# Effect of Alkaline pH on the Optical Properties of Native and Modified Erythrocyte Membranes†

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ABSTRACT: In circular dichroism (CD) spectra of erythrocyte membranes, the intensity of the 208-nm transition was found to increase with pH, in the region above 9.5. This effect, which in native membranes is only partially reversible, was not observed if the membranes had previously been exposed to 70° at neutral pH, or treated with formaldehyde; in both these cases, the intensity of the whole CD spectrum was decreased. Using chloroform–methanol-treated membranes, which from their infrared (ir) characteristics appeared to be partially in a $\beta$ conformation, it was possible to correlate the alkali-induced variations of the CD spectrum to modifications of the ir spectrum. The results are discussed in terms of a depolymerization of the membranes at high pH, or of a pH-dependent transition of membrane proteins toward a larger amount of random coil conformation.

Optical methods have been widely used, in recent years, to assess the conformation of proteins in biological membranes. Infrared (ir) spectroscopy, although limited by the high absorption of water, has generally indicated the absence of $\beta$ structure in plasma membranes from mammalian cells (Maddy and Malcolm, 1965; Wallach and Zahler, 1966). Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra of membranes from various sources have also, by comparison to spectra of pure proteins of known conformation, been interpreted as indicating a large $\alpha$-helix contribution (Lenard and Singer, 1966; Wallach and Zahler, 1966; Urry *et al.*, 1967). Deviations from the typical polylysine $\alpha$-helix pattern, such as the low amplitude of the CD signals and the red shift of the CD extremum around 222 nm, have been discussed in terms of optical artifacts due to the particulate nature of the membrane preparations (Urry and Ji, 1968; Urry and Krivacic, 1970; Ottaway and Wetlaufer, 1970; Schneider *et al.*, 1970; Glaser and Singer, 1971). The magnitude of the spectral anomalies due to these artifacts is however still a matter of discussion, so that estimates of $\alpha$ helix from CD measurements vary from 80 to 35% or even less (Gordon *et al.*, 1969; Choules and Bjorklund, 1970; Glaser and Singer, 1971).

The present study shows some modifications of CD spectra induced by alkaline pH in native erythrocyte membranes. Optical density measurements were performed in order to estimate the anomalies due to optical artifacts. The use of membranes which had been modified by exposure to high temperatures, to formaldehyde or to chloroform–methanol allowed some interesting comparisons. Ir spectra yielded an independent, though only semiquantitative, method to verify the assumptions about protein conformation.

## Materials and Methods

Beef red blood cells were collected by centrifugation at 800*g* from freshly drawn blood, and washed with isotonic saline. Erythrocyte membranes were prepared essentially according to Dodge *et al.* (1963), by several washings with 6 mM phosphates (pH 7.8) until the supernatant at 25,000*g* was free of hemoglobin. In some cases, the preparations, which contained around 30 $\mu$g of hemoglobin/mg of protein, were used as such; in most cases, however, two washings with distilled water and two more with isotonic NaCl containing 10% isotonic phosphates, pH 6.8–6.9 (buffer A), were subsequently performed. This procedure was effective in causing a further reduction of the hemoglobin content of the ghosts, to less than 10 $\mu$g/mg of protein. CD spectra were usually performed with membranes suspended in buffer A. For infrared measurements, and also for some CD spectra, the membranes were extensively dialyzed against distilled water. Ten different preparations were used, and were found to behave in a very similar, if not identical, manner.

Protein concentration was determined according to Gornall *et al.* (1949), and checked by using a Coleman nitrogen analyzer. Hemoglobin was determined by the pyridine hemochromogen method of De Duve (1948).

The buffers used were generally prepared by mixing isotonic solutions of NaCl, $KH_2PO_4$, and glycine, in the volume ratio 8:1:1; the appropriate pH was then reached by addition of isotonic HCl or NaOH. For infrared measurements and for a few CD experiments, pH values above 11 were reached by addition of concentrated ammonia.

Exposure to high temperature was usually performed in a Dubnoff bath, under constant but mild agitation.

Formaldehyde treatment of intact erythrocytes or of erythrocyte ghosts was effected essentially according to Butler (1963). Anyhow since the original procedure caused transformation to methemoglobin of hemoglobin within the erythrocytes, the time of contact of 3% (v/v) formaldehyde with the erythrocytes or the membranes was reduced to 3 hr at 37°. By this modification, it was possible to obtain erythrocytes which, though resistant to lysis by detergents or by sonication, retained the ability to bind oxygen in a reversible fashion (A. Scioscia Santoro and R. Strom, unpublished results).

Treatment with chloroform–methanol involved shaking the aqueous membrane suspension with three volumes of

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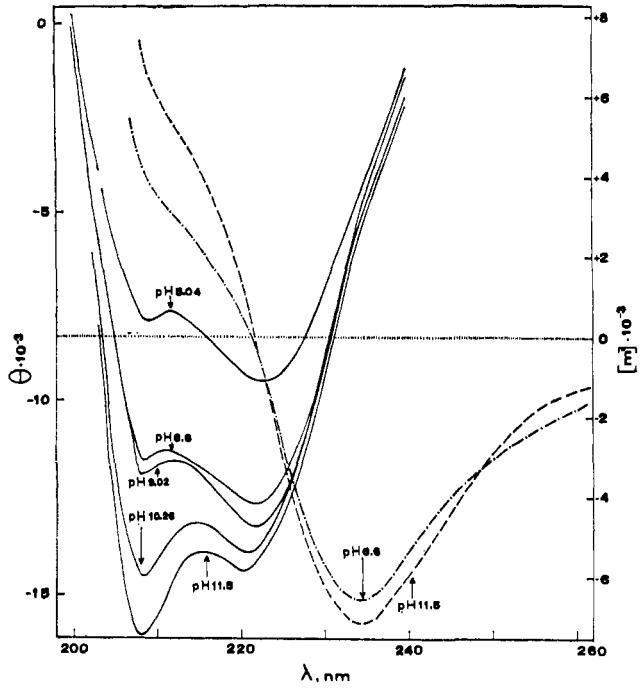


FIGURE 1: CD and ORD spectra of native erythrocyte membrane suspension at various pH values (full lines, CD spectra; broken lines, ORD spectra).

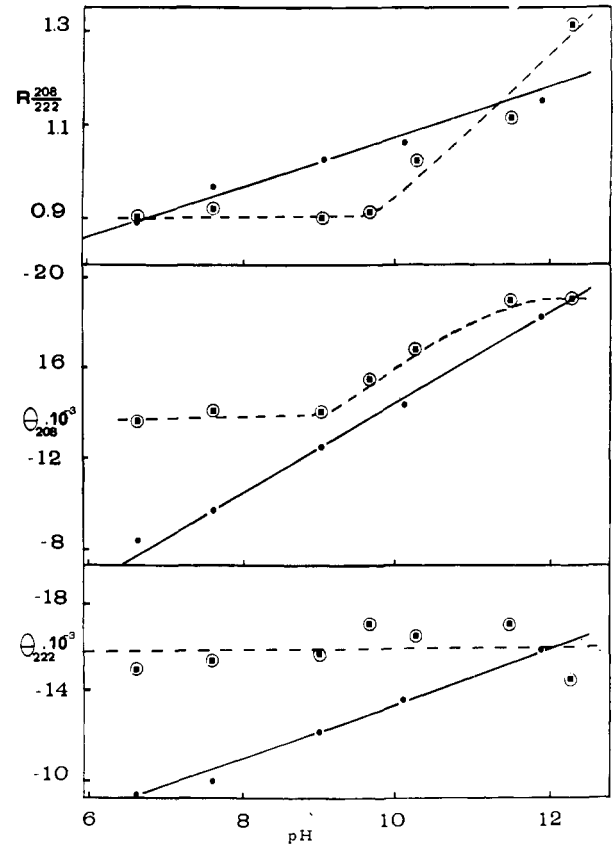


FIGURE 2: pH dependence of CD parameters of erythrocyte membrane suspension. (○) The membranes were brought to the desired pH value and the spectra performed within 60 min. (●) The membranes, after having been brought to the desired pH value, were exposed for 30 min to 70°.

chloroform–methanol (3:1, v/v) at room temperature, with separation of the phases; the treatment was then repeated twice more on the aqueous phase, all phases reassembled, evaporated almost to dryness in a rotatory evaporator under suction, lyophilized, and finally resuspended in water or in buffer by sonication with a MSE 100-W sonifier.

Erythrocyte lysis was estimated by following, in a Beckmann DB spectrophotometer thermostated at 25°, the variation of optical density at 740 nm, using 1-cm path-length cuvets. The erythrocyte suspension in isotonic NaCl was mixed at zero time with ten volumes of isotonic buffer of the desired pH, and the recorder started.

Ir spectra were obtained on a Perkin-Elmer Model 521 spectrometer, using films cast on Irtran 2 disks by slow vacuum evaporation of aqueous suspension of erythrocyte membranes.

CD and ORD measurements were performed at room temperature, using 1-mm path-length cells, on a Cary Model 60 spectropolarimeter with or without CD attachments. Although several scans were generally taken and averaged, the values in the region below 200 nm had a very low signal to noise ratio (<4:1), and were therefore neglected. The baseline, given by the buffer without the membranes, was substracted. The mean residue ellipticity, $[\theta]$, and the mean residue rotation, $[m']$, were calculated using a mean residue molecular weight of 114. The performance of the instrument was checked with an aqueous solution of *d*-10-camphorsulfonic acid.

Optical density was measured in a Beckman DK2 spectrophotometer, using 1-cm and 1-mm path-length cells.

## Results

*Effect of Alkaline pH on Optical Properties of Native Erythrocyte Membranes.* At neutral pH, the CD spectrum of beef erythrocyte ghosts exhibited, in the 300- to 200-nm region, two negative extrema, a major one around 222 nm and a smaller one at 208 nm (Figure 1). Different membrane preparation showed some variations on the position of the 222-nm extremum, with oscillations between 221 and 225 nm, and in the magnitude of the 208-nm band, which could be present either as a well-defined peak of intensity up to 80% of that of the 222-nm one, or as a mere indenture, approximately half way in the ascending branch of the 222-nm peak. As already noticed by Ji and Urry (1969) and by Glaser and Singer (1971), the ellipticity at 208 nm was less marked and the 222-nm peak was more shifted toward the red in membrane preparations which, probably because of extensive washing and of successive cycles of freezing and thawing, exhibited the highest turbidity.

Increasing the pH up to 9.2–9.5 did not modify the CD spectrum. Above this value, a progressive deepening of the 208-nm extremum was seen, so that the amplitude of this peak became greater than that at 222 nm (Figure 1). In all membrane preparations examined, the $[\theta_{208}]:[\theta_{222}]$ ratio increased steadily from pH 9.5 up to pH values as high as 12.3 (Figures 1 and 2). ORD spectra showed only minor differences between pH 6.6 and 11.5. The effects of alkalinization on ORD spectra were less evident, consisting in a sharpening of the 234-nm negative trough and an increase in the positive value of the shoulder around 210 nm. These modifications corresponded fairly well to the alterations of the CD spectra. The intersection of the ORD spectrum with the zero rotation line was, both at neutral and at alkaline pH, at 221.5 ± 0.3 nm. The high optical density of alkaline solutions in the far-uv region prevented exploration above this pH.

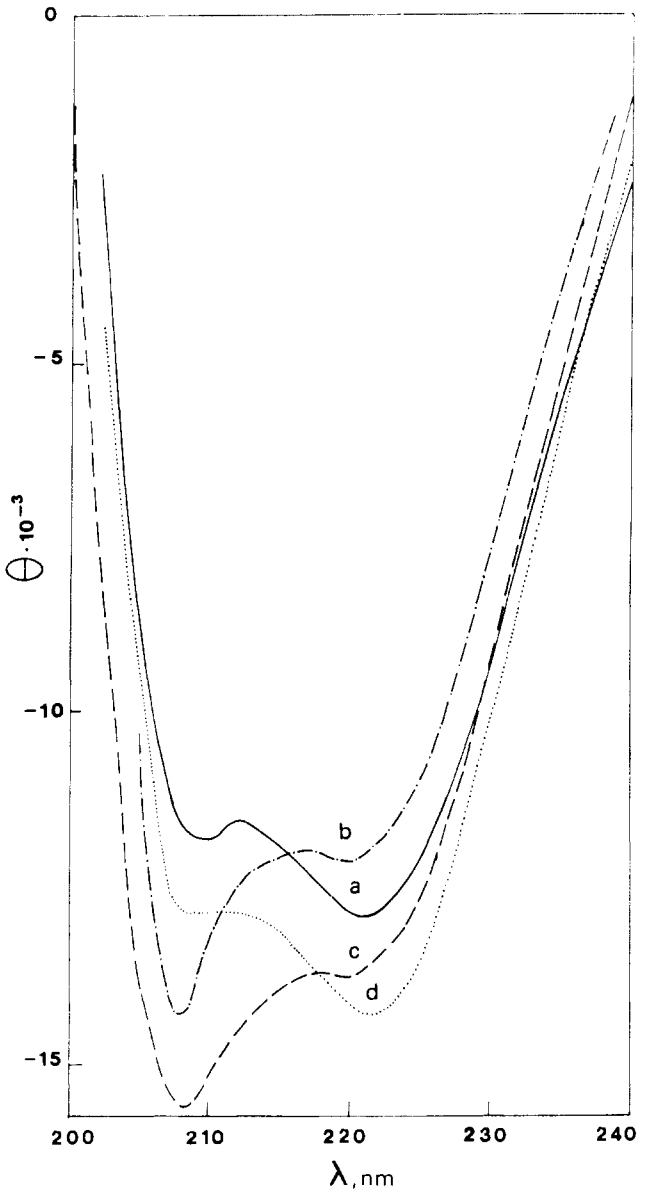


FIGURE 3: Irreversibility of CD modifications due to alkaline pH. (a) Native membranes, pH 7.1; (b) membranes brought to pH 11.7; (c) the membranes, brought to pH 11.7, were after 30 min brought back to pH 7.1, by addition of the required amount of isotonic $HCl$–$KH_2PO_4$ (1:1, v/v) mixture; (d) the native membranes were added to the pH 11.7 solution which had been neutralized to pH 7.1.

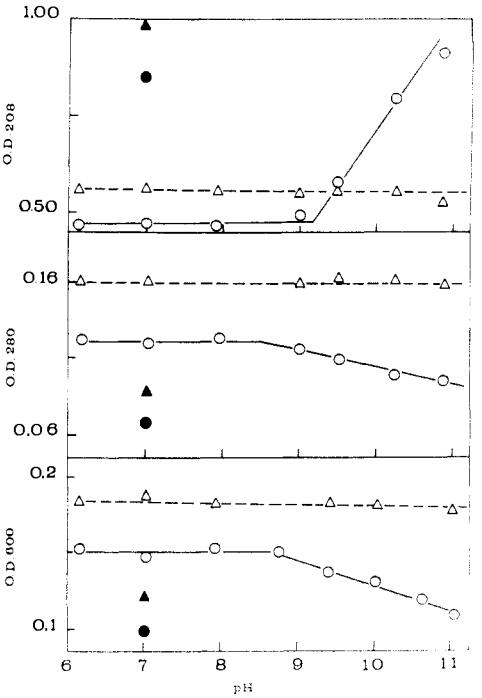


FIGURE 4: pH dependence of turbidity and of uv absorbancy of erythrocyte membrane suspensions. The data were obtained either on 1-mm cuvets (for measurements at 208 or at 280 nm) or on 1-cm cuvets (for measurements at 600 nm). Concentration of membrane protein was 110 μg/ml. (○) native membranes; (●) the same, after sonication; (△) membranes exposed for 30 min to 70°; (▲) heat-treated membranes after sonication.

The ellipticity at 222 nm also changed somewhat upon alkalinization, increasing at first and then decreasing at pH > 12. The magnitude of this effect varied among different membrane preparations; it was however always small as compared to the increase of the ellipticity at 208 nm.

The acid pH region was not thoroughly investigated, since visible flocculation occurred. At pH 5, the CD spectrum had a shape similar to that of neutral pH, but was shifted toward smaller ellipticity values.

The changes brought about by exposure to high pH were only partially reversible (Figure 3). Similar results were obtained if the membranes were suspended in $H_2O$ and the pH raised by addition of concentrated ammonia. Addition of 70% (v/v) glycerol, either at neutral or alkaline pH, did not modify the CD spectra significantly. As described by other authors (Lenard and Singer, 1966; Choules and Bjorklund, 1970), addition of 90% (v/v) 2-chloroethanol caused instead a conspicuous change in the CD spectrum, which showed two very intense bands of almost equal ellipticity, at 220–221 nm and at 208 nm. The CD characteristics were the same, whether or not the membranes had been exposed to high pH.

Variations of optical density were also checked. There was a background absorption, due to turbidity, at wavelengths above 260 nm; at lower wavelengths the optical density increased rapidly, with a shoulder around 220 nm and a maximum below 200 nm. Upon alkalinization, the absorbancy at 208 nm remained constant up to pH 9.3–9.5, then increased progressively; turbidity, estimated either at 280 nm or at 600 nm, decreased instead somewhat (Figure 4).

Sonication, at neutral pH, caused a large decrease of turbidity, to levels well below those reached at high pH values, while the absorption curve below 230 nm became sharper and somewhat more intense. Concomitantly, while the ellipticity at 222 remained practically unchanged except for a 2-nm blue shift, the $[\theta_{208}]:[\theta_{222}]$ ratio increased, as if the membranes had been exposed to a pH value around 10.5–11.0. These results were similar to those obtained by Schneider *et al.* (1970) and by Glaser and Singer (1971); they differed from those of Urry *et al.* (1971), who found upon sonication a conspicuous increase of ellipticity in the whole 240- to 200-nm region. According to Urry (private communication) the reasons for such differences may reside in a more extensive aggregation of his membrane preparations, due to freezing of the membranes in concentrated suspension.

In the ir spectra of films cast from aqueous suspension of erythrocyte membranes, the amide I band, attributed to the stretching modes of hydrogen-bonded peptide C=O bonds, appeared as formed by a narrow peak at 1650 $cm^{-1}$ (Figure

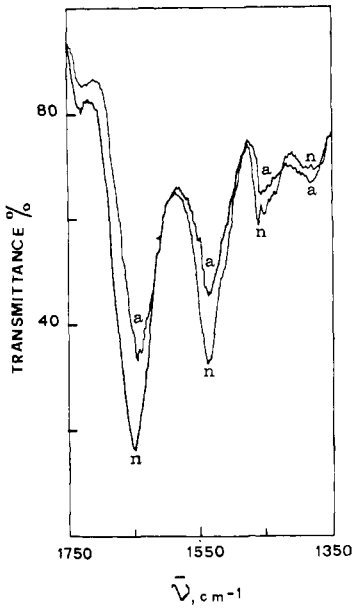


FIGURE 5: Ir spectra of films cast from an erythrocyte membrane suspension in $H_2O$. n: pH around neutrality; a: alkaline pH, obtained by addition of 0.2 ml of concentrated ammonia per ml of suspension.

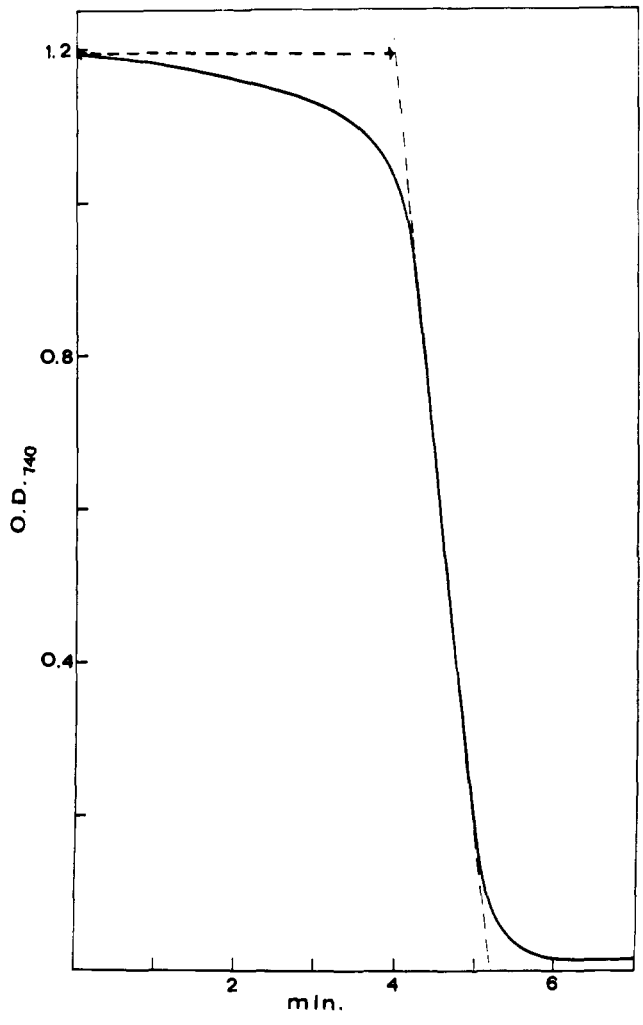


FIGURE 6: Kinetics of hemolysis by alkaline pH. Beef erythrocytes in isotonic NaCl were added to 20 volumes of an isotonic buffer, pH 12.2, and the variations of optical density at 740 nm recorded as a function of time.

5). The sharpness of these peaks, and the absence of stretching modes in the 1630-cm$^{-1}$ region, indicate the absence of significant amounts of $\beta$ structure (Maddy and Malcolm, 1965; Chapman *et al.*, 1968), but do not allow any distinction between $\alpha$ helix and random coil conformations. Similar conclusions can also be drawn from the amide II band, which was located at 1540 cm$^{-1}$. Identical ir spectra were obtained whether or not the erythrocyte suspension had been brought to pH >11, prior to evaporation, by addition of concentrated ammonia.

*Alkaline pH-Induced Lysis.* When beef erythrocytes in isotonic saline were brought to pH $\geq$ 11, after a certain time lysis occurred. Figure 6 shows the time course of this phenomenon, and indicates how the lag time and the rate of lysis can be graphically estimated. Hemoglobin was generally still in the oxy form when lysis was complete, and became denatured only afterwards. The duration of the lag time was shortest at highest pH values, being, in the pH 11–12.5 range, a linear function of hydrogen concentration. The relation, if the lag time $\tau$ was expressed in seconds, could apparently be described by the formula $\tau = 4 \times 10^{14} \times [H^+]$. Also the rate of lysis followed a similar pH dependence, becoming faster at higher pH.

*Effect of Heat and of Formaldehyde on Optical Properties of Isolated Membranes.* After exposure of the membrane suspension, at neutral pH, for 30–60 min to temperatures ranging from 25 to 80°, their CD spectra, taken at room temperature, had similar shapes, but differed in the ellipticity values, lowest signals corresponding to highest temperatures (Figure 7). The decrease of ellipticity occurred almost throughout the whole temperature range explored, without any indication of a critical point. The $[\theta_{208}]:[\theta_{222}]$ ratio remained practically constant up to 55°, and diminished somewhat afterwards. Even membranes which had been exposed, sealed in nitrogen-filled vials, to 130° for 30 min had a CD spectrum of similar shape. Also the ORD signals became of lower amplitude upon exposure of the membranes to heat, but the shape of the spectrum remained the same, except for a 1-nm red shift of the zero-rotation intercept at 221–222 nm (Figure 7).

If the membranes were exposed to heat at pH values higher than neutrality, the ellipticity at 208 nm decreased less than that at 222 nm, so that, at pH >7.4, the $[\theta_{208}]:[\theta_{222}]$ ratio assumed values above those of the native membranes (Figure 2). Membranes which had been heat treated at high pH (~11.0) did not differ sensibly from native membranes at the same pH. The changes observed were quite irreversible.

Heat treatment at neutral pH caused the membranes to lose the ability to be modified by alkalinization of the medium. As shown in Table I, the difference in the $[\theta_{208}]:[\theta_{222}]$ ratio between membranes at pH 6.8 and pH 10.8 decreased as the preincubation temperature was increased, and became nil for temperatures above 48°.

Spectrophotometric control of the absorbancy of heat-treated membranes showed that, at neutral pH, both turbidity (at 280 or 600 nm) and optical density at 208 nm were slightly higher than those of the corresponding native membranes. Alkalinization did not significantly modify either of these parameters (Figure 4).

Upon sonication, heat-treated membranes exhibited a decrease in turbidity and an increase in far-uv absorbancy similar to that occurred in native membranes. Their CD spectrum

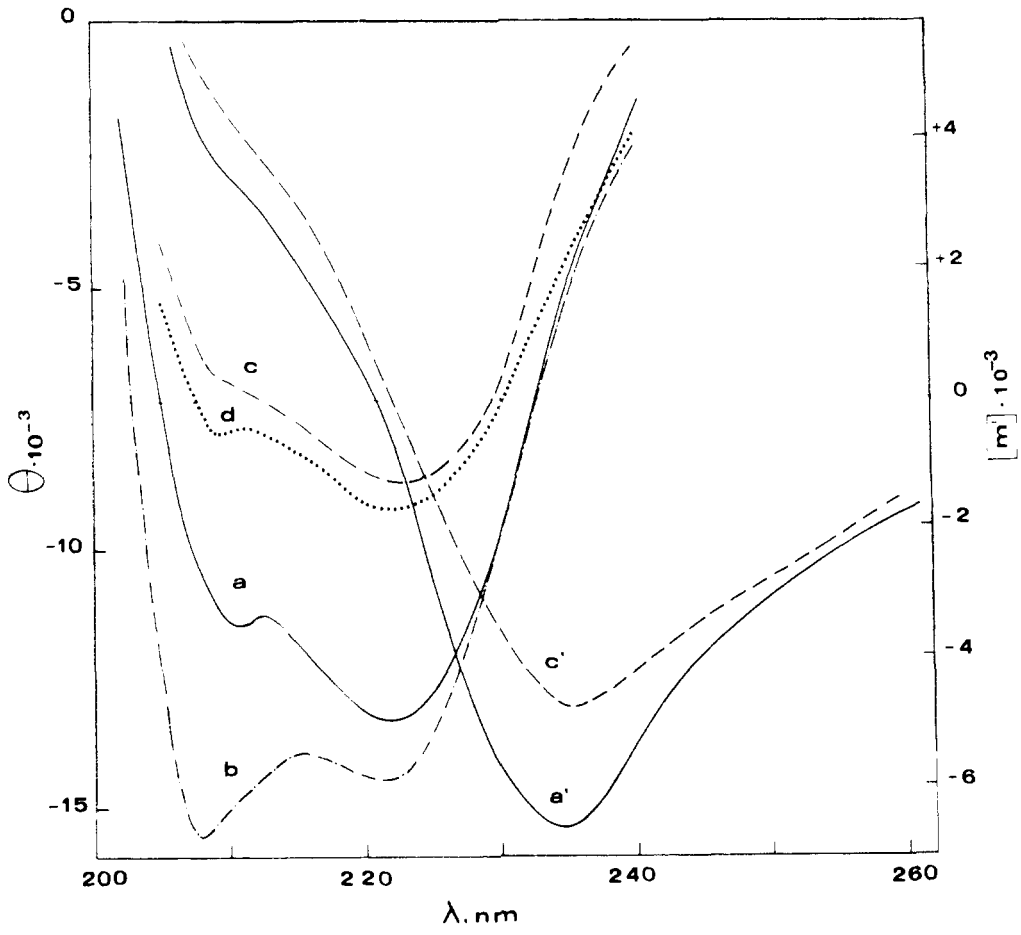


FIGURE 7: CD and ORD spectra of native and heat-treated erythrocyte membrane suspensions. a and a': native membranes, pH 6.9; b: native membranes, pH 11.3; c and c': membranes exposed for 30 min to 70° at pH 6.9; d: membranes exposed for 30 min to 70° at pH 6.9 then brought to pH 11.3.

remained unaffected, *i.e.*, the 208-nm extremum did not increase selectively. On the other hand, heat treatment of previously sonicated membranes, which caused only a minor increase of turbidity, caused a uniform "flattening" of the whole CD spectrum of the same magnitude as in native unsonicated membranes.

Careful examination of the ir spectra of membrane which had been exposed to 70° for 30 min at neutral pH showed a small but significant shift of the lower frequency ascending branches of both amide I and amide II bands, so that both bands appeared wider (Figure 8). These changes suggest the presence, in heat-treated membranes, of small amounts (*i.e.*, around 10–15%) of protein having a $\beta$ conformation. These results are somewhat at variance with those obtained by Chapman *et al* (1968), who exposed the membranes to the high temperature as dry films, and not as aqueous suspensions.

The effects of formaldehyde treatment (at neutral pH) of native membranes were practically indistinguishable from the effects of high temperatures: the CD spectrum at neutral pH was of normal shape but with low ellipticity values, and was not significantly modified by alkalinization; the ir spectrum showed a slight widening of the amide I and amide II bands, the amount of proteins in the $\beta$ conformation being approximately the same (10–15%) as in heat-treated membranes.

*Effect of Chloroform–Methanol Extraction.* Shaking with chloroform–methanol at room temperature caused a partial flocculation of membrane proteins at the interface. After evaporation of the solvent and sonication in aqueous buffer, a homogeneous and slightly opalescent suspension was obtained. The CD spectrum at neutral pH was similar to that of the native membranes, except that the ellipticity values were somewhat lower and the 222-nm band was shifted of approximately 3 nm toward the red. The $[\theta_{208}]:[\theta_{222}]$ ratio reached, despite the sonication, a value only slightly above 0.75. Upon alkalinization, this ratio increased, in a manner similar to that which occurred in native membranes, but did not reach values above unity (Figure 9); ir spectra of films cast from these modified membranes at neutral pH clearly showed the presence, in the amide I region, of peaks not only at 1650 $cm^{-1}$ but also around 1630 $cm^{-1}$; a small shoulder around 1670–1680 $cm^{-1}$ might indicate that the pleated-sheet configuration obtained is anti-parallel (Miyazawa and Blout, 1961). Also the amide II band was wider, confirming the presence of an appreciable amount (40–50%) of $\beta$ structure (Figure 10). Upon alkalinization, the ir spectra became more similar to those of native membranes, the amount of $\beta$ structure becoming negligible.

In preliminary experiments, similar results were obtained if the membranes were modified by addition of the aqueous suspension of 0.1 volume of dimethyl sulfoxide. The amide I band became very wide, with a main $\beta$-structure contribution. If the suspension was brought to pH >11 after the addition of dimethyl sulfoxide, the spectrum was markedly reverted toward a more "native" appearance.

TABLE I: Effect of Exposure to High Temperature on CD Parameters of Erythrocyte Membranes.[a]

| Temp | $[\theta_{222}]$ | | $[\theta_{208}]$ | | $[\theta_{208}]:[_{222}]$ | |
|---|---|---|---|---|---|---|
| (°C) | pH 6.9 | pH 10.8 | pH 6.9 | pH 10.8 | pH 6.9 | pH 10.8 |
| 25 | −13,900 | | −11,200 | | 0.806 | |
| | | −14,200 | | −13,900 | | 0.980 |
| 35 | −14,000 | | −11,200 | | 0.801 | |
| | | −14,200 | | −13,800 | | 0.973 |
| 43 | −12,600 | | −10,400 | | 0.826 | |
| | | −12,000 | | −10,400 | | 0.868 |
| 50 | −11,600 | | −9,400 | | 0.810 | |
| | | −11,200 | | −9,100 | | 0.812 |
| 60 | −10,400 | | −8,100 | | 0.779 | |
| | | −9,900 | | −7,800 | | 0.788 |
| 70 | −9,100 | | −6,700 | | 0.736 | |
| | | −9,000 | | −6,000 | | 0.733 |

[a] The membrane suspension was kept at pH 6.9, for 30 min at the desired temperature, then diluted either to pH 6.9 or to 10.8, and CD spectra performed at room temperature.

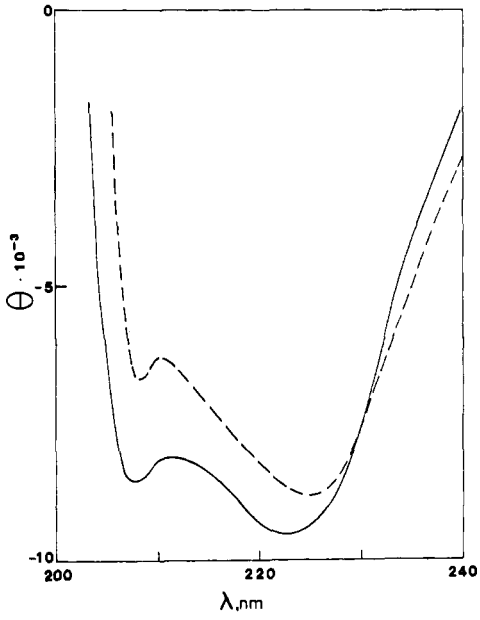


FIGURE 9: CD spectra of chloroform–methanol treated membranes. - - - - - -: at pH 6.9; ——: at pH 11.1.

## Discussion

The presence of optical artifacts makes the interpretation of CD spectra of particulate systems difficult and often doubtful. As indicated by Urry (1970) and by Glaser and Singer (1971), three sources of optical artifacts are to be investigated: light scattering on the particles, which causes a fraction of incident light to be unavailable for interaction with the chromophores; high local absorption, due to concentration of the chromophores in particles, so that a fraction of the chromophores is masked and becomes unavailable for interaction with incident light; differential light scattering, due to a different refractive index of the particles for left and for right circularly polarized beams, and resulting in a distortion of the CD spectrum.

The first artifact, the magnitude of which is indicated by the turbidity of the suspension, causes a decrease of intensity of specific absorption bands and of ellipticity bands. The second one has a similar "flattening" effect. The third one, which exerts its effects on CD spectra but which is obviously related to the general light-scattering properties of the suspension, modifies the shape of the CD spectrum by contributions which have a distribution similar to an ORD spectrum (Urry, 1970); it causes therefore a red shift of the long-wavelengths branch of the 222-nm band, and makes a

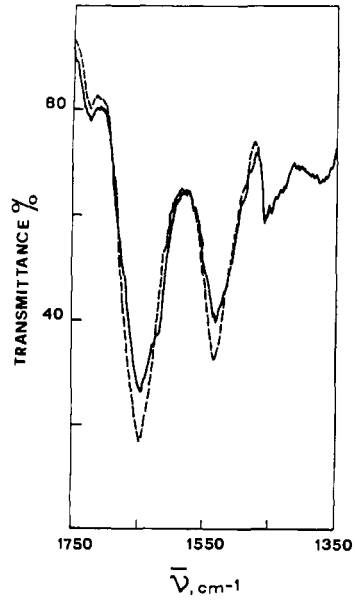


FIGURE 8: Ir spectrum of a film cast from a membrane suspension exposed for 30 min to 70° at neutral pH (full line). The dotted line indicates the spectrum of the native membranes at neutral pH, taken from Figure 5.

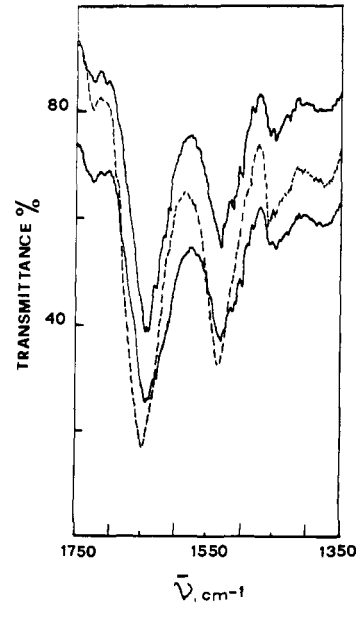


FIGURE 10: Ir spectra of chloroform–methanol-treated membranes. Lower continuous curve: at neutral pH; upper continuous curve: at alkaline pH (for conditions, see Figure 5). The dotted curve represents the spectrum of native membranes at neutral pH, taken from Figure 5.

positive contribution in the region below 210 nm (Urry and Krivacic, 1970; Gordon and Holzwarth, 1971). It should be noted that, according to Schneider *et al.* (1970), the $[\theta_{208}]$:$[\theta_{222}]$ ratio of hemoglobin is not altered when this protein is confined within erythrocyte membranes, although extensive flattening occurs. The ratio is sensibly decreased only in conditions of extremely high scattering, such as in water–silicone oil emulsions. More recently, application of Mie scattering theory to erythrocyte membranes, considered as spherical shells, has been shown to explain the red shift in the 230-nm region, but only partially the low value of the $[\theta_{208}]$:$[\theta_{222}]$ ratio (Gordon and Holzwarth, 1971).

Since hydroxyl ions have a high absorbancy in the 200-nm region, variations of the refractive index of the solvent should also be taken into consideration. The pH insensitivity of heat-treated membranes indicates anyhow that, in the range considered, this effect is negligible.

The increase of ellipticity at 208 nm observed by us upon exposure of native membranes to high pH corresponded to a decrease of light scattering, as indicated by the lower turbidity and the higher optical density at 208 nm. Similar effects have been shown to occur upon addition to the membranes of sodium dodecyl sulfate, which converts the suspension to an optically clear solution (Lenard and Singer, 1966; Gordon and Holzwarth, 1971), or upon fragmentation of the membranes (Schneider *et al.*, 1970; Glaser and Singer, 1971). On the other hand, a protein-bound spin label has been shown to lose its mobility restrictions at pH >9.5 (Chapman *et al.*, 1969). A partial solubilization of the membrane can therefore be assumed to occur at high pH. Also the occurrence of hemolysis upon exposure of intact erythrocytes to high pH could be due to modifications of membrane architecture leading to enhanced membrane permeability; it could be explained also by other hypotheses, such as osmotic inbalance, oxidation of sulfhydryl groups, or other kinds of metabolic damage (Whittam, 1964).

No attempt was made to correct CD spectra for optical artifacts, since the various theories (Glaser and Singer, 1971; Gordon and Holzwarth, 1971; Urry *et al.*, 1971) are not in full agreement, and moreover our measurements of absorption and of optical activity were performed on instruments having a different geometry. In our hands, solubilization or fragmentation procedures caused, as compared to high pH, larger effects on turbidity and smaller ones on the $[\theta_{208}]$:$[\theta_{222}]$ ratio.

Moreover, when heat-treated membranes were sonicated, although turbidity was considerably decreased, the CD spectrum was not modified. The possibility of a transition, at pH >9.5, of membrane proteins toward a different conformation, most probably a random coil, should therefore be taken into consideration. This hypothesis is substantiated by the irreversibility of the phenomenon (especially if the membranes are exposed to the alkaline pH at high temperature), and by the shift, at alkaline pH, of the ir spectrum of membranes which had been partially converted to a $\beta$ conformation. The two possibilities—conformational change of the membrane proteins and depolymerization of the membrane—are not mutually exclusive, and could instead be intimately related.

The intensity of the CD signals of membrane suspensions, which is already low in native membranes, became even weaker after exposure to heat. A similar result, which was not further investigated, had been obtained by Glaser *et al.* (1970). Neither in native nor in heat-treated membranes did the signal get stronger after sonication, though fragmentation occurred. Although other possibilities may also be invisaged, we may consider membranes as particles which, irrespective of their size, have a high local density of peptide chromophores; upon heat treatment or formalinization—as well as upon treatment with other fixatives (Lenard and Singer, 1968)—this density may increase, possibly because of formation of interchain rigid bonds, leading to a more marked flattening of all optical signals. The experimental data do not support the hypothesis (Glaser *et al.*, 1970) of a decrease, after heat treatment, of the amount of $\alpha$ helix.

Comparison of ir and CD spectra of modified membranes poses an intriguing problem about the optical activity of membrane proteins when in $\beta$ conformation. Even when, *e.g.*, after shaking with chloroform–methanol, a conspicuous amount of $\beta$ structure appeared to be present, the CD spectrum was not grossly modified. It should be noted that mitochondrial membranes exhibit a certain amount of $\beta$ structure as judged from ir (Graham and Wallach, 1969), but this does not appear in the CD or ORD spectra (Urry *et al.*, 1967; Ji and Urry, 1969). Even membranes cast from 2-chloroethanol, which according to the CD spectra are definitely in a $\alpha$-helix conformation, when examined by ir show evidence of $\beta$ structure (Wallach and Zahler, 1966). Further investigations are needed to elucidate this point.

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# Binding of Aminoacyl Transfer Ribonucleic Acid Synthetases to Ribosomes from Rabbit Reticulocytes†

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ABSTRACT: Some, but not all, of the aminoacyl-tRNA synthetases present in lysates of rabbit reticulocytes are recovered in high proportion with ribosomes collected by high-speed centrifugation. Up to 90% of the phenylalanyl-tRNA synthetase activity in the lysate is recovered in the ribosomal fraction and appears to have been physically bound to the ribosomes in intact cells. Synthetase activity for lysine and arginine is bound to the ribosome but also appears as a high molecular weight complex or aggregate that sediments at approximately 14 S. Aminoacyl-tRNA synthetases are removed from the ribosomes by 0.5 M KCl but may be rebound to the ribosomes or ribosomal subunits under suitable conditions in solutions of moderate ionic strength. The synthetases for phenylalanyl-tRNA, lysyl-tRNA, and arginyl-tRNA bind preferentially to the large subunit.

We have reported previously the existence of a factor in the salt-wash fraction from reticulocyte ribosomes which promotes binding of *N*-AcPhe-tRNA or Phe-tRNA to nitrocellulose filters or to ribosomes (McKeehan *et al.*, 1970). This factor was identified as Phe-tRNA synthetase on the basis of cochromatography of the activity for binding and the synthesis of Phe-tRNA plus the specific inhibitory effect of phenylalanine and ATP on *N*-AcPhe-tRNA binding. It was concluded that a phenylalanyl adenylate complex was formed on Phe-tRNA synthetase bound to ribosomes. Here, we extend these studies and demonstrate an interaction of other synthetases with ribosomes.

## Experimental Procedure

*Materials.* CHEMICALS. All labeled amino acids were obtained from New England Nuclear, except for cysteine and tryptophan obtained from Amersham-Searle Co. Sephadex G-200 was purchased from Pharmacia.

SOLUTIONS. Solution A contained 2 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 1 mM EDTA, and 5% glycerol (v/v). Solution B contained 20 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.25 M sucrose. Solution C contained 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol.

*Methods.* The preparation of rabbit reticulocytes, regular ribosomes, ribosomal supernatant, rabbit liver tRNA, and aminoacyl-tRNA was as previously described (Hardesty *et al.*, 1971). In the procedure used, ribosomal pellets collected by centrifugation from lysates of reticulocytes are rinsed gently with solution B before the ribosomes are resuspended. This washing procedure removes loosely packed material from the surface of the ribosomal pellet.

RIBOSOMAL SUPERNATANT AMINOACYL-tRNA SYNTHETASES. Ribosomal supernatant protein was precipitated at 74% saturation of ammonium sulfate (466 g of ammonium sulfate per l.) without pH adjustment in the cold and isolated by centrifugation for 15 min at 15,000 rpm in the Sorvall SS-35 rotor. The precipitate was dissolved in 0.01 the original supernatant volume of solution A and dialyzed overnight against about 50 volumes of the same solution at 4°. The dialyzed enzyme fraction was stored at −90°. Generally, the final protein concentration of this fraction was about 50 mg/ml. The hemoglobin concentration was about 8 mg/ml.

SALT-WASHED RIBOSOMES. Regular ribosomes suspended in solution B were washed by a procedure modified from that of Miller and Schweet (1968). The ribosomal solution containing 20 mg/ml of ribosomes was adjusted to 0.5 M KCl by addition of 4 M KCl with stirring at 0°. The mixture was stirred for 15 min, and then centrifuged for 2.5 hr at 60,000 rpm in the 65 rotor (Beckman Instruments, Inc.). The supernatant from the centrifugation was removed and treated to give the salt-wash fraction as described below. The ribosomal pellet was rinsed three times with solution B, dissolved in the same solution, and adjusted to 20 mg of ribosomes per ml using a 1 mg/ml extinction coefficient for 260-

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