groups are abnormal. All phenolic groups appear equally abnormal from the alkaline spectrophotometric titration.

The alkaline spectrophotometric titration of the phenolic groups does not appear to be reversible from pH 12 (Figure 1). This is unexpected, since the solvent perturbation results, taken together with the large change in absorption at acid pH, suggest that all the phenolic groups are partially exposed to solvent. The irreversibility of the alkaline titration can be accounted for in at least two ways. (1) Not all the phenolic groups are exposed to solvent, but one or more buried phenolic groups are in a portion of the molecule which changes conformation in alkali. (2) All the phenolic groups are partially exposed, but titration to alkaline pH is accompanied by a small irreversible conformational change which further exposes the partially exposed chromophores. Measurements of intrinsic viscosity and optical rotation at alkaline pH are planned.

Comparison of Solvent Perturbation Experiments. The results of the D_2O solvent perturbation experiments resolve apparent inconsistencies between the solvent perturbation experiments of Herskovits and Laskowski (1962) and the present observations of the acid difference spectrum. Herskovits and Laskowski showed that the exposure of phenolic chromophores in ovomucoid to methanol, ethylene glycol, and dimethyl sulfoxide decreased as the pH was lowered from 6 to 3. They suggested that phenolic chromophores become buried as the pH is decreased. A change in absorption of these chromophores is expected if they become buried, but no change in absorption is observed in this pH range (Figure 5). This observation, however, is consistent with the constant exposure to D_2O throughout this pH range (Table I).

In ovomucoid, there must be marked differences in exposure of the phenolic chromophores to D_2O and H_2O compared to methanol, ethylene glycol, and dimethyl sulfoxide. With a change in pH from 5 to 3, methanol molecules are excluded from the neighborhood of some of the phenolic chromophores (Herskovits and Laskowski, 1962), but water molecules are not. It is possible, as suggested by Herskovits and Laskowski, that some of the phenolic groups are in crevices near the surface of the ovomucoid molecule, and a narrowing of the crevices prevents entry of methanol or larger molecules, while water molecules are still admitted. The reduction in size of the crevice opening, if this is the correct interpretation of this change in exposure of phenolic groups to methanol, ethylene glycol, and dimethyl sulfoxide, occurs at higher pH than the acid difference spectrum (Figure 5).

Quenching of Fluorescence of Phenolic Chromophores. The quantum yield of 0.006 for fluorescence at neutral pH from the phenolic chromophores in ovomucoid is one-half that reported by Teale (1960). This difference probably results from the higher purity of the present ovomucoid preparation. This quantum yield, while quite low, is comparable to the quantum yield of 0.007 reported by Cowgill (1963) for pancreatic trypsin inhibitor, and might be produced by proton transfer to, or hydrogen bonding to, carboxylate anions (Weber and Rosenheck, 1964; Cowgill, 1966). Comparison with the quantum yield of 0.21 for the phenolic chromophore in the amino acid tyrosine (Teale and Weber, 1957) makes it appear that the phenolic chromophores in ovomucoid are strongly quenched. However, the presence of peptide bonds can markedly reduce the fluorescence efficiency of phenolic chromophores. Cowgill (1967) observed a quantum yield of 0.05 for insulin B chain, oxytocin, reduced ribonuclease A, and uncharged Gly-Tyr-Gly-amide. If these are suitable models for unquenched phenolic fluorescence in a protein, then the observed quantum yield of 0.025 for ovomucoid at pH 1 suggests that the phenolic chromophores in ovomucoid are still quenched at this pH, but much less than at neutral pH. Since the fluorescence yield of the ester derivative of ovomucoid is the same as that of ovomucoid at pH 1 (Figure 7), the major source of quenching of the phenolic groups is either the direct interaction with carboxylate anions of aspartic and glutamic acid residues in the same molecule, or another intramolecular interaction controlled by the ionization of carboxyl groups. Such an interaction might be quenching of the phenolic groups by the peptide amide bonds (Cowgill, 1965) in a conformation of the molecule determined by the state of ionization of one or more abnormal carboxyl groups.

Conformation of Ovomucoid. The hydrodynamic properties of ovomucoid are relevant to the interpretation of the experiments described above. Estimates of the hydrodynamic parameter β (Scheraga and Mandelkern, 1953), based on the sedimentation and diffusion coefficients ($s_{20,w} = 2.62$ S; $D = 6.01 \times 10^{-7}$) reported by Deutsch and Morton (1961) and the intrinsic viscosity reported above (5.46 ml/g), were made using the partial specific volume of 0.685 (Fredericq and Deutsch, 1949) and the sedimentation–diffusion molecular weight 31,500 (Deutsch and Morton, 1961). β values of 1.92×10^6 (calculated from s and $[\eta]$)[2] and 1.78×10^6 (calcu-

[2] There is a remarkable amount of disagreement on the sedimentation coefficient of ovomucoid. $s_{20,w}$ values, extrapolated to zero concentration, range from about 2.3 (Montreuil *et al.*, 1965) to 2.8 (Chatterjee and Montgomery, 1962). If this latter value is chosen for calculations, a β value of 2.07×10^6 is obtained. The reported molecular weights of ovomucoid vary from 27,000 (Fredericq and Deutsch, 1949) to 31,500 (Deutsch and Morton, 1961). Amino acid analysis and osmotic pressure measurements on purified ovomucoid carried out in this laboratory are in better agreement with the higher molecular weight (J. G. Davis and J. W. Donovan, manuscript in preparation).

lated from D and $[\eta]$) were obtained. The minimum theoretical value of β is 2.12×10^6, corresponding to an axial ratio of unity. Thus, ovomucoid is probably not compact and highly asymmetric, but instead, highly hydrated. Some confirmation for this interpretation is obtained from gel filtration experiments. Whitaker (1963) has observed that ovomucoid is eluted from Sephadex G-100 at a volume corresponding to a molecular weight of 45,000.

Attached to the protein moiety of ovomucoid, of molecular weight about 22,000, are three similar carbohydrate chains, containing about 13 monosaccharide units each, of chain weight about 2500 (Montgomery and Wu, 1963). It is conceivable that these side chains could act as perturbants of the chromophores of the protein portion of ovomucoid. However, the hydrodynamic and gel filtration results both suggest that these chains are not in close contact with the protein moiety throughout their length, but extend outward into the solvent, and so probably do not act as perturbants. Removal of these side chains by enzymes splitting glycosidic bonds would clarify this point, and also show that the polysaccharide side chains are not buried in the interior of the protein moiety. Such enzymatic experiments have not yet been carried out since purified preparations of enzymes splitting these particular side-chain bonds are not available.

The Nondenaturation Blue Shift. At acid pH, the absorption spectrum of the phenolic groups of ovomucoid shifts to a shorter wavelength. This blue shift is not accompanied by a change in exposure of phenolic chromophores to solvent, and, as stated above, cannot be termed a denaturation blue shift. Two other examples of such a blue shift are known. Kronman *et al.* (1965) and Kronman and Holmes (1965) showed that the blue shift of the spectrum of α-lactalbumin observed at acid pH was accompanied by a conformational change in which the exposure of indole chromophores to solvent was unaltered. Bigelow and Sonenberg (1962) have shown that binding of dodecyl sulfate to bovine serum albumin produces a blue shift in its absorption spectrum. Although not accompanied by a change in exposure of chromophores to solvent (Ray *et al.*, 1966), this blue shift is accompanied by a change in optical rotation (Reynolds *et al.*, 1967).

These three blue shifts all have two features in common: (1) no change in exposure of chromophores to solvent, and (2) a change in conformation small enough to be detectable by one experimental technique, but not by another. A common source of these three blue shifts may be small alterations in the positions of charged groups close to the affected chromophores. Bigelow and Sonenberg (1962) consider this the most likely cause of the blue shift observed for bovine serum albumin. However, charge effects upon the spectra of chromophores of proteins and amino acids (Donovan *et al.*, 1961) ordinarily are not as large as observed here for ovomucoid. Perhaps a change in dielectric constant near the charge (but not near the chromophore) is responsible. Obviously, further work is needed.

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References

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

Bettelheim, F. A. (1963), *Ann. N. Y. Acad. Sci. 106*, 247.

Bier, M., Terminiello, L., Duke, J. A., Gibbs, R. J., and Nord, F. F. (1953), *Arch. Biochem. Biophys. 47*, 465.

Bigelow, C. C., and Sonenberg, M. (1962), *Biochemistry 1*, 197.

Chatterjee, A. K., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 99*, 426.

Chervenka, C. H. (1959), *Biochim. Biophys. Acta 31*, 85.

Cowgill, R. W. (1963), *Arch. Biochem. Biophys. 100*, 36.

Cowgill, R. W. (1965), *Biochim. Biophys. Acta 109*, 536.

Cowgill, R. W. (1966), *Biochim. Biophys. Acta 112*, 550.

Cowgill, R. W. (1967), *Biochim. Biophys. Acta 133*, 6.

Deutsch, H. F., and Morton, J. I. (1961), *Arch. Biochem. Biophys. 93*, 654.

Donovan, J. W. (1965), *Biochemistry 4*, 823.

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

Fraenkel-Conrat, H. (1951), *in* Amino Acids and Proteins, Greenberg, D. M., Ed., Springfield, Ill., C. C Thomas, Chapter IX.

Fredericq, E., and Deutsch, H. F. (1949), *J. Biol. Chem. 181*, 499.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 3418.

Klotz, I. M., and Franzen, J. S. (1962), *J. Am. Chem. Soc. 84*, 3461.

Kronman, M. J., Cerankowski, L., and Holmes, L. G. (1965), *Biochemistry 4*, 518.

Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526.

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

Lineweaver, H., and Murray, C. W. (1947), *J. Biol. Chem. 171*, 565.

Melamed, M. D. (1966), *in* Glycoproteins, Gottschalk, A., Ed., Amsterdam, Elsevier, p 317.

Melamed, M. D. (1967), *Biochem. J. 103*, 805.

Mihalyi, E. (1965), *Arch. Biochem. Biophys. 110*, 325.

Monsigny, M., and Montreuil, J. (1966), *Compt. Rend. 262*, 1780.
Montgomery, R., and Wu, Y.-C. (1963), *J. Biol. Chem. 238*, 3547.
Montreuil, J., Castiglioni, B., Adam-Chosson, A., Caner, F., and Queval, J. (1965), *J. Biochem. (Tokyo) 57*, 514.
Ray, A., Reynolds, J. A., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2606.
Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6*, 937.
Schellman, J. A. (1955), *Compt. Rend. Trav. Lab. Carlsberg 29*, 223.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Stevens, F. C., and Feeney, R. E. (1963), *Biochemistry 2*, 1346.
Tanford, C. (1955), *J. Phys. Chem. 59*, 798.
Teale, F. W. J. (1960), *Biochem. J. 76*, 381.
Teale, F. W. J., and Weber, G. (1957), *Biochem. J. 65*, 476.
Tomimatsu, Y., and Gaffield, W. (1965), *Biopolymers 3*, 509.
Turner, G. K. (1964), *Science 146*, 183.
Weber, G., and Rosenheck, K. (1964), *Biopolymers Symp. No. 1*, 333.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.
Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.

Synthesis of Selenium-Containing Peptides*

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ABSTRACT: A general and convenient method for the synthesis of Se-benzyl-L-selenocysteine derivatives, including Se-benzyl-L-selenocysteine peptides, is described. The method involves the nucleophilic displacement of the *O*-tosyl moiety in *O*-tosylated serine derivatives and *O*-tosylated serine residues within peptides by sodium benzyl selenolate. The applicability of this method was demonstrated in the synthesis of the protected C-terminal tetrapeptide of selenooxytocin and selenoglutathione. This work suggests the feasibility of replacing serine residues with selenocysteine residues in other biologically significant peptides.

In connection with our investigations (Walter and Chan, 1967; Theodoropoulos *et al.*, 1967) concerning the replacement of sulfur by selenium moieties within biologically active peptides and proteins, it became apparent that a general method for the synthesis of optically active selenium-containing amino acids and peptides had to be developed. Adopting Painter's (1947) synthesis of Se-benzyl-DL-selenocysteine, Frank (1964a) recently prepared the methyl Se-benzyl-L-selenocysteinate. The carboxyl group of this ester was liberated hydrolytically by treatment with boiling aqueous hydrochloric acid, thus providing the Se-benzyl-L-selenocysteine. In view of the necessity for such drastic reaction conditions it is not surprising that we and others[1] were unable to repeat this preparation of Se-benzyl-L-selenocysteine with consistent results.

In contrast, a consistent and convenient procedure for the preparation of Se-benzyl-L-selenocysteine is provided by the nucleophilic displacement of the *O*-*p*-toluenesulfonate moiety of L-serine by the benzyl selenolate anion. This method has the additional advantages of operating under very mild reaction conditions and of being applicable to the synthesis of Se-benzyl-L-selenocysteine compounds which bear selectively removable protecting groups. It should be noted that the susceptibility of the *O*-*p*-toluenesulfonate group of *O*-tosylated derivatives toward nucleophiles was demonstrated in the course of the synthesis of L-cysteine derivatives and peptides (Zervas and Photaki, 1960; Photaki, 1963; Photaki and Bardakos, 1965; Zioudrou *et al.*, 1965).

In preliminary experiments we have found that benzyl *N*-carbobenzoxy-*O*-tosyl-L-serinate indeed re-

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