Indicator Dyes as Probes of Electrostatic Potential Changes on Macromolecular Surfaces†

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ABSTRACT: An indicator dye attached to an electrostatically charged macromolecular surface generally has a pK value (pK_b') different from that of uncombined dye (pK_f'). The question if changes in (pK_b' − pK_f'), designated as $\Delta\Delta$pK, records changes in the electrostatic potential at the binding site has been examined in spectrophotometric and binding experiments, using the interaction of Chlorophenol Red and Phenol Red with human serum albumin and cationic micelles as examples. (1) In serum albumin solutions $\Delta\Delta$pK is decreased by a reduction of pH. The decrease is correlated with the increase in positive charges on the protein molecule, and the response is attenuated by high ionic strength in accordance with electrostatic theory. (2) Opposite changes in binding affinity to serum albumin and $\Delta\Delta$pK as a function of pH are observed; the binding of basic (bivalent anion) dye is more susceptible to a change in pH than is the acidic (univalent anion) form. (3) Preferential uptake of the basic as compared to the acidic form of dye is observed by binding to cetyltrimethylammonium chloride and cetylpyridinium chloride micelles (μ = 0.033, [Cl^-] = 0.033 M). An increase in the ionic strength is accompanied by a positive value of $\Delta\Delta$pK. The results are consonant with the view that the observed $\Delta\Delta$pK values reflect changes in the electrostatic potential at the binding site with consequently little, if any, effect on the intrinsic pK. The extension of the method to measure changes in the electrostatic potential at binding sites on cell membranes is briefly discussed.

Binding of indicator dyes by macromolecular particles such as proteins and ionic micelles is often accompanied by a color change, resulting from a difference in the proportion of the acidic and basic form of bound dye, compared with that existing in the surrounding solution. The apparent pK of protein–dye complexes is pH dependent, suggesting that electrostatic interactions of bound dye with the macromolecule contribute to the changes in the protrotropic equilibria (Danielli, 1937; Kragh-Hansen and Moller, 1973b). The pK changes observed by uptake of indicator dyes by ionic micelles are also consonant with an effect of electrostatic interactions on this process (Hartley, 1934; Hartley and Roe, 1940). These findings suggest the use of indicator dyes as spectroscopic probes, for instance in the evaluation of electrostatic interactions between ligands bound on the same macromolecule. Indicator dyes may also be useful as spectroscopic probes in the investigation of membrane phenomena. At the present time fluorescent dyes are mostly used for this purpose. The attractive prospect of including indicator dyes in such investigations stems from the possibility of using them to monitor the movement of electrostatic charges accompanying important membrane phenomena like nerve conductance and transport of inorganic ions. Generation of membrane potentials also appears to be important in mitochondrial energy metabolism (Mitchell, 1966; Liberman and Skulachev, 1971) and in the active uptake of many organic substances by bacterial membranes (West and Mitchell, 1972; Hirata et al., 1974).

Before attempting to apply indicator dyes as probes on complicated systems such as cell membranes it is important to establish if pK changes do reflect changes in the electrostatic potential at the binding site. An evaluation of this kind is possible by subjecting a simple system like a protein or a micelle dissolved in an aqueous electrolyte medium to changes in electrolyte composition and pH which will lead to predictable effects on the electrostatic potential at the surface of the macromolecular ion. A further check is possible by relating the observed pK values to changes in binding as described in one of our previous papers on the interaction of Phenol Red with human serum albumin (Kragh-Hansen and Moller, 1973b). In this paper we report the result of more extensive experiments in which we have studied the uptake of Chlorophenol Red and Phenol Red by proteins and cationic micelles. The results indicate that although other factors than electrostatic interactions affect the pK value of bound dye, changes of this parameter induced by various manipulations of pH and ionic strength are adequately described by the ensuing changes in the electrostatic potential.

Theory

The change in the proportion of the acidic and basic form of an indicator dye after combination with a macromolecular particle can be represented by

$$\Delta pK = pK_b' - pK_f' = (pK_{int,b} - pK_f') + 0.434\psi\Delta Z_D/kT \quad (1)$$

Here ΔpK is the difference between the apparent pK values of bound and free dye, pK_b' and pK_f'. The other symbols in the equation are as follows: p$K_{int,b}$, representing the intrinsic (or thermodynamic) dissociation constant of bound dye; ψ, the electrostatic potential at the location of the prototropic group of the indicator dye; ΔZ_D, the difference in the valency of the electrostatic charge of the acidic and basic form of the indicator dye which is −1 electronic unit; k, Boltzmann's constant; T, the absolute temperature. It should be noted that pK_b' = p$K_{int,b}$ + $0.434\psi\Delta Z_D/kT$, the last term measuring the electrostatic interactions between dye and macromolecular particle. For theoretical calculations of the electrostatic interactions of the surface of pro-

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teins $0.434\psi\Delta Z_D/kT$ is often replaced by $0.868w\Delta Z_D Z_p$, where w is the electrostatic interaction factor and Z_p is the average net charge of the protein (Tanford, 1961). An overall value of w may then be calculated on the basis of the Debye-Hückel theory of electrostatic interactions as adapted to proteins by Linderstrom-Lang (1924), assuming that the net charge is evenly distributed over the protein surface and that the protein can be represented by a sphere

$$W = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \quad (2)$$

where e is the electronic charge unit, D is the dielectric constant of the solution, a is the radius of the protein, b is the distance between the center of the protein and bound ligand, and κ has the same meaning as in the Debye-Hückel theory. The calculation of w does not take into account the effect of particular configurations of positive and negative charges that may exist at different locations of the protein surface. However, eq 2 will be used in this paper to calculate approximately the effect of removal or uptake of protons and Cl^- by human serum albumin on the electrostatic surface potential. Notwithstanding the approximate nature of the assumptions underlying eq 2 (Tanford and Roxby, 1972), evidence has been obtained in the case of serum albumin that w values calculated in this way give quantitatively correct results as indicated by the increase of pH of isoionic serum albumin solutions in the presence of Phenol Red (Kragh-Hansen and Moller, 1974).

From an experimental standpoint ΔpK of eq 1 can be calculated by the use of the Henderson-Hasselbalch equation from the pH of the solution and from spectrophotometric measurements of the absorbances of the acidic and basic form of free and combined dye (Kragh-Hansen and Moller, 1973b). The right side of eq 1 then contains two unknowns, $pK_{int,b}$ and ψ, which are not in general subject to independent measurements. However, a perturbation of the system primarily leading to a change in the electrostatic interactions may not affect $pK_{int,b}$ appreciably. As an example suppose that the electrostatic potential at a dye bound to the lipid bilayer of a cellular membrane assumes a new value as a result of changes in the potential difference between the two sides of the membrane. Or that a charged ligand is bound on the macromolecular particle in the vicinity of the dye binding site. Experimentally the new value of the electrostatic potential at the prototropic group of the indicator dye should then be discernible as a change in ΔpK according to

$$\Delta pK_1 - \Delta pK_o = 0.434(\psi_1 - \psi_o)\Delta Z_D/kT \quad (3)$$

where subscript o refers to the original state, and subscript 1 to the perturbed state. Equation 3 may also be formulated as

$$\Delta\Delta pK = -0.434e\Delta\psi/kT \quad (3a)$$

inserting $\Delta Z_D = -1$ e (electronic charge unit) and letting $\Delta\Delta pK$ and $\Delta\psi$ designate the change in ΔpK and electrostatic potential induced by the ligand. It will be noted that the underlying assumption of eq 3 and 3a is that ($pK_{int,b} - pK_f$) remains constant. It is the purpose of this paper to discuss the practical usefulness of eq 3 on the basis of experiments which primarily are expected to affect the electrostatic interactions of bound indicator dye.

Materials and Methods

Protein Preparations and Other Reagents. Human serum albumin (lyophilized, grade A) was obtained from AB Kabi, Sweden. The protein was deionized by chromatographing aqueous solutions of it on a mixed-bed resin (AG 501-X8, Bio-Rad). The eluted material was freeze-dried and stored in a desiccator at 4° before use.

The micelle forming substances cetyltrimethylammonium chloride (CTAC)[1] and cetylpyridinium chloride (CPC) were obtained from Eastman Kodak Co. Rochester, N.Y., and Fluka A.G., Switzerland, respectively, and were used without further purification. The indicator dyes Phenol Red, Chlorophenol Red, and Bromothymol Blue were from Merck A.G., Darmstadt, W. Germany. Methylphenol Red was synthesized from Phenol Red according to the method described by Orndorff and Sherwood (1923). No change in the absorption curve of the synthetized compound was observed in the pH interval 4–10, indicating that the preparation was not contaminated by Phenol Red. The inorganic salts used in this study were all analytical grade.

Interaction Studies. Among the various phenolsulfophthalein dyes tested only Phenol Red and Chlorophenol Red proved to be suitable as candidates for measurements of changes in the electrostatic interactions. Most of the experiments reported in this paper were carried out with Chlorophenol Red, since the characteristics of the interaction of Phenol Red with serum albumin and other proteins have already been published (Kragh-Hansen and Moller, 1973a,b). In most cases the concentration of both dye and serum albumin was 0.070 mM, assuming a molecular weight of the protein of 69,000. Sodium salts of phosphate and Cl^- were used as supporting electrolyte as mentioned in connection with the individual experiments. Protein-free solutions having the same concentration of dye and pH were termed reference solutions. After accurate checking and adjustment of the pH of protein and reference solutions, spectral curves were drawn immediately at 25° on a Unicam SP 1800 spectrophotometer equipped with a Unicam AR 25 Linear recorder. The binding of dye in the presence of protein was determined at 25° by ultrafiltration as previously described (Kragh-Hansen et al., 1972). The absorbances of 100% bound dye, A_B, i.e., the absorbances which would have been observed by complete binding of dye to the protein, were calculated on the basis of the following equation

$$A_B = (A_P - fA_R)/(1 - f) \quad (4)$$

where A_P and A_R are the measured absorbances at any given wavelength of the protein and reference solution, respectively, and f is the filtrable fraction of dye in the protein solution. The equation does not include a correction for the Donnan effect on the filtered fraction of dye. Such a correction would be insignificant because of the rather tight binding of Chlorophenol Red by serum albumin.

Experiments on the uptake of Phenol Red and Chlorophenol Red by CTAC and CPC micelles were performed in a similar way as the protein experiments. The concentration of dye was 0.070 mM, and those of CTAC and CPC 0.70 and 0.38% (w/v), respectively. These concentrations aimed at producing a molar concentration of micelles similar to that of the dye and were based on a molecular weight of CTAC micelles of 100,000 (Birdi, 1972) and 54,000 for CPC micelles (Birdi, personal communication). The micellar solutions contained 0.033 M Tris (tris(hydroxymeth-

[1] Abbreviations used are: CTAC, cetyltrimethylammonium chloride; CPC, cetylpyridinium chloride; Tris, tris(hydroxymethyl)aminomethane.

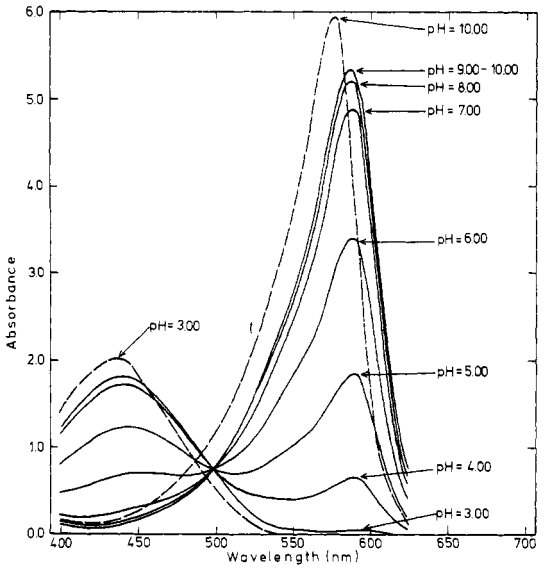

FIGURE 1: Effect of pH on the spectral curves of Chlorophenol Red in the presence and absence of human serum albumin. The solid lines illustrate the spectral curves of bound dye as calculated from absorption and binding measurements according to eq 4. The dashed lines represent absorption measurements in the absence of serum albumin at pH 3 and 10. Chlorophenol Red concentration, 0.070 mM; serum albumin concentration, 0.070 mM. The medium contained 0.033 M sodium phosphate. Temperature, 25°. Absorbances have been converted to a path length of 10 mm.

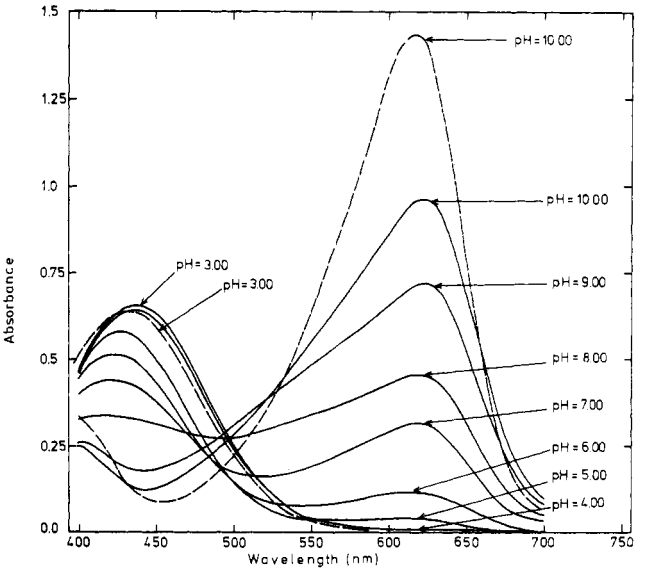

FIGURE 2: Spectral curves of Bromothymol Blue bound to human serum albumin (—) at different pH in 0.033 M sodium phosphate (25°). Bromothymol Blue concentration, 0.036 mM; serum albumin concentration, 0.36 mM. The dashed lines show the curves of uncombined Bromothymol Blue at pH 3 and 10. Absorbances have been converted to a path length of 10 mm.

yl)aminomethane), and K^+ and Cl^- to produce ionic strengths of either 0.033, 0.233, or 4.233.

H^+ Titration Curves. The protonic charge of human serum albumin as a function of pH was determined in 0.005 M NaCl by the procedure previously described (Lind et al., 1974).

Results

Spectrophotometric Data on the Interaction of Phenolsulfophthalein Dyes with Human Serum Albumin. An example of the spectral curves of Chlorophenol Red combined with serum albumin at different pH values is shown in Figure 1. The electrolyte medium was in this case 0.033 M phosphate, and the pK value for the interconversion of the acidic (univalent anion) and the basic (bivalent anion) form of the indicator dye is 6.15 at 25° in the reference media, i.e., in the absence of protein. The dashed curves showing the spectral curves in the reference solutions at pH 3 and 10 therefore represent the acidic and basic component of the indicator dye, respectively. The curves of bound Chlorophenol Red at the same pH values are characterized by a lower A_{max} and a higher λ_{max}. It should be noted that the spectral curves of bound indicator dye obtained at different pH have an isosbestic point at around 500 nm. This finding suggests that the perturbation of the spectral curve of the acid and basic component of the indicator dye, induced by binding to serum albumin, is not appreciably affected by pH. However, λ_{max} of the basic peak is slightly reduced at high pH (589 nm at pH 5, and 586 nm at pH 10). But the sum of the concentration of acidic and basic component when calculated on the basis of the maximal absorption coefficient at pH 3 and 10 turns out to be constant at all pH values. This presumably means that the maximal absorption coefficient of acidic and basic component is not affected by pH. Similar results were previously obtained in the case of Phenol Red (Kragh-Hansen and Moller, 1973b). The constancy of the spectral curves of the acidic component at low pH is rather remarkable since the conformation of serum albumin changes markedly here: at pH values of 4–4.5 the N → F transition occurs, and at pH values below 4 an expansion of the whole molecule sets in (Foster, 1960).

In contrast to Chlorophenol Red and Phenol Red, the spectral curves of other phenolsulfophthalein dyes (Bromothymol Blue and Bromocresol Purple) reveal marked pH dependent changes in the shape of the acidic and basic component of the spectral peak. As an example consider Figure 2 in which we have depicted the spectral curves of Bromothymol Blue as a function of pH. In this case no isosbestic point is observed, and the spectral curve at, e.g., pH 8, is characterized by a very shallow trough between the acidic and basic peak which clearly cannot be formed by superposition of the spectral curves observed at the extreme pH values. Since a constant value for the absorption coefficients at different pH of both acidic and basic component is a prerequisite for calculation of pK values of bound dye, Chlorophenol Red and Phenol Red were the only dyes used for further spectrophotometric experiments in this study.

Table I summarizes pK differences between bound and free Chlorophenol Red in media of different pH and electrolyte composition. The figures in parentheses show that at pH 7.0 the values of ΔpK ($pK_b' - pK_f'$) range from 0.10 to −0.33, depending on the electrolyte composition and ionic strength of the medium. The remaining figures in the table show changes of ΔpK ($\Delta\Delta pK$) at other pH values, using pH 7.0 as the reference state. It is seen that $\Delta\Delta pK$ assumes increasingly negative values as the pH is lowered. This is as predicted from electrostatic theory since the increase in the net charge of the protein at low pH values should result in preferential binding of the basic component of Chlorophenol Red. The composition of the electrolyte medium affects the response of the dye to a lowering of pH, the most pronounced changes of ΔpK being observed in media of low ionic strength (compare 0.005 M phosphate with 0.033 M phosphate, and the different NaCl solutions). These obser-

Table I: Effect of pH on $\Delta\Delta pK$ of Chlorophenol Red in Different Electrolyte Media at 25°.

	$\Delta\Delta pK$[a]				
pH	Sodium Phosphate 0.005 *M*	Sodium Phosphate 0.033 *M*	NaCl 0.005 *M*	NaCl 0.150 *M*	KCl 2 *M*
7.0	0 (−0.23)[b]	0 (−0.19)[b]	0 (−0.33)[b]	0 (−0.33)[b]	0 (0.10)[b]
6.0	−0.32	−0.22	−0.23	−0.09	−0.06
5.0	−0.83	−0.68	−0.67	−0.20	−0.09
4.0	−1.35	−1.09	−1.22	−0.30	[c]

[a] Taking pH 7.0 of the different electrolyte media as the reference state. [b] Figures in parentheses refer to ΔpK values at pH 7.0. [c] Not determined, due to precipitation of serum albumin.

Table II: Calculated $\Delta\Delta pK$ Values of Chlorophenol Red Bound to Human Serum Albumin in 0.005 *M* NaCl.

pH	ΔZ_p[a]	$\Delta\psi_{calcd}$[b] (mV)	$\Delta\psi_{obsd}$[c] (mV)
7.0	0	0	0
6.0	5.6	21	14
5.0	10.4	38	40

[a] Calculated as $(\Delta\bar{\nu}_{H^+} - \Delta\bar{\nu}_{Cl^-})$, taking pH 7.0 as the reference state, with $\bar{\nu}_{H^+}$ and $\bar{\nu}_{Cl^-}$ indicating the average number of moles of H^+ and Cl^- bound per mole serum albumin. $\bar{\nu}_{H^+}$ was estimated from H^+ titration data, and $\bar{\nu}_{Cl^-}$ from the data of Scatchard and Yap (1964). [b] $\Delta\psi$ (millivolts) calculated according to eq 3a, using a value of −59.2 mV for the conversion factor $(-kT/0.434e)$ at 25°; $\Delta\Delta pK$ calculated as $-0.868w\Delta Z_p$. The value of the electrostatic interaction factor, w, as determined from eq 2 was 0.072. [c] Experimental data from Table I, using a value of −59.2 mV for the conversion factor $(-kT/0.434e)$ at 25°.

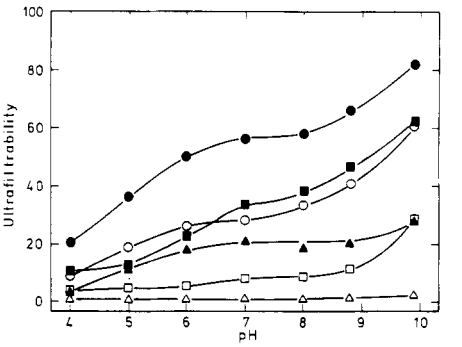

FIGURE 3: Effect of pH on ultrafiltrability of various phenolsulfophthalein dyes in the presence of human serum albumin. The dyes were: Phenol Red (●, ○); Methylphenol Red (▲, △); Chlorophenol Red (■, □). The dye/serum albumin concentrations were: (●) 0.35 m*M*/0.090 m*M*; (○) 0.35 m*M*/0.36 m*M*; (▲) 0.68 m*M*/0.18 m*M*; (△) 0.34 m*M*/0.36 m*M*; (■) 0.28 m*M*/0.070 m*M*; (□) 0.070 m*M*/0.070 m*M*. The medium contained 0.033 *M* sodium phosphate. Temperature, 25°.

vations are also as expected for two reasons: (1) the change in net charge of serum albumin is smaller at high ionic strength due to binding of anions by the protein, and (2) the electrostatic interactions between macromolecule and ligand are attenuated at high ionic strength according to eq 2.

In addition to these qualitative considerations we have attempted to calculate the expected magnitude of the change of the surface potential ($\Delta\psi$) in the case of the 0.005 *M* NaCl experiments (Table II). The change in net charge of the protein, ΔZ_p, was evaluated from titration curves of human serum albumin in 0.005 *M* NaCl, and from literature data on the binding of Cl^- by human serum albumin (Scatchard and Yap, 1964). The electrostatic interaction factor, w, was calculated according to eq 2, using values of b = 30 Å and a = 32.5 Å (Scatchard and Yap, 1964). It is seen from the table that the $\Delta\psi$ values calculated in this way agree well with those obtained experimentally. We consider literature data on Cl^- binding at 0.15 *M* KCl to be too unreliable to permit a similar calculation. However, on the basis of the empirical determination of w from the titration curve reported by Tanford et al. (1955) one would expect that the electrostatic interactions at 0.15 *M* KCl are about ⅓ of those at the low ionic strength. A reduction of $\Delta\Delta pK$ of this order at 0.15 *M* KCl is apparent from Table I. Thus, $\Delta\Delta pK$ reflects the expected change in surface potential as far as can be ascertained from this kind of analysis.

Comparison of Protein Binding and ΔpK of Phenolsulfophthalein Dyes. Changes in the electrostatic potential at the binding site, apart from resulting in variations in ΔpK of protein-bound dye, should also manifest themselves as changes in the affinity of the dye for protein. Results obtained on the ultrafiltrability of Phenol Red, Chlorophenol Red, and Methylphenol Red in serum albumin solutions as a function of pH are assembled in Figure 3. The experiments were carried out at two different dye and serum albumin concentrations as described in the legend of the figure. In all cases ultrafiltrability increases as pH is raised, indicating less binding of the dyes at high pH values. However, the curves are characterized by a plateau region at pH 7–8. Chlorophenol Red is bound more strongly than Phenol Red, but due to the different concentrations of dye and protein employed two of the binding curves are comparable. The only noticeable difference between the two comparable curves is that a jump in binding of Chlorophenol Red occurs at pH 7–5, followed by a levelling off at lower pH values. Methylphenol Red, in which one of the hydroxyl functions of Phenol Red is blocked by O-methylation, is bound by serum albumin with a much higher affinity than is Phenol Red. Methylphenol Red only exists in the univalent anion form at pH 4–10, and it is interesting to note that the decrease of binding of this compound in alkaline media is much less pronounced than in the case of Phenol Red and Chlorophenol Red. Although caution should be exercised when comparing the binding behavior of dyes, even if they are structurally similar, the fact that binding of Phenol Red and Chlorophenol Red at pH 8–10 is affected to a larger extent than Methylphenol Red definitely suggests a negative electrostatic potential at the binding sites in this pH interval. A negative electrostatic potential would have a more pronounced repellant effect on Phenol Red and Chlorophenol Red than on Methylphenol Red, since the former compounds predominantly exist in the bivalent anion form at pH 8–10. Conversely, the relatively higher binding of Chlo-

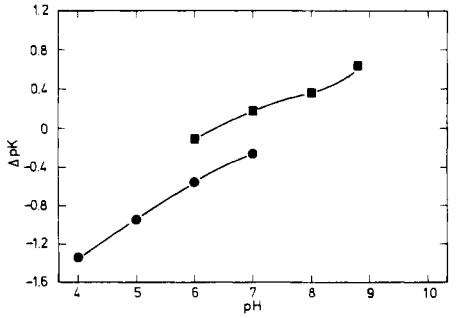

FIGURE 4: Effect of pH on ΔpK for Chlorophenol Red and Phenol Red bound to human serum albumin. (●) Results in 0.033 *M* sodium phosphate as given in Table I. (■) Similar results for Phenol Red, taken from Kragh-Hansen and Moller (1973b).

rophenol Red at pH 7–5 is consonant with a positive electrostatic potential at pH <7.

p*K* values of Phenol Red and Chlorophenol Red in 0.033 *M* phosphate are shown in Figure 4. The measurement of ΔpK values for a particular dye is limited to 3–4 pH units, because the pH must be rather close to pK_b' for accurate measurement of both acidic and basic component of the dye. However, by combining the results obtained on Chlorophenol Red (Table I) with those previously reported on Phenol Red (Kragh-Hansen and Moller, 1973b) it is possible to depict ΔpK changes in the pH interval 4–9. Figure 4 shows a parallel course of the two curves in the common region at pH 6–7. The difference may be attributed to different values of ($pK_{int,b} - pK_f'$) of the two indicator dyes. However, ΔpK in both cases is close to zero at pH 7–8. Since the electrostatic potential apparently also changes sign at pH 7–8 it would appear that $pK_{int,b}$ does not differ substantially from pK_f'. The small ΔpK values in 2 *M* KCl as indicated by Table I also support this conclusion, since the effect of electrostatic interactions on pK_b' is minimized at high ionic strength.

Spectrophotometric Data on the Uptake of Chlorophenol Red and Phenol Red by Cationic Micelles. The spectral curves of Chlorophenol Red at different pH in the presence of CTAC is depicted in Figure 5. Ultrafiltration studies indicated that both Chlorophenol Red and Phenol Red were almost completely taken up by CTAC and CPC (binding percentages higher than 99%) under the conditions of these experiments. It appears from the figure that λ_{max} of the basic peak is increased by incorporation into the micelles while a blue shift is observed for the acidic peak compared to that of free Chlorophenol Red. Furthermore, an isosbestic point is observed around 485 nm. Very similar results were obtained for the uptake of Phenol Red by CTAC micelles, and also for the uptake of both dyes by CPC micelles (not shown).

A summary of the ΔpK values of Chlorophenol Red and Phenol Red in different electrolyte media is shown in Table III. All media contained Tris-KCl (μ = 0.033), supplemented as indicated by further addition of KCl to raise the ionic strength to 0.233 or 4.033. The ΔpK values in a given electrolyte medium were independent of pH, which is to be expected, since the cationic groups of the detergents are not titrated in the pH interval 4–10. Table III shows that at an ionic strength of 0.033, negative values of ΔpK are obtained for both Chlorophenol Red and Phenol Red. At μ = 0.233 a distinct increase of the ΔpK values is observed, agreeing with the reduction of the electrostatic potential at the surface of the micelle that must occur by an increase of the

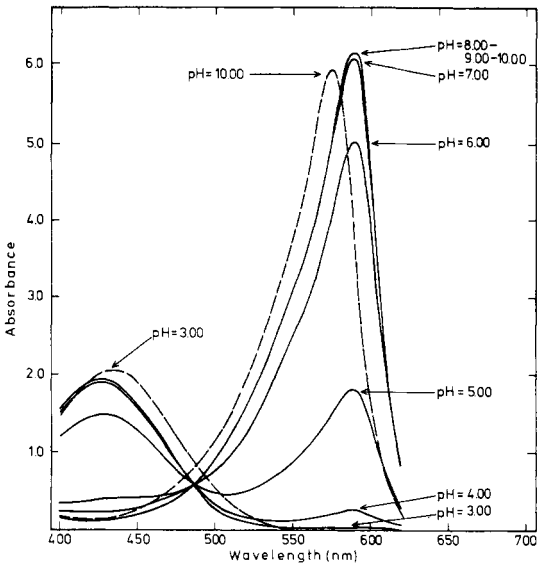

FIGURE 5: Spectral curves of Chlorophenol Red bound to CTAC micelles (—) at different pH in Tris-Cl (μ = 0.033). Chlorophenol Red concentration, 0.070 m*M*; CTAC concentration, 0.70% (w/v). The dashed lines show the curves of uncombined Chlorophenol Red at pH 3 and 10. Temperature, 25°. Absorbances converted to a path length of 10 mm.

Table III: p*K* Changes Accompanying the Uptake of Phenol Red and Chlorophenol Red by CTAC and CPC Micelles at 25°.

Dye	Micelle	Ionic Strength[a]	ΔpK^b	$\Delta\Delta pK^c$
Phenol Red	CTAC	0.033	−0.58	0
	CTAC	0.233	0.24	0.82
	CTAC	4.033	1.08	1.66
Chlorophenol Red	CTAC	0.033	−0.69	0
	CTAC	0.233	−0.03	0.66
	CTAC	4.033	0.85	1.54
Phenol Red	CPC	0.033	−0.68	0
	CPC	0.233	0.12	0.80
Chlorophenol Red	CPC	0.033	−0.99	0
	CPC	0.233	−0.22	0.77

[a] The ionic strength given in the table refer to media of the following composition: ionic strength 0.033; 0.033 *M* Tris-KCl as described under Materials and Methods; ionic strength 0.233: Same medium as at ionic strength 0.033 *plus* 0.2 *M* KCl; ionic strength 4.033, same medium as at ionic strength 0.033 *M plus* 4 *M* KCl. [b] The values given are based on measurements at pH 7.0 and 8.0 for Phenol Red, and at pH 5.0 and 6.0 for Chlorophenol Red. [c] The values refer to changes of ΔpK, using the 0.033 ionic strength medium as the reference state.

electrolyte concentrations of the solutions. ΔpK of Chlorophenol Red is consistently below that of Phenol Red. However, the last column of Table III indicates only small differences in $\Delta\Delta pK$ of the two dyes, taking μ = 0.033 as the reference state. In the case of CTAC it proved possible to keep the detergent in solution in the presence of 4 *M* KCl, while CPC micelles were "salted-out" under these conditions. Uptake of the dyes by CTAC micelles in 4 *M* KCl is accompanied by large positive values for ΔpK. Therefore in all likelihood $pK_{int,b}$ is larger than pK_f' following uptake by cationic micelles.

Discussion

The spectroscopic properties of Phenol Red and Chlorophenol Red combined with human serum albumin and cat-

ionic micelles as reported here and in a previous paper (Kragh-Hansen and Moller, 1973b) show that these dyes probably can be used to monitor changes in the electrostatic potential at binding sites on macromolecular surfaces. The results suggest that $pK_{int,b}$ of these dyes is not appreciably affected by changes in pH and ionic strength. The same conclusion regarding the electrostatic origin of ΔpK changes has been drawn by Fromherz (1973) and Fromherz and Masters (1974) in studies on the uptake of the fluorescent dye, 4-heptadecylumbelliferone, in monomolecular layers of a lipid nature. However, larger differences between $pK_{int,b}$ and pK_f' were reported in these studies than is apparent from the results obtained on the interaction of Phenol Red and Chlorophenol Red with human serum albumin and cationic micelles.

The applicability of phenolsulfophthalein dyes in the study of membrane phenomena has in particular been examined in mitochondrial preparations. The effect of pH on ΔpK of Bromothymol Blue following conversion of mitochondria from a nonenergized to an energized state were discussed by Chance and Mela (1966) and by Mitchell et al. (1968). Other studies suggest that changes in ΔpK also occur in the absence of pH changes (Jackson and Crofts, 1969; Colonna et al., 1972), but the nature of these alterations remains to be clarified. Spectroscopic perturbations of Bromocresol Purple bound to sarcoplasmic reticulum of skeletal muscle have also been observed following interaction of the membranes with Ca^{2+} and ATP (Nakamaru and Schwartz 1973; Nakamaru and Sugii, 1974). Our own experience on the interaction of serum albumin with alkylated phenolsulfophthalein dyes such as Bromothymol Blue and Bromocresol Purple indicates that the spectral peaks of the acidic and basic forms are very susceptible to conformational changes of the protein. By contrast the absorption spectrum of the acidic form of Chlorophenol Red and Phenol Red is not significantly altered by the drastic expansion of the albumin molecule that occurs by lowering of the pH of the protein solution to 3. It would appear that Chlorophenol Red and Phenol Red are more likely candidates as probes of changes in the electrostatic potential of cellular membranes than alkylated phenolsulfophthalein dyes.

The question whether indicator dyes would sense the potential difference across a cellular membrane or whether they would rather indicate localized charge changes may depend on experimental conditions and cannot be evaluated now. It is of interest in this connection that Cohen et al. (1974) have found that the fluorescence intensity of several dyes changes in parallel with the membrane potential in the squid axon. Fluorescent compounds like 8-anilino-1-naphthalenesulfonate and cyanine dyes have also by other authors been shown to respond to the potential across the squid axon (Tasaki et al., 1972) and *Amphiuma* erythrocytes (Hoffman and Laris, 1974). The basis for the fluorescence changes is uncertain, but an important factor may be variations in the extent of binding in relation to the membrane potential (Cohen et al., 1974). In the case of cyanine dyes Sims et al. (1974) have proposed that formation of dye aggregates during hyperpolarization may play an important role for the fluorescence changes observed under these conditions. Whatever the exact nature of polarization dependent fluorescence changes it is evident that the response reflects changes in the state of the dye which are only indirectly related to the transmembranal potential. On the other hand, suitable indicator dyes would appear to afford a more direct measure of electrostatic potential differences. For the purpose of using indicator dyes to measure changes in this parameter in cellular membranes the distribution of the dye between the two sides of the membrane, the effect of transmembranal pH differences, and the nature of the binding sites have to be taken into account.

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Electron Microscopy and Physical Characterization of the Carcinoembryonic Antigen†

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ABSTRACT: Carcinoembryonic antigen (CEA), a glycoprotein material purified from human tumors, has been visualized by electron microscopy. At neutral pH, it consists largely of relatively homogeneous, morphologically distinctive twisted rod or cruller shaped particles, with dimensions 9 × 40 nm. The particle length is considerably diminished at pH 4.0, which correlates with a known diminution of charge. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated a molecular weight of 180,000 in the peak region of the CEA band for both 10 and 15% acrylamide. When native CEA was treated with neuraminidase, reduced, and alkylated, a relatively compact random coil was produced, whereas reduction and alkylation without neuraminidase treatment produced a less compact random configuration, as determined by sedimentation studies and by electron microscopy. Electrophoretic migration, however, was apparently unaffected by reduction and alkylation. Thus the characteristic CEA particle appears by several lines of evidence to be substantially folded into a recognizable tertiary structural arrangement.

Carcinoembryonic antigen (CEA)[1] is a macromolecular glycoprotein antigen isolated from adenocarcinomas of the digestive system (Gold and Freedman, 1965a,b). The CEA produced by tumor cells passes into the circulation where it can be detected by radioimmunoassay (Thomson et al., 1969; Egan et al., 1972). These findings suggested that CEA might be of value in the diagnosis of cancer. The literature in this area has been reviewed by Terry et al. (1974).

Although the carbohydrate composition of CEA varies for different preparations (Terry et al., 1974), the amino acid composition is quite constant for the approximately one-third of the molecule which is protein (Terry et al., 1972, 1974). CEA has a sedimentation coefficient of 6.8 S (Coligan et al., 1972).

Since a molecule of this size is amenable to molecular electron microscopic techniques, we attempted to characterize preparations of this material morphologically, using techniques recently applied to other glycoproteins (Slayter and Codington, 1973; Slayter et al., 1974). At the same time, in order to obtain precise information about size and physical dispersity, sedimentation and electrophoretic studies were undertaken on the same material utilized for electron microscopy. Native, neuraminidase treated, and reduced and alkylated materials were analyzed in the study reported here.

Materials and Methods

Preparation of CEA. CEA used in these studies was purified as described by Coligan et al. (1972).

Radioimmune Assay for CEA. CEA was measured by double antibody radioimmune assay (Egan et al., 1972; Egan, 1974).

Neuraminidase Digestion. Vibrio cholerae neuraminidase (500 units/ml; 1.6 units/μg; General Biochemicals, Chagrin Falls, Ohio) was added to lyophilized CEA in a ratio of 1 unit/35 μg of CEA. For each milliliter of neuraminidase, 100 μl of sodium citrate buffer (0.2 *M* sodium citrate–0.01 *M* $CaCl_2$, pH 5.5) was added. After approximately 48 hr at 37°, the reaction mixture was dialyzed against deionized H_2O at 4°, lyophilized, and stored at 4°.

Amino acid analysis was performed on CEA samples that had been hydrolyzed with *p*-toluenesulfonic acid by the method of Liu and Chang (1971). A Beckman Model 121 H amino acid analyzer was used.

Carbohydrate Analysis. Values for neutral sugars were obtained by the gas chromatographic procedure of Clamp et al. (1972). Amino sugars were determined on the amino acid analyzer during the amino acid analysis. Sialic acid was measured by the method of Warren (1959).

Reduction and Alkylation. Samples to be reduced and alkylated were dissolved at a concentration of 6–7 mg/ml in 0.1 *M* Tris-HCl buffer (pH 8.3) containing urea (9 *M*) and EDTA (2 m*M*). After deaeration with a stream of nitrogen (30 min), sufficient 1.0 *M* dithiothreitol was added to make the concentration 10 m*M*. After 4 hr at room temperature under a stream of nitrogen, the reaction mixture was cooled

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[1] Abbreviations used are: CEA, carcinoembryonic antigen; the subscripts mi and na after CEA designate the individuals from whose tumors the CEA was isolated. Both preparations were isolated from liver metastases of primary adenocarcinomas of the colon. These preparations are chemically very similar, but not identical, although they are antigenically indistinguishable (Terry et al., 1974).