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THE EFFECTS OF pH AND TEMPERATURE ON THE CIRCULAR DICHROISM OF HUMAN ERYTHROCYTE MEMBRANES *

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The effects of pH and temperature on the structure of human erythrocyte membranes were studied by circular dichroism (CD). The results obtained demonstrate that the membrane CD spectra undergo significant changes when the pH of the solution deviates from its native pH range of 7 to 8. Spectral changes in the acidic pH region include drastic reductions and slight shifts in the CD signal which may reflect a decrease in α-helical content of the proteins and/or an increase in optical artifacts, both of which are irreversible. In the alkaline pH region, dramatic increases in ellipticity and blue-shifts in the spectra are observed between pH 8 and 10. In addition, the spectra more closely resemble those reported for membrane samples where the spectral distortions have been removed. The changes in the alkaline region are demonstrated to be only partially reversible and may be due to conformational alterations in the membrane proteins and/or to a reduction in optical distortions. Thermal stability studies reinforce the irreversible behavior of the membrane samples.

1. Introduction

The proteins in the erythrocyte membrane *in situ* have been extensively studied by circular dichroism (CD) [1–6] since the carbohydrate and lipid components do not contribute significantly to the CD spectrum [7–9]. The particulate nature of membrane suspensions, however, poses a number of problems in the interpretation of the CD spectra [1,2,9,10]. The origins of the flattening of the 210 nm trough due to absorption statistics [11,12] and the distortion and red-shift of the spectrum that originate from differential light scattering [2] are now well documented and corrections are feasible [2,5,13]. Other possible artifacts have also been shown to be negligible in interpreting the CD spectra of membranes [9].

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Several attempts have been made to reduce the turbidity of membrane suspensions and, therefore, to remove the spectral distortions. Exposure of erythrocyte membranes to such solvents as n-pentanol, 2-chloroethanol, glycerol, and sodium dodecyl sulfate has resulted in CD spectra with varying degrees of reduced flattening and red-shifting compared to the typical membrane patterns [2,14,15]. Similar spectral changes have been observed with erythrocyte and plasma membranes following sonication [1,2,10] and fragmentation with a French press [3]. The spectra achieved in most of these reports were more characteristic of solubilized proteins possessing substantial amounts of α-helical structure. The amount of observed secondary structure induced as a result of the individual treatments is not known at this time.

In several reports, the contributions of the optical artifacts have been determined theoretically and corrected membrane CD spectra have been generated [2,15,16]. Determinations of secondary structure from these corrected spectra provide evidence for considerable α-helical content in the proteins of the erythrocyte membrane [2,5].

The present report demonstrates the effects of pH

and temperature on the circular dichroism of human erythrocyte membranes. The CD spectra of the membrane suspensions appear to be sensitive to changes in both environmental parameters. The possibility of conformational transitions in the membrane proteins over the pH range studied is discussed in light of the observation that exposure to extreme acidic and alkaline pH results in irreversible or partially reversible alterations in the CD spectra. Similar irreversible behavior is also observed upon heat denaturation.

2. Materials and methods

Human erythrocyte membranes were isolated from freshly drawn blood of normal adults according to the method of Dodge et al. [17]. The pH of the sodium phosphate buffer was adjusted to 7.8 for maximal hemoglobin removal. Erythrocyte membrane samples were stored in isotonic sodium phosphate solution (310 ideal milliosmolar) as a wet pellet at 4° for immediate use. The absence of residual hemoglobin and the characterization of the polypeptide components were ascertained by detergent-polyacrylamide gel electrophoresis.

Circular dichroism spectra were obtained from a JASCO Model J-20 automatic recording CD spectropolarimeter at 26 ± 1°. The absorbance of solutions was adjusted to 1.00 at 222 nm. The results are expressed in terms of mean residue weight ellipticity, $[\theta]_{MRW}$, in units of deg • cm^2/dmole using the equation: $[\theta]_{MRW} = (\theta \cdot MRW)/(d \cdot c)$: where, θ is the observed ellipticity in degrees, c is the concentration of total protein in g/ml, d is the optical pathlength in decimeters, and MRW is the mean residue weight of total membrane proteins which is assumed to be 114. The CD spectropolarimeter was calibrated with a 1 mg/ml solution of d-10-camphorsulfonic acid to give a $[\theta]$ value of +7260 at 290.5 nm [18]. This calibration is found to be unchanged after several months. Estimations of secondary structural information contained in far ultraviolet circular dichroism spectra were obtained both by the method of Chen et al. [19] using a BMD073 computer program (Biomedical Computer Programs, University of California Press, 1972) and an IBM 270/145 computer, and by the method of Chen and Yang [20], using the mean residue weight ellipticity value at 222 nm.

CD spectra of the membrane samples at various pH values were measured 30 minutes after the pH adjustment, and in some cases, again after several days of storage at 4° to monitor possible time dependent changes. Following each CD measurement, the exact total protein concentrations of the membrane samples were determined, and the absorbances remeasured.

The reversibility after exposure to extreme acidic and alkaline pH was also studied. Membrane samples in phosphate buffer at neutral pH were adjusted to pH 3.0 and 11.7 with 0.1 N HCl and NaOH, respectively, after which they were readjusted to the initial neutral pH values. CD spectra were measured 30 minutes after each pH value was reached. In addition, the sodium phosphate buffer was also adjusted to the same pH extremes and restored to the initial pH values. Fresh membranes were then suspended in these buffers and CD scans were made as controls for the studies.

Temperature studies were performed using a Lauda K-2/R temperature bath. Membrane samples, heated for 30 minutes at the desired temperature, were cooled to 26° and their CD spectra obtained.

Ultraviolet absorbance measurements were performed with a Cary 14 Spectrophotometer. All pH measurements were made with a Radiometer Model 26 pH meter equipped with combined glass electrodes (GK2302C). The pH meter was standardized against pH 4.01 and 7.00 standard buffers. Total protein concentration of membrane samples was determined by the Hartree modification [21] of the Lowry method [22]. Detergent-polyacrylamide gel electrophoresis of membrane proteins was performed according to the procedure of Fairbanks et al. [23], using "sequanal" grade SDS obtained from Pierce Chemical Company. All other chemicals used were reagent grade. The water used in all experiments was double-deionized and distilled.

3. Results

The human erythrocyte membrane prepared for this study was characterized by SDS-polyacrylamide gel electrophoresis. A typical spectrophotometric scan of such a gel is shown in fig. 1 which agrees very well with published data [23,24]. The absence of significant amounts of the hemoglobin polypeptide chains (molecular weight of approximately 16 000) suggests

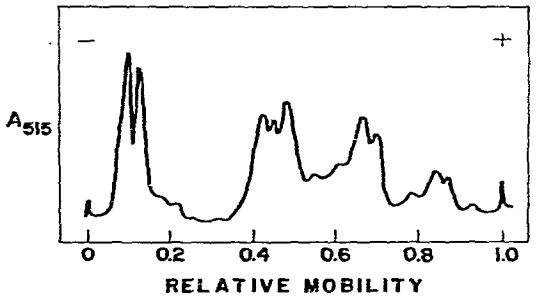

Fig. 1. Spectrophotometric scan of SDS polyacrylamide gel demonstrating the typical distribution of polypeptide components from normal human erythrocyte membranes.

that the membrane samples used are essentially free of hemoglobin. The pH- and temperature-dependent studies were performed at an ionic strength of 0.1 which is within the range of maximal stability for the human erythrocyte.

3.1. Effect of pH

The effect of pH on the CD spectra of membrane proteins was studied in the range of pH 3.0 to 11.7. The results are shown in fig. 2. Precipitation of the membrane below pH 3.0 and the high optical density of the solutions in the far UV region at pH above 12.0 prevented spectral measurements outside the pH range 3.0 to 11.7. The membrane suspensions at different pH values were prepared from the stock native membrane sample at 7.8 by addition of HCl or NaOH to the desired pH and equilibrated for 30 minutes. No further change of the CD spectra was observed when the samples were allowed to stand for 2 to 4 days at 4°C. CD spectra over the measurable pH range showed the typical double-trough pattern, but displayed a wide variation in both magnitude and shape of the CD bands. In the pH range from 7 to 9, CD CD spectra maintained similar shapes with ellipticity values increasing slightly with increasing pH. The double troughs, originating from the π—π^* and the n—π^* electronic transitions of the polypeptide backbone occurred at 210 nm and 223 nm, respectively. Above and below this pH range, however, different changes in the CD spectra were observed. With decreasing pH, the 210 nm trough decreased to a larger extent than the 223 nm trough. In addition, the latter

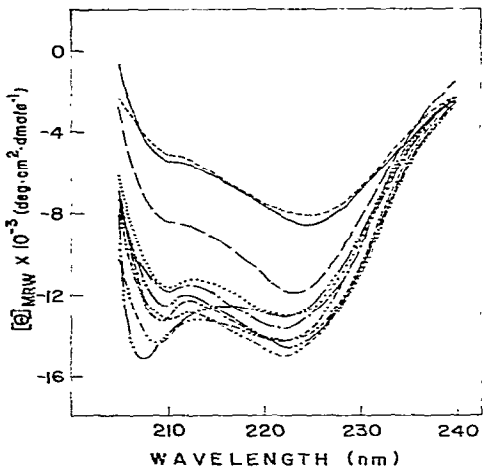

Fig. 2. Far ultraviolet CD spectra of erythrocyte membrane at various pH values. - - - - - 3.0; ——— 5.0; — — — 6.0; . . . . . 7.05; . — . — 7.8; .. — .. 8.65; . — — . — — . 9.25; .. — — .. — — 9.8; ... — — — ... 10.7; ... — ... 11.7. Conditions: temperature, 26° ± 1°C, in 310 ideal milliosmolar sodium phosphate adjusted with either 0.1 N HCl or NaOH.

trough red-shifted to 225 nm at pH 3.0. With increasing pH from 7 to 11.7, the 210 nm trough increased gradually while the 223 nm trough increased initially, followed by a decrease in ellipticity above pH 10. Both troughs displayed blue-shifts with increasing pH so that they appeared at 208 nm and 222 nm at pH 11.7. The changes in ellipticity at 210 and 223 nm as a function of pH are shown in fig. 3. The magnitude of the

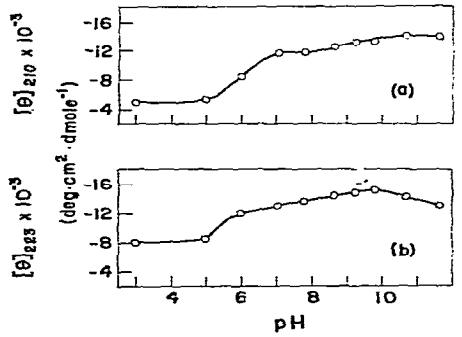

Fig. 3. The change in CD parameters of erythrocyte membrane as a function of pH. Conditions: temperature, 26° ±1°C, in 310 ideal milliosmolar sodium phosphate adjusted with either 0.1 N HCl or NaOH.

210 nm trough increased significantly from pH 5 to 7 and was followed by a more gradual and almost linear increase from pH 5 to 6 followed by a gradual increase from pH 6 to 10, and a significant decrease above pH 10. The change in ratios of the two troughs, $[\theta]_{210}/[\theta]_{223}$, occurred mainly between pH 5 to 7 and above pH 10.

Analyses of the CD spectra by the methods of Chen et al. [19] and of Chen and Yang [20] gave estimations of the amount of secondary structure and α-helical content, respectively, of proteins in the membrane at various pH values. The results are shown in table 1. At pH 7.8, membrane protein CD spectra revealed average α-helical contents of 37% and 45%, depending upon the method of analysis. Estimates of α-helical content over the pH range of 3.0 to 11.7 varied from 25 to 50% using the method of Chen et al. [19] and from 18 to 42% when the analysis according to Chen and Yang [20] was employed.

It should be noted that the method of analysis described by Chen et al. [19] is based on reference spectra of proteins in solution. Since we have not evaluated the effects of differential scattering and absorption flattening on the membrane protein spectra, caution should be exercised concerning the quantitative aspects of these conformational changes. However, an approximate relative trend apparent from both methods of secondary structural estimation, which reflects some pH denaturation effects on the membrane proteins, is of some interest.

Table 1
Estimation of secondary structure of human erythrocyte membrane proteins at various pH values

pH	Based on entire CD spectrum [a]		Based on $[\theta]_{222}$ [b]
	% α-helix	% β-structure	% α-helix
3.0	25	2	18
5.0	26	3	19
6.0	36	8	31
7.05	43	6	36
7.8	45	7	37
8.65	48	4	39
9.25	49	3	40
9.8	50	5	42
10.7	49	7	40
11.7	46	8	35

[a] Estimation by the method of Chen et al. [19].
[b] Estimation by the method of Chen and Yang [20].

3.2. Reversibility of acid and alkaline denatured membrane

Native erythrocyte membrane at pH 7.8 was subjected to alkaline denaturation by addition of NaOH to pH 11.7 and equilibrated for 30 minutes. The pH was then readjusted back to 7.8 by HCl, and the CD spectrum of the renatured membrane was measured. The results are shown in fig. 4. As seen from the curves, reversibility after exposure to pH 11.7 was not complete. Analyses of the CD spectra revealed that the α-helical content of the renatured membrane decreased slightly from 45 to 42% when the entire spectrum was analyzed [19] and from 37 to 33% when the mean residue weight ellipticity at 222 nm only was considered [20]. The β-structural content remained essentially the same.

Exposure of the membrane to pH 3.0 resulted in drastic changes in the CD spectra shown in fig. 5. The change was not reversible since the CD spectrum for the renatured sample was not the same as that of the native but rather resembled that of the denatured membrane. The membrane sample which had been

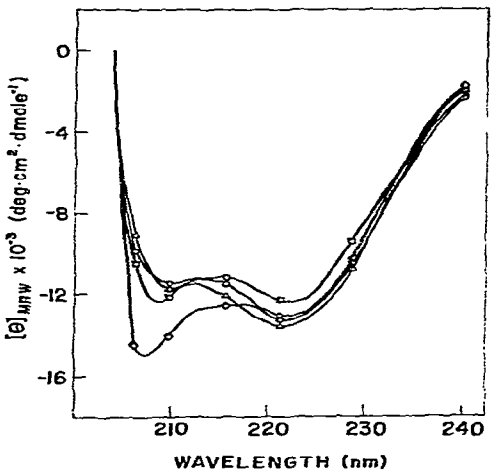

Fig. 4. Alkaline denaturation of erythrocyte membrane, —△—△— pH 7.8; —◇—◇— pH 11.7; —□—□— pH 7.8, readjusted; —○—○— Buffer adjusted from pH 7.8 to 11.7 to 7.8 + membrane (see text for experimental details).

Table 2
Estimation of protein secondary structure of alkaline and acid denatured and renatured human erythrocyte membranes

Erythrocyte membrane	Based on entire CD spectrum [a]		Based on $[\theta]_{222}$ [b]
	% α-helix	% β-structure	% α-helix
Alkaline denaturation:			
Native, pH 7.8	45	7	37
Denatured, pH 11.7	46	8	35
Renatured, readjusted to 7.8	42	8	33
Native membrane + buffer adjusted pH 7.8 → 11.7 → 7.8	44	6	36
Acid denaturation:			
Native, pH 7.05	43	6	35
Denatured, pH 3.0	25	2	18
Renatured, readjusted to 7.05	25	6	18
Native membrane + buffer adjusted pH 7.05 → 3.0 → 7.05	44	6	35

[a] Estimation by the method of Chen et al. [19].
[b] Estimation by the method of Chen and Yang [20].

exposed to pH 3.0 and readjusted back to pH 7.05 resulted in a CD spectrum which was very similar to that of the denatured membrane at pH 3.0. It is of interest to note that while the α-helical content of the renatured membrane sample remained irreversibly altered, the small but significant amount of β-structure was virtually restored. The results of these estimations are summarized in table 2. Again, due to the optical artifacts inherent in membrane CD, only the qualitative trend of the change is significant and the quantitative aspect of these values is subject to large uncertainty.

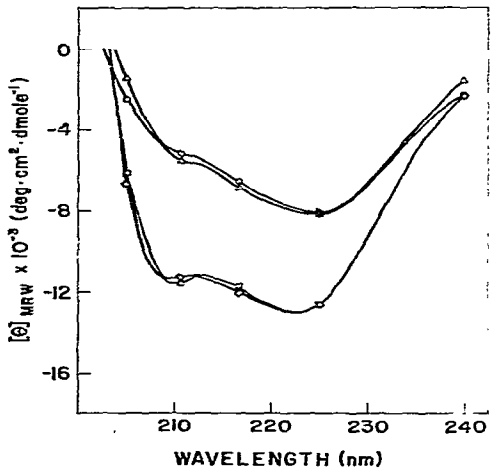

Fig. 5. Acid denaturation of erythrocyte membrane. —□—□— pH 7.05; —○—○— pH 3.0; —△—△— pH 7.05, readjusted; —◇—◇— Buffer adjusted from pH 7.05 to 3.0 to 7.05 + membrane (see text for experimental details).

3.3 Thermal stability

When native membrane was heated for 30 minutes at an elevated temperature and then cooled to room temperature, significant denaturation of membrane proteins occurred as shown by the CD spectra in fig. 6. A gradual flattening and decrease in ellipticity of the CD curves at both the 210 and 223 nm troughs were observed when the membrane was exposed to increasingly higher temperature from 25° to 65°C. As shown in fig. 7, the ellipticity at both 210 and 223 nm decreased slightly from 25° to 45°C, but changed more drastically from 45° to 65°C. Structural estimations of the membrane proteins reflected in these relative changes suggested a decrease in α-helical content, based on both methods of analysis, and virtually no change in β-structure. These results are summarized in table 3.

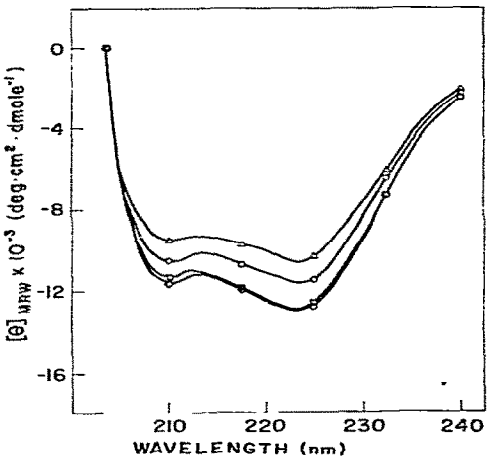

Fig. 6. CD spectra of erythrocyte membrane at various temperatures, —◇—◇— 37°; —□—□— 45°; —○—○— 55°; —△—△— 65°; Conditions: pH 7.8, in 310 ideal milliosmolar sodium phosphate.

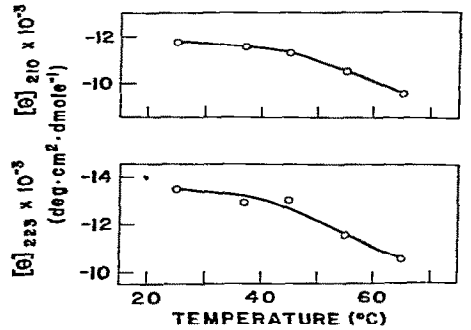

Fig. 7. The change in CD parameters of erythrocyte membrane as a function of temperature. Conditions: pH 7.8, in 310 ideal milliosmolar sodium phosphate.

Table 3
Estimation of protein secondary structure in renatured human erythrocyte membranes after exposure to various temperatures

Temperature, °C	Based on entire CD spectrum[a]		Based on $[\theta]_{222}$[b]
	% α-helix	% β-structure	
25	45	7	37
37	43	5	35
45	43	5	35
55	38	6	30
65	35	7	27

[a] Estimation by the method of Chen et al. [19].
[b] Estimation by the method of Chen and Yang [20].

4. Discussion

The purpose of this work was to study the denaturation effects of pH and temperature on the human erythrocyte membrane proteins by circular dichroism. The membrane circular dichroism spectra obtained at pH values of physiological significance displayed the typical distortions observed for erythrocyte membrane suspensions. Double minima were red-shifted, relative to spectra of typical soluble proteins containing considerable amounts of α-helical structure, so that they occurred at 210 and 223 nm. The membrane spectra also appeared to be flattened over the observed spectral range, especially at the region of the 210 nm trough. The optical artifacts which contribute to the observed distortions have been analyzed and theoretical calculations of the absorption flattening and differential scattering terms have been published [2,5]. The analysis of the terms indicated that the red-shifts of the observed membrane spectra were due to differential scattering alone while the flattening was attributed to both optical terms. It was apparent, however, that the optical terms caused flattening to a considerably greater degree at the 210 nm trough than at the 223 nm minimum. In fact, further analysis indicated that the differential scattering term probably did not contribute significantly to the magnitude of the latter trough. While the 210 nm trough is considerably altered by the optical artifacts, then, the 223 nm trough may remain reasonably unaffected in its magnitude. The ellipticity of this trough has been demonstrated to show very little dependence on particle size since the magnitude varied only slightly when comparing erythrocyte membrane CD spectra with those of the same membrane samples in which turbidity effects had been removed with either sonication or exposure to sodium dodecyl sulfate [2]. Similar results have been reported for plasma membrane CD spectra [10], where the mean residue weight ellipticity value at 222 nm was suggested to yield reliable estimates of helicity. In the present report, estimates of secondary structure are based on both the entire CD spectrum as well as the mean residue weight ellipticity value at 222 nm and should at best be considered as rough approximations. The relative changes in secondary structure reflected in either method of estimation, however, are of interest.

The results obtained in this study show that erythro-

cyte membrane circular dichroic spectra undergo significant alterations when the pH of the sample environment deviates from its physiological pH range. The increase in spectral flattening and the further red-shift of the spectra which is observed as the pH is lowered are probably indicative of increased optical artifacts. The approximately fifty percent decrease in estimated α-helical content at the acidic extreme is not an entirely accurate assessment because of the increased optical distortions. Some permanent loss of secondary structure has probably occurred, however, since the spectrum obtained at pH 3.0 remains irreversibly distorted upon restoration to the native pH.

Below pH 3, the membrane suspension coagulates into large precipitates. One source of this phenomenon could be attributed to the charged nature of the erythrocyte membrane. The membrane particles are highly charged at pH higher than 3 because of the negatively charged sialic acid residues located on the surface which have pK's of about 2.6. The repulsion among the membrane particles keeps them suspended. When the pH is much lower than 3, however, the negative charges diminish and aggregation begins to occur.

As the pH is increased from the physiological range, a gradual increase in the trough magnitudes and a disappearance in the red-shifts are evident. In the alkaline extremes, the CD troughs are reversed in magnitude so that the lower wavelength trough is now the deeper of the two. The alkaline CD spectra actually resemble those published in the literature where spectral distortions have been removed [1,2,3,10]. The possibility exists that the erythrocyte ghosts are partially solubilized under alkaline conditions and that these spectra more accurately reflect the secondary structural content of the membrane. The estimates of approximately 40 to 50% α-helix calculated from membrane spectra in the alkaline environment agree very well with α-helical estimation made from sonicated and detergent-solubilized erythrocyte membranes [2] and from intact erythrocyte membranes whose CD spectra had undergone detailed calculational corrections [5]. The partial reversibility from the spectrum obtained under alkaline conditions probably reflects the decrease in membrane solubility when the physiological pH is restored. Small alterations in membrane protein secondary structure, however, cannot be ruled out.

By maintaining a constant pH and varying a different environmental factor, as in the temperature studies, the observed spectral changes are not the same. With increasing temperature, the ratio of the trough ellipticities remains relatively constant and no spectral shifts to longer or shorter wavelengths are evident. The changes are merely in overall magnitude of ellipticity. The CD spectra maintain the same shape indicating the possibility of membrane protein secondary structural changes without drastic alterations in optical distortions.

The spectral alterations of the intact erythrocyte membrane found in this pH-dependent study are irreversible in the acidic range and only partially reversible in the alkaline region. The observed changes are probably attributable to both magnifications and reductions in optical artifacts as well as conformational changes in some of the erythrocyte membrane proteins. The thermal stability studies reinforce the irreversible behavior in that no complete recovery of original CD spectra was attained after heating.

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