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Calorimetric Study of Protein Transitions in Human Erythrocyte Ghosts†

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ABSTRACT: Using differential heat capacity calorimetry, several well-defined thermal transitions can be detected for human erythrocyte ghosts at pH 7.4. These structural transitions are totally irreversible and are not seen for vesicles formed from lipid extracts of erythrocyte membranes and therefore must be due to thermal denaturation of membrane-bound proteins. It is shown that one bound enzyme (ATPase) does in fact lose its activity in the temperature region where the calorimetric transitions are observed. The denaturation pattern shows a strong dependence on ionic strength and this might be correlated with changes in the degree of protein–protein association on the membrane, which also shows a marked ionic strength dependence as judged by the freeze–fracture electron micrographs of Pinto da Silva (*J. Cell Biol. 53*, 777 (1972)). At an ionic strength of 0.07 M, calorimetric scans show four sharp and well-defined transitions which suggest that the bulk of the membrane protein may be organized into only a few different types of structural units.

With the fairly general acceptance of the bilayer as the basic structural framework of lipids in membranes, the major questions which are now posed concern the structure of membrane-bound protein molecules, the way in which proteins are organized in and on the membrane, and the identification of any alterations in the basic bilayer structure which might occur as a result of protein–lipid interactions. These are difficult questions to answer since solution methods applicable to membrane suspensions generally do not provide very detailed information about absolute structure and organization of protein molecules. One meaningful way of approaching the problem is to study changes in structure which occur in response to changes in solvent conditions. Hopefully, some conclusions about absolute structure can then be deduced. This approach has, in fact, been moderately productive of new information when applied to the phase transitions of lipid vesicles and of the lipid phase in membranes, but has not been widely applied to order–disorder transitions of membrane-attached proteins. Perhaps the reason for this is that the membrane proteins constitute a less cooperative system and transitions are therefore more difficult to study.

The most general technique for investigating structural transitions is calorimetry, since any process having a significant energy change may be detected. The utility of differential scanning calorimetry for detecting thermally induced phase changes in biological membranes has been demonstrated with studies of the plasma membrane of *Mycoplasma laidlawii* (Steim *et al.*, 1969; Melchoir *et al.*, 1970). For whole membranes two thermal transitions were observed; a reversible transition attributed to a phase change in the lipid bilayer and a smaller irreversible transition at higher temperature attributed to unfolding of the membrane protein.

Human erythrocyte ghosts have previously been examined by calorimetry (Chapman, 1968; J. Steim, personal communication) but no thermal transitions could be detected when using conventional DSC instruments which are adequate for studying lipid phase changes in other membranes. Using a more sensitive differential heat capacity calorimeter (Jackson and Brandts, 1970; Jackson, 1970) we have studied thermal transitions in human erythrocyte membranes as a function of solvent conditions and have examined the thermal behavior of lipid extracts from these membranes. In addition, we have investigated the thermal stability of a membrane bound enzyme function (ATPase).

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Experimental Section

Preparation of the Membranes. Membranes were isolated from freshly drawn human blood by the method of Dodge *et al.* (1963) at pH 7.4 in 20 mosm phosphate buffer. Changes in the suspending buffer composition were accomplished by overnight dialysis at 4°. The final concentration of the membranes was determined by drying to constant weight at 105°. Protein content of membranes was estimated by measuring the difference in optical density at 223 and 350 nm in a two wavelength spectrophotometer (Perkin-Elmer 356) with the optical cell mounted about 3 cm from the phototube to minimize scattering loss. This method was calibrated with protein determinations by the method of Lowry *et al.* (1951).

Membrane Fractionation. Membrane protein and lipid were separated by the butanol–water partitioning procedure of Rega *et al.* (1967). Lipids were recovered from the butanol phase by evaporating to dryness under nitrogen and reextracting the residue with chloroform. This extract was dried under nitrogen and the lipids were suspended in phosphate buffer by sonication. Microscopic examination of the suspension revealed well-defined vesicles.

Cholesterol was removed from the lipid fraction by acetone extraction according to Andreoli (1967). Cholesterol determination by the method of Rosenthal *et al.* (1957) indicated 81% removal.

Heat Capacity Measurements. Temperature-dependent heat capacities were measured in a high sensitivity differential calorimeter (Jackson and Brandts, 1970; Jackson, 1970). The twin 5-ml cells were filled with equal weights of sample and reference buffer and heated at a rate of 18°/hr. After subtracting an instrument base line determined in a separate experiment, the heat capacity per gram of suspended sample was calculated using an instrument constant checked by heat capacity measurement of KCl solutions. As described earlier, a small uncertainty in the amount of sample in the calorimeter cell leads to a relatively large uncertainty in the absolute value of heat capacity but has only a small effect on the temperature dependence. For this reason the reported heat capacities are shown on a relative rather than an absolute scale.

Measurement of ATP Hydrolysis. The amount of added ATP hydrolyzed by a membrane preparation in the calorimeter cell was estimated by sampling an identical preparation held in the calorimeter water bath which was always within 0.1° of the cell temperature. The enzymatic hydrolysis in these samples was stopped by the addition of 0.1 volume of 50% trichloroacetic acid, and the ADP concentration was determined by a pyruvate kinase procedure described by Wampler and Westhead (1968).

CD Measurements. Circular dichroism measurements on whole membranes were carried out in a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichorism attachment, using a thermostated 0.1-mm path-length optical cell. The ellipticity at 223 nm was calculated using a solvent base line determined in a separate series of experiments with 300 nm as the coincident reference point. Protein concentrations were determined by the method of Lowry *et al.* (1951) and a mean residue weight of 135 daltons was used.

Results

Heat capacity measurements of human erythrocyte membrane suspensions were carried out at pH 7.4 in several different phosphate buffers. The membrane concentrations ranged from 0.0085 to 0.017 g of membrane/g of suspension with protein content of the membranes ranging from 53 to 58% by weight. The membranes exhibit well-defined thermal transitions which show significant dependence on the composition of the suspending buffer as shown in Figure 1. Increasing the ionic strength from 0.018 (Figure 1a) to 0.071 (Figures 1b and 1c) by the addition of phosphate or NaCl causes increased resolution of the thermal transitions. The effect appears to be partially ion specific as can be seen by comparison of heating curves b and c, which are at identical ionic strengths. The small transition near 75° shows little dependence of buffer composition. A second heating of these suspensions showed that all of the observed transitions were *completely irreversible* although no gross morphological changes could be detected with the light microscope.

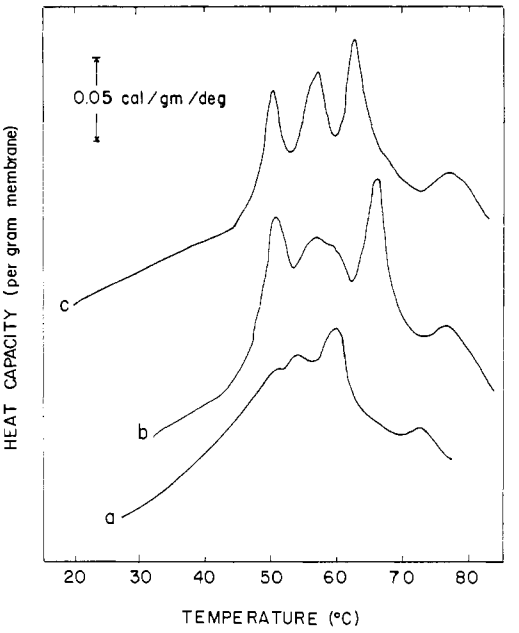

FIGURE 1: The heat capacity of human erythrocyte membranes, as a function of temperature. The heating profiles have been arbitrarily adjusted on the heat capacity axis. (a) Membranes suspended in 20 mosm sodium phosphate buffer (ionic strength = 0.018 M) (pH 7.4). (b) Membranes suspended in 20 mosm phosphate buffer with 53 mM sodium chloride added (ionic strength = 0.071 M) (pH 7.4). (c) Membranes suspended in 77 mosm sodium phosphate buffer (ionic strength = 0.071 M) (pH 7.4).

The total enthalpy change, ΔH, for the observed thermal transitions in each of these membrane preparations may be estimated by taking the integral of the experimental heat capacity in the transition region and subtracting a base line contribution arising from the normal temperature dependent heat capacity of the membranes that would be expected in the absence of a transition (Jackson and Brandts, 1970). It is this latter subtraction which introduces the greatest uncertainty in the determination of the enthalpy primarily because of the lack of a well-defined posttransition heat capacity. Using a linear base line which connects the experimental heat capacity at the beginning of the transition near 40° with the heat capacity minimum near 70°, the ΔH values for the experiments shown in Figure 1 were found to be of the order of 1.5–2 cal/g of membrane or 3–4 cal/g of protein. This represents a minimum estimate of the total ΔH, since the small high temperature transition near 75° is not included.

Sonication of membranes suspended in 0.018 M ionic strength phosphate buffer at pH 7.4 for 20 sec was sufficient to cause a large reduction in the average ghost diameter and fragmentation of about 25% of the original intact ghosts, but this treatment had only a small effect on the temperature

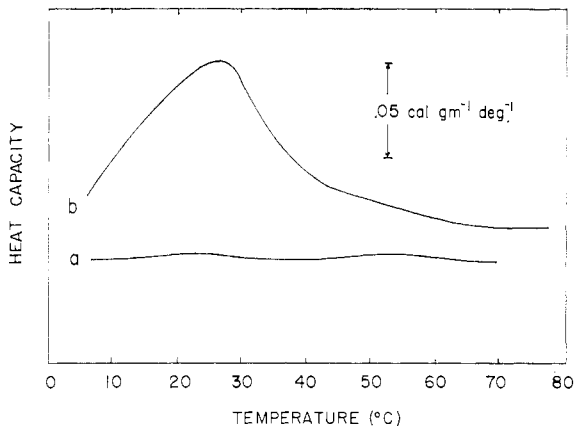

FIGURE 2: The heat capacity of erythrocyte membrane lipids, as a function of temperature. The suspending buffer is 77 mosm sodium phosphate buffer. (a) Total lipid extract; (b) membrane lipids after removal of 81% of the original cholesterol.

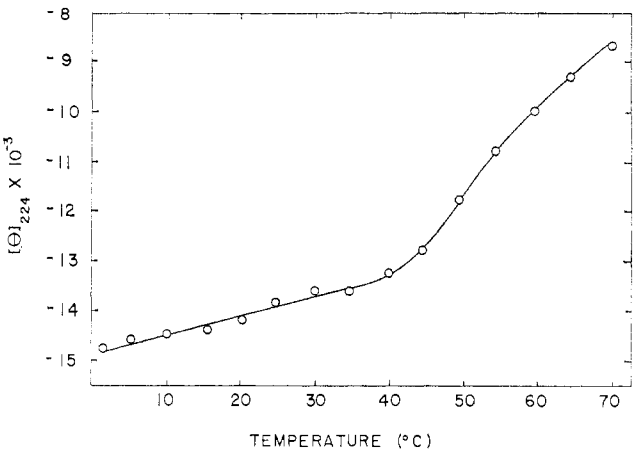

FIGURE 3: Temperature dependence of the ellipticity at 224 nm for an erythrocyte membrane suspension in 20 mosm sodium phosphate buffer (pH 7.4).

dependence of the heat capacity (data not shown). The broad transition occurred over the same temperature interval while the small high temperature transition was shifted to 67° compared with 77° for the unsonicated membranes shown in Figure 1.

In an attempt to determine whether the thermal transitions observed in whole membranes arise from protein conformational changes or from lipid phase changes, heat capacity measurements were carried out on suspensions of the membrane lipid obtained by butanol extraction. Although the lipid in these suspensions was about twice as concentrated as in the whole membrane suspensions, no thermal transition could be detected as shown in curve a of Figure 2. This result is in agreement with that reported by Ladbrooke *et al.* (1968). The lack of a characteristic lipid phase change can be attributed to the presence of a high concentration of cholesterol, which interferes with the formation of a highly cooperative bilayer. When 81% of the cholesterol was removed by acetone extraction, the remaining lipid fraction showed a well-characterized phase change centered at about 25° as shown in curve b of Figure 2. This transition was reversible as determined by heating the sample through the transition region a second time. Although the temperature-dependent heat capacity is qualitatively similar to that obtained by Ladbrooke *et al.* (1968), their transition for a phospholipid extract was centered at about 37°, probably reflecting the higher degree of cholesterol removal in their extraction procedure.

From these results we conclude that it is highly unlikely that any of the thermal transitions observed in whole membranes arise from a cooperative phase change associated *only with the lipid* and that this is because of the high concentration of cholesterol in the intact membrane.

In a further attempt to determine the origin of the thermal transitions observed in whole membranes, the temperature dependence of the circular dichroism was measured. Since the optical activity of the erythrocyte membrane is known to arise from the bound protein, any changes in the ellipticity should reflect changes in protein conformation. Figure 3 shows the change in ellipticity at 224 nm for membranes suspended in 0.018 M ionic strength phosphate buffer at pH 7.4. Although it is uncertain as to what extent the errors due to light scattering (Glaser and Singer, 1971; Urry and Krivacic, 1970; Urry and Ji, 1968) influence the ellipticity values at each temperature, the break in the curve at 40° coincides with the onset of the calorimetrically observed transition shown in Figure 1, curve a.

Similar results were obtained for human erythrocyte membranes under nearly identical solvent conditions by Sheetz and Chan (1972) using proton magnetic resonance. They found that the area of the protein methyl resonance began increasing significantly at about 40°, indicating a protein conformational change, while the area of the lipid choline methyl resonance underwent a monotonic change with temperature, demonstrating the absence of a cooperative lipid phase transition.

Since it appears highly likely that the observed thermal transitions in erythrocyte membranes arise from protein conformational changes, an attempt was made to correlate this behavior with membrane function. The hydrolysis of ATP is a conveniently measured function since the large heat of reaction is easily detected in the calorimeter. This system is also of interest in the light of experiments carried out by Penniston and Green (1968) in which erythrocyte membranes from cow, pig, and rabbit blood were observed by electron microscopy to undergo significant morphological changes when incubated with ATP in the presence of Mg^{2+}, K^+, and Na^+. In a follow up study of this effect using human erythrocyte membranes, Graham and Wallach (1971) found that ATP induced a protein conformational change as detected by infrared spectroscopy.

Using pH 7.0 TES buffer with added Mg^{2+}, K^+, and Na^+ as described by Penniston and Green (1968) we have determined the temperature dependence of the heat capacity of membranes in the presence (curve b in Figure 4A) and absence (curve a in Figure 4A) of ATP, with other conditions identical. Since the hydrolysis of ATP is strongly exothermic, the reaction makes a negative contribution to the measured heat capacity. At a given temperature, the magnitude of this contribution will be proportional to the *rate* of hydrolysis. The large changes in heat capacity in the temperature region from 30 to 60°, seen in the presence but not in the absence of ATP, are therefore a reflection of the large temperature dependence in the rate of ATP hydrolysis. The difference between the two curves in Figure 4A should then be proportional to the rate of hydrolysis, and this difference curve is shown in Figure 4B. This is plotted as the negative or exothermic contribution. The total area under this heat capacity difference curve is 0.21 cal, which is a measure of the total amount of ATP hydrolyzed during the experiment. Using a value of −4.7 kcal/mol for the heat of hydrolysis (Podolsky and Morales, 1956) and after

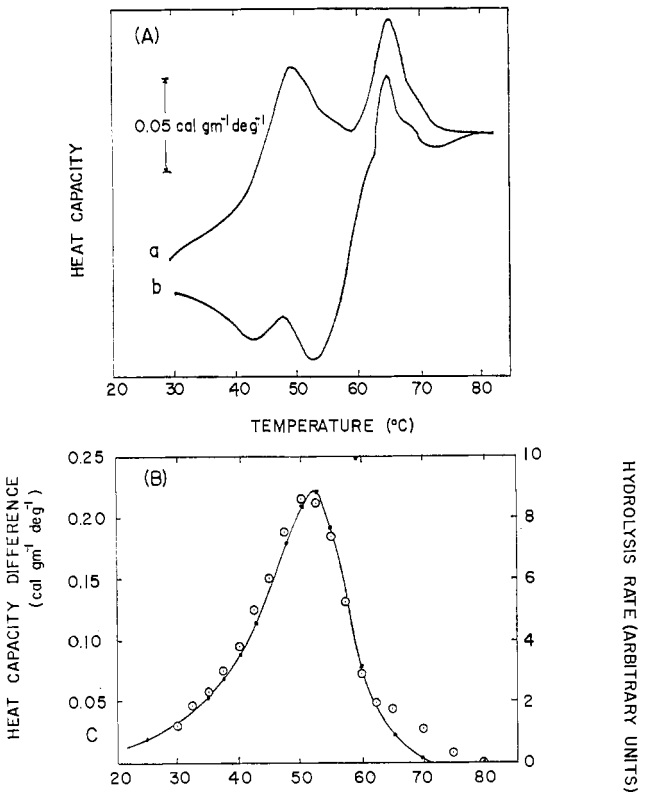

FIGURE 4: Estimate of the rate of ATP hydrolysis by calorimetry. (A) Heat capacity of membranes (1.07% by weight) suspended in 20 mM TES buffer (pH 7.04) with 100 mM NaCl, 20 mM KCl, and 4 mM $MgCl_2$: (a) with no added ATP; (b) same as in a, but with 12.3 mM ATP added. (Curves a and b have been adjusted on the heat capacity axis, so that they coincide at temperatures above 80°.) (B) Estimate of that part of the apparent heat capacity of curve b, Figure 4A, which is due to the hydrolysis of ATP. The points were obtained by subtracting curve b of Figure 4A from curve a of Figure 4A. The solid line passing through the points represents calculated values of the hydrolysis rate, based on eq 2 (see text).

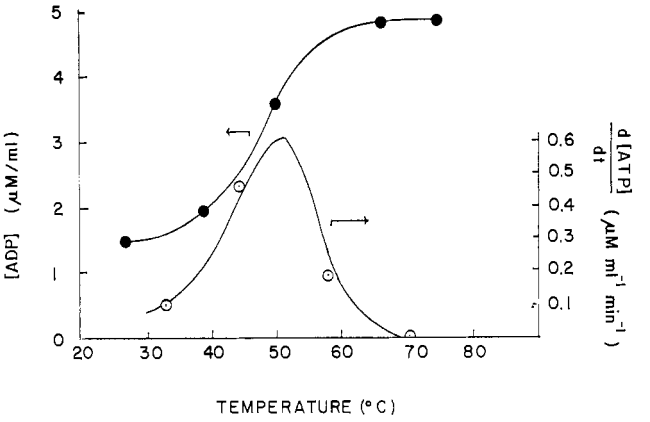

FIGURE 5: Direct measurement of ATP hydrolysis. The suspending buffer was the same as in Figure 4, but with initial ATP concentration of 11.8 mM and membrane concentration of 0.96 wt %. (a) The extent of ATP hydrolysis determined by the chemical analysis of the ADP liberated. The heating rate was 18°/hr. (b) The rate of ATP hydrolysis, determined from the data in curve a and the known heating rate of 18°/hr.

subtracting the enthalpy contribution from the changes in buffer ionization due to proton liberation resulting from hydrolysis of ATP, we estimate that about 40% of the ATP initially present was hydrolyzed. This shows that excess substrate is always present so that the decrease in rate at high temperatures cannot be due to the substrate depletion.

These results suggest that the activity of ATPase goes through a sharp maximum centered at about 50°. In order to substantiate this conclusion obtained by calorimetry, the rate of hydrolysis of ATP was measured directly by a chemical analysis for ADP production. Under almost identical conditions and with the same heating schedule used in the calorimetric experiment, the concentration of ADP as a function of temperature is shown as curve a of Figure 5. The initial value of the ADP concentration at 27° represents the total accumulation of product during the time interval between addition of ATP and commencement of heating, which was about the same time as for the calorimetric experiment of Figure 4. At high temperatures, the ADP concentration levels off at 4.8 μmol/ml (corresponding to hydrolysis of 40% of the initial ATP), suggesting that catalyzed hydrolysis of ATP is no longer occurring. Curve b shows the rate of hydrolysis as a function of temperature, calculated from the data in curve a. It shows a pronounced maximum centered near 50°, thus confirming the type of behavior which was found from the analysis of the calorimetric data in Figure 4.

The bell-shaped rate profile suggests that thermal denaturation of ATPase occurs at temperatures above 50°. The rate for an enzyme-catalyzed reaction can be expressed (Brandts, 1967) as

$$\text{rate} = [\text{N}]c \exp(-E_a/RT) \quad (1)$$

where E_a is the apparent activation energy, [N] is the concentration of *native* enzyme, and c is a factor which depends on the concentration of reactants and the Arrhenius frequency factor. If it is assumed that ATP was always present in sufficient excess during the course of the experiment so that all of the catalytic sites were saturated, c may be taken as a constant. If the enzyme is thermally labile, then [N] will be temperature dependent. Treating the denaturation reaction as a two-state process, [N] = [D], then eq 1 becomes

$$\text{rate} = \frac{[\text{N}_0]}{1 + \exp(\Delta S/R - \Delta H/RT)} c \exp(-E_a/RT) \quad (2)$$

where $[\text{N}_0]$ is the concentration of native enzyme at a temperature below the transition region, and where ΔH and ΔS are the enthalpy and entropy change for the denaturation. The solid line passing through the experimental points of Figure 4B is a plot of eq 2 using E_a = 20 kcal/mol, ΔH = 83 kcal/mol, and ΔS = 253 eu/mol. The latter two thermodynamic parameters are in the range usually found for thermal denaturation reactions and suggest that the transition occurs with a midpoint T_m = 55°. This coincides approximately with the small shoulder between the two main heat capacity peaks in Figure 4A, curve a. The apparent activation energy E_a of 20 kcal is somewhat higher than the 12–13 kcal reported for myosin ATPase catalyzed hydrolysis (Ouellet *et al.*, 1952; Levy *et al.*, 1959). However, erythrocyte ATPase is known to be located on the inner surface of the membrane (Marchesi and Palade, 1967) so that temperature dependence of the transport process could conceivably contribute to the apparent activation energy and the above estimate cannot be regarded as a fundamental parameter. The thermodynamic parameters ΔH and ΔS must also be regarded as phenomenological estimates, since the ATPase denaturation is essentially irreversible. Nevertheless, the comparison of eq 2 with the experimental data does suggest, at a qualitative level, that the increase in activity below 50° may occur from the normal thermal enhancement

of the hydrolysis reaction while the decrease in activity above 50° results from denaturation of the enzyme.

It should be pointed out that calorimetric data on red cell ghosts are not as reproducible as calorimetric data on purified proteins. Scans on aliquots of the same preparation on successive days lead to good reproducibility generally, but we have found some variability in the relative magnitudes and temperatures of the protein thermal transitions for aliquots of the same sample with different storage times. We have also failed to achieve complete reproducibility with independently prepared suspensions, usually from different donors. This was particularly true under low salt conditions (*e.g.*, Figure 1, curve a) where the transitions are poorly defined. However, for samples in the 77 mosm phosphate buffer (Figure 1, curve c) where the thermal transitions are very welld efined, excellent reproducibility was observed for the five separate experiments carried out on different preparations at different times.

Discussion

This study demonstrates the utility of heat capacity calorimetry for detecting thermal transitions in human erythrocyte membranes. The observed transitions appear to be associated with membrane-bound protein rather than with lipid phase changes, in contrast to the results obtained for *Mycoplasma laidlawii* (Steim *et al.*, 1969; Melchoir *et al.*, 1970). In the latter case, the heat capacity curves were dominated by a reversible lipid phase transition, making it difficult to study the contribution from the protein component. The absence of a lipid transition for erythrocyte membranes probably results from the higher cholesterol content, which disrupts the cooperative interactions between the phospholipid molecules.

The observed thermal transitions are most likely due to denaturation of the erythrocyte membrane protein. This view is supported by the apparent decrease in α-helical content as measured by the change in ellipticity at 224 nm and by the proton magnetic resonance studies of Sheetz and Chan (1972). In the latter case, the sharpening of the protein resonances at temperatures above 40° indicates a significant increase in protein side chain mobility which would be expected if a protein unfolding reaction occurred. Also, it was demonstrated that at least one protein (ATPase) does in fact undergo denaturation in the temperature region where the calorimetric transitions are seen. Our calorimetric results show that there is a minimum enthalpy change of 3–4 cal/g of membrane protein. Since the denaturation of most soluble proteins involves an enthalpy change of 4–7 cal/g at these same temperatures, a large fraction of the membrane protein is apparently being denatured.

The heat capacity curves for whole membranes show a very strong dependence on the ionic strength of the suspending buffer. This is particularly apparent when comparing the transitions in the 77 mosm phosphate buffer (Figure 1, curve c) with those in the 20 mosm phosphate buffer (Figure 1, curve a). The three sharp, well-defined transitions which are seen in the high salt case in the 40–65° region are merged together and poorly resolved in the low salt buffer. This suggests that ionic strength plays an important role in determining the state of protein molecules in the native membrane. The sensitivity of erythrocyte membrane protein to changes in ionic strength has also been demonstrated in the recent study of Pinto da Silva (1972). Using the freeze–fracture technique, he observed by electron microscopy that the protein particles in the plane of the membrane were extensively aggregated when the membranes were suspended in pH 5.5 buffer at low ionic strength while at higher ionic strengths the aggregation did not occur. The same pattern of results was observed at pH 6.2, although the low ionic strength aggregation was less extensive than at pH 5.5. However, at pH 7.5, which most closely corresponds to the conditions of our experiments, Pinto da Silva found no unusual association of the protein particles at low ionic strength and no detectable change as salt was added. Thus, the large effect of ionic strength on the state of membrane protein molecules observed in our calorimetric experiments is apparently not detectable by freeze–fracture studies. It nevertheless seems possible, based on the results obtained by electron microscopy at slightly lower pH, that the ionic strength effect observed calorimetrically at pH 7.4 is caused by changes in the degree of protein–protein association and that these changes are either too subtle to be detected by or too fragile to withstand the preparative treatment of the freeze–fracture technique.

There is also independent evidence in the literature which suggests that red cell membranes undergo very cooperative changes in certain functional properties as the ionic strength (NaCl) is lowered below about 30 mosm at room temperature (Rubalcava *et al.*, 1969). Perhaps the most striking change is that, below this value, the membranes become "leaky" to cations and to some large uncharged solutes such as sucrose, *i.e.*, they no longer function as osmometers. Although this alteration in transport characteristics had previously been thought to be caused by structural changes in the lipid phase (Rubalcava *et al.*, 1969), our results suggest that the protein component may be a more important participant since there is a very large change in the denaturation pattern as the ionic strength passes through the range noted above (Figure 1, curve a *vs.* curve b or c).

This study is not sufficiently complete to permit any conclusions regarding the detailed structure of the erythrocyte membrane, but the fact that only four well-defined thermal transitions could be observed under certain buffer conditions (Figure 1, curve c) is particularly noteworthy when one considers the enormous multiplicity of protein mediated functions associated with the membrane. Although many of the functional proteins may be present in such low concentration that their thermal denaturation is below the range of detection in our calorimeter, at least 17 different molecular weight polypeptides are in sufficient concentration to be easily detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Trayer *et al.*, 1971). Therefore it seems likely that each of the calorimetrically observed transitions represents the denaturation of more than a single type of protein molecule. This suggests that the structural transitions of chemically different proteins are coupled together either because of direct physical association into high molecular weight complexes or by some other cooperative mechanism working through the lipid phase. Because of the narrowness and relatively high degree of separation that could be attained for these transitions at 77 mosm, it seems improbable that the heat capacity curves result from the *accidental* superposition of a large number of uncoupled transitions at a few discrete points along the temperature axis.

Grouping of the multiplicity of membrane polypeptide chains into a relatively small number of classes has also been demonstrated by gel chromatography. Using a neutral detergent (Brij 36T) which can solubilize erythrocyte membrane protein in the native state, Gitler (1971) obtained four well-separated protein bands from a 4% agarose column. Although the correlation of this result with our calorimetric data is not yet clear, it at least indicates that a highly heterogeneous mixture of membrane proteins can demonstrate simplified phys-

ical behavior. Clearly, however, more experimental results are needed before it can be definitely concluded that a large fraction of the total protein of the erythrocyte membrane is indeed organized into a few well-defined structural units under certain solvent conditions.

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Comparison of Human Hemoglobin A Carrying Glutathione as a Mixed Disulfide with the Naturally Occurring Human Hemoglobin A_3†

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ABSTRACT: A method for the quantitative preparation of a mixed disulfide between native human hemoglobin A and glutathione (Hb ASSG) without formation of methemoglobin is described. Properties of the uniformly modified Hb ASSG are presented. One glutathione molecule was found to be bound to the cysteinyl residue in position β-93 (F-9). The failure to find such a hemoglobin in normal hemolysates and in the blood from two patients with Heinz body anemia suggests that this compound does occur, if at all, in less than 0.1% of the total hemoglobin. The naturally occurring minor component of human blood, Hb A_3, with chromatographic and electrophoretic properties nearly identical with the ones of Hb ASSG, is not a mixed disulfide. Hb ASSG is converted *in vitro* to Hb A by GSH but not by glutathione reductase from human erythrocytes, when both are used at physiological concentrations.

In contrast to most cells, the mammalian reticulocyte loses its ability to synthesize proteins after being present for 1 or 2 days in circulating blood. Beyond the reticulocyte stage the human erythrocytes survive for over 100 days with their original protein complement and therefore appear to be a favorable system for studying the "aging" of protein molecules. Oxidative processes in erythrocytes are considered to be such aging phenomena. Indeed, hemoglobin A carrying a glutathione molecule as a mixed disulfide on the β chains (Hb ASSG)[1] has been found in undialyzed hemolysates and

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[1] Nonstandard abbreviations used are Hb ASSG, hemoglobin carrying as a mixed disulfide 1 mol of glutathione/mol of β chain; CM-Hb A, hemoglobin with the thiol groups at position β-93 carboxyamidomethylated; GSH, reduced glutathione; GSSG, oxidized glutathione; *p*-ClHgBzO, *p*-chloromercuribenzoate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate).