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pH-Dependent Changes in Mitochondrial Membrane Structure

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Abstract

The pH-dependent changes in structure of submitochondrial vesicles prepared from rat liver have been investigated by a variety of structural "probes". The main changes are: (a) the volume of the vesicles as assessed by electron microscopy and packed volume is dependent upon pH, being a minimum at pH 5. Between pH 5 and pH 9 the changes are reversible; (b) the accompanying light-scattering changes are also sensitive to divalent cations; (c) the binding characteristics of 8-anilinonaphthalene-1-sulfonic acid indicate pH-dependent changes in the amount of net charge on the membrane; (d) above pH 4, circular dichroism spectra show alterations characteristic of changes in quaternary protein structure; (e) below pH 4, infrared studies indicate changes in protein secondary conformation are also taking place. From these results, the nature and limits of conformational (molecular) and configurational (morphological) changes in mitochondrial membranes following changes in H^+ activity are better defined. In the physiological range, pH-dependent conformational changes are confined to reversible changes in quaternary structure resulting from alterations in membrane charge.

Introduction

Mitochondria are known to pass through a hierarchy of structural changes in response to the transduction of energy accompanying electron transport or ATP hydrolysis. These structural changes can be divided into three main categories: (a) volume (or size) changes resulting, for example, from osmotic ion gradients; (b) configurational (or shape) changes. These may result not only from osmotic volume changes which affect the shape of membrane systems, but also from internal structural changes in the membrane itself; (c) conformational changes of secondary, tertiary or quaternary nature in the molecular components of the membrane. A major objective of current research is to identify the detailed nature of the structural changes accompanying energy transduction and to determine the sequence of these changes following the primary energetic event brought about by electron transport or ATP hydrolysis.

Two views on the mechanism of energy coupling have recently been receiving considerable attention. The first, developed by Mitchell and coworkers,[1] proposes that energy from electron transport is conserved in the form of an electrochemical gradient of protons which can be utilized to drive energy-linked reactions such as ion transport and ATP synthesis. In the second scheme, the process of electron transport leads, by means of conformational changes[2] to the conservation of energy in the form of a chemical intermediate[3–5] or is directly conserved in the form of conformational changes in the mito-

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chondrial membranes.[6] The conserved energy can then be utilized to drive ion transport, including protons, and ATP synthesis. The main controversy surrounds the question of whether or not the primary energy-conserving event brought about by electron transport is H^+ gradient or a conformational change. (The latter is distinguished from any conformational changes accompanying the H^+ transfer reactions of the chemiosmotic theory. These do not comprise the high-energy intermediate of energy coupling which, in that scheme, resides in the H^+ gradient.) Neither the measurement of pH changes in the external medium nor the use of electron microscopy are sensitive enough techniques in time or resolution to afford critical answers to this question.

The present investigation was undertaken with the view of examining the relationship between H^+ gradients and conformational changes in mitochondria by examining the influence of H^+ ions on conformational, configurational, and volume changes in submitochondrial vesicles. Knowledge of the effects of H^+ activity on structural changes in mitochondria is needed before a more complete understanding of electron transport-dependent changes in H^+ gradients can be evaluated in terms of mitochondrial membrane function.

Materials and Methods

Mitochondria were isolated from rat liver in 300 mM sucrose, 1 mM Tris methyl-2-amino ethane sulfonic acid (TES) buffer as previously described.[8] Submitochondrial vesicles were prepared from mitochondria, frozen and thawed once, by sonicating in 50 mM sucrose (using a Branson sonifier set at maximum output for 45 sec) and collecting, by centrifugation, the fraction between 10,000 × *g* for 10 min and 104,000 × *g* for 40 min. The submitochondrial vesicles were suspended in distilled water for use. Antimycin A (270 nM) was added to inhibit respiration and exclude any metabolically linked structural changes.

Ninety degree light scattering was monitored at 546 nm. Packed volume was measured on samples placed in capillary tubes as described perviously[9] using a centrifugal force of 104,000 × *g* for 20 min. Circular dichroism spectra were obtained at room temperature on a Cary, Model 60, Spectropolarimeter with circular dichroism attachment using a 1 mm cell. For infrared measurements, the samples were frozen, lyophilized to constant weight, and resuspended in mineral oil (Parke, Davis and Co., Detroit). Spectra were obtained on a Perkin-Elmer Grating Spectrophotometer, Model 257. Front-face fluorescence between 455 nm and 678 nm was detected at an angle of approximately 20° to the incident exciting beam (290–404 nm). Details of the apparatus have been given previously.[10]

Protein concentrations were measured on unfixed samples by the Folin method.[11] The sodium salt of 8-anilinonaphthalene-1-sulfonic acid (ANS) was recrystallized from water. Redistilled glutaraldehyde was obtained as an 0·8 M aqueous solution (pH 7·0), stored in ampoules under N_2, from Polysciences, Inc., Warrington, Pennsylvania.

For electron microscopy, the samples were prefixed for 30 min with glutaraldehyde (final concentration of 60 mM at the required experimental pH), after which osmium tetroxide was added to a final concentration of 1% and the particles immediately centrifuged at 30,000 × *g* for 10 min, washed in distilled water, recentrifuged, and placed in 2% osmium tetroxide for 2 h. After dehydration in ethanol, the samples were embedded in Epon for thin sectioning.

Results

pH-dependent Light Scattering and Volume Changes

The pH dependence of light scattering and packed volume of submitochondrial vesicles is shown in Fig. 1. As the pH of the suspending medium is decreased from 11 to 5·5, the light scattering level increases and the packed volume decreases. At approximately pH 5·5 both measurements reach an extremum after which the light scattering level falls and the volume becomes greater as the acidity is increased.

Glutaraldehyde-fixed vesicles did not show any light scattering change within ±5% over the range pH 6 to pH 10. The effect of pH on the interstitial space in the packed volume measurements was measured using glutaraldehyde-fixed vesicles where structural changes, as shown by the light scattering experiment above, do not occur. Fixed vesicles were centrifuged at different pH values. The packed volume over the range pH 6·5 to pH 9·0 increased only by 5%. Hence changes in packed volume in unfixed vesicles are mainly due to the volume changes occurring in the vesicles and are not due to a variation in interstitial space by pH.

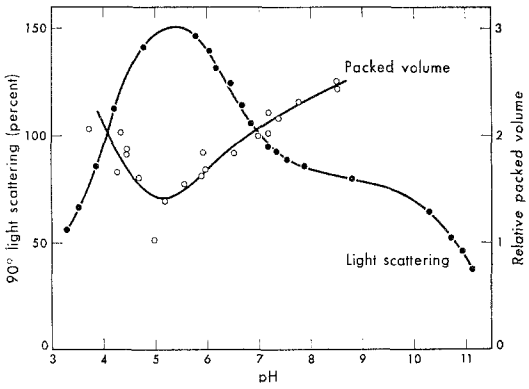

Figure 1. pH dependence of packed volume and light scattering of submitochondrial vesicles. Measurements were done in distilled water at concentrations of 5–10 mg protein/ml for packed volume (○) and 0·2 mg protein/ml for light scattering (●). pH of the suspension was changed by adding small quantities (generally less than 5λ) of either 10 mM NaOH or 10 mM HCl to the rapidly stirred reaction mixtures. Light scattering is expressed relative to its value at pH 7·0.

Electron micrographs taken at pH 4, 6, and 9 (Fig. 2) show the striking difference in average vesicle size over this pH range. Between pH 6 and 9, the *maximum* diameter increases from approximately 0·2 μ to 0·35 μ (Fig. 2 b and c, inset). Below pH 6 flocculation of the particles can be seen (Fig. 2a). This effect is also apparent in light scattering changes below pH 6 when high concentrations (greater than 0·5 mg protein/ml) are used.[12]

Effect of Ionic Concentration on Light Scattering

The effect of various monovalent and divalent ions on the light scattering level of submitochondrial vesicles is shown in Table I. At pH 7·0 the addition of 100 mM

TABLE I. Effect of inorganic ions on submitochondrial vesicle light scattering

Salt added	Light scattering (%)
None	100
NaCl, 100 mM	85 ± 3
KCl, 100 mM	84 ± 3
Tris-EDTA, 0·14 mM	93 ± 2
$MgCl_2$, 0·7 mM	119 ± 4
$CaCl_2$, 0·7 mM	123 ± 4

Conditions of measurement were: submitochondrial vesicles, 0·7 mg protein/ml; antimycin A, 270 nM; in a total volume of 7·0 ml at pH 7·0.

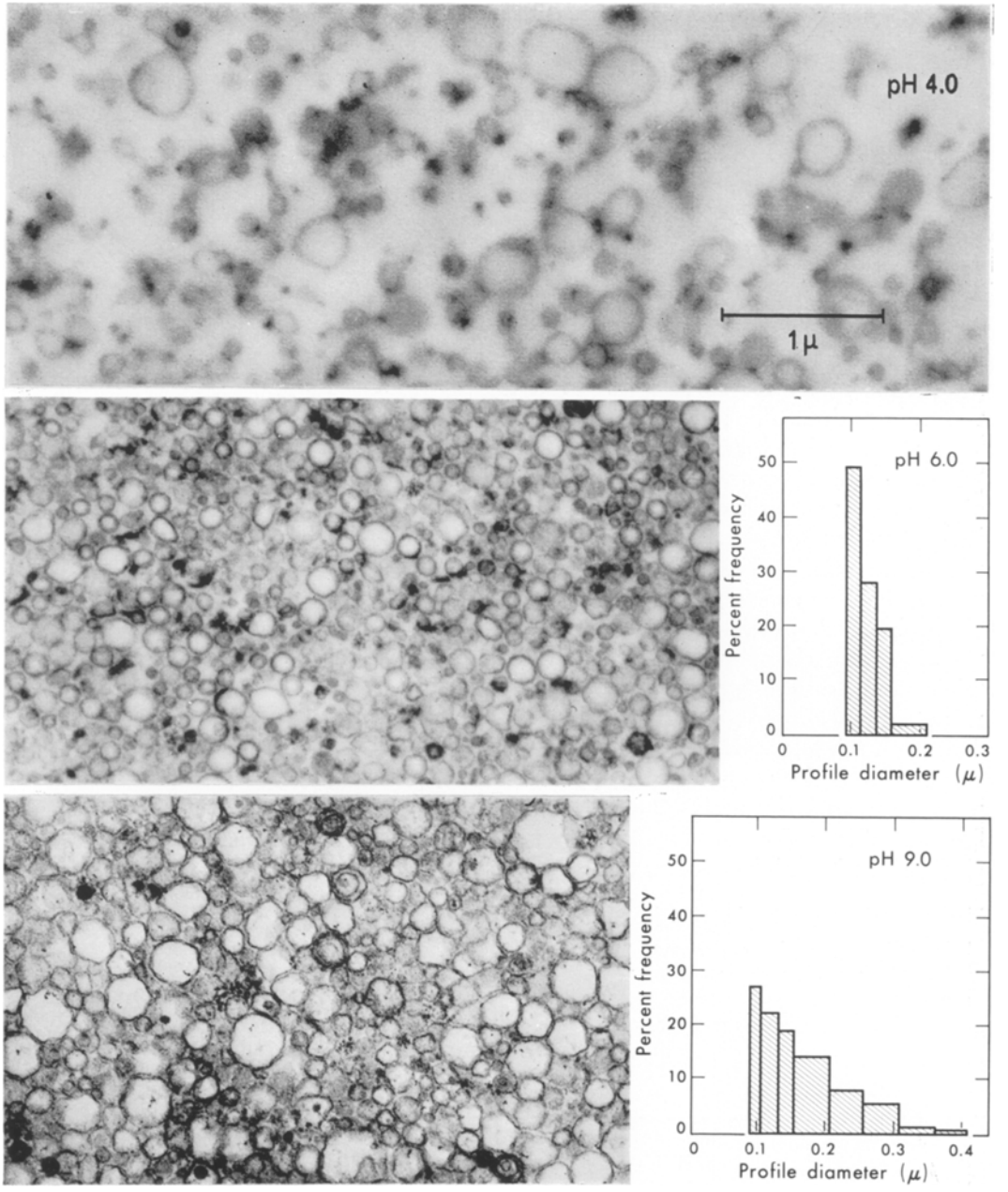

Figure 2. Electron micrographs of submitochondrial vesicles fixed at pH 4, 6, and 9. Conditions of fixation were: submitochondrial vesicles (4 mg protein/ml) and glutaraldehyde (0·6% for 30 min) adjusted to the appropriate pH with HCl or NaOH. Insets: frequency distribution of profile diameters at pH 6 and 9. Diameters lower than 0·1 μ are not included.

monovalent ions causes a 15% decrease in scattering. The effect of divalent ions, however, becomes apparent at much lower concentrations. Below 1 mM both Mg^{2+} and Ca^{2+} cause a 20% increase in light scattering. The opposite effect of EDTA at similarly low concentrations presumably reflects the removal, by chelation, of any endogenous divalent ions present in the vesicles.

ANS Fluorescence

The pH-dependent change in fluorescence of ANS when in interaction with submitochondrial vesicles is shown in Fig. 3. A marked enhancement of fluorescence is observed as the pH of the suspension is lowered. Both a change in quantum yield and an alteration in the number of molecules bound to the membrane could contribute to this observed fluorescence dependence on pH.[13] To test these alternatives, the fluorescence of a fixed concentration of dye was followed as the submitochondrial vesicle concentration was increased. The fluorescence intensity when all the dye molecules are bound can then be derived from a double reciprocal plot. It can be seen from Fig. 4 that, at infinite protein concentration, the relative fluorescence intensities at pH 6, 7, and 8 are the same within ±6%. Thus any fluorescence change due to an alteration in hydrophobic environment must be less than 12% of that due to changes in binding properties of the molecules. To measure the binding of the dye molecules to the membrane, the method of Daniel and Weber[14] was used. The results in Table II show, as would be expected from Fig. 4, that a reduction in pH leads to an increase in the amount of dye bound to the membrane. At pH 6·0, the presence of 150 mM KCl was also found to cause an increase in dye binding by 35%.

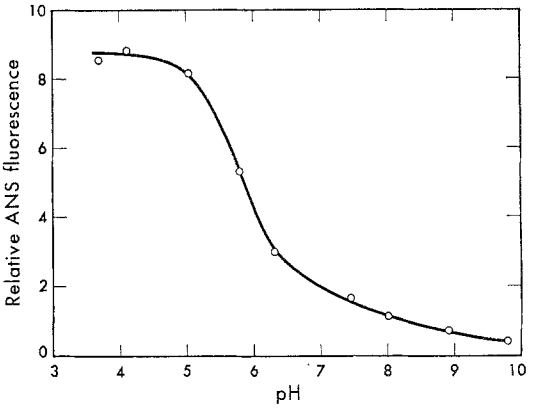

Figure 3. pH dependence of ANS fluorescence in submitochondrial vesicles. Conditions were 2–3 mg protein/ml, 42 μM ANS and 270 nM antimycin A in a total volume of 3·7 ml. Excitation was between 290 nm and 404 nm, and detection was made between 455 nm and 578 nm.

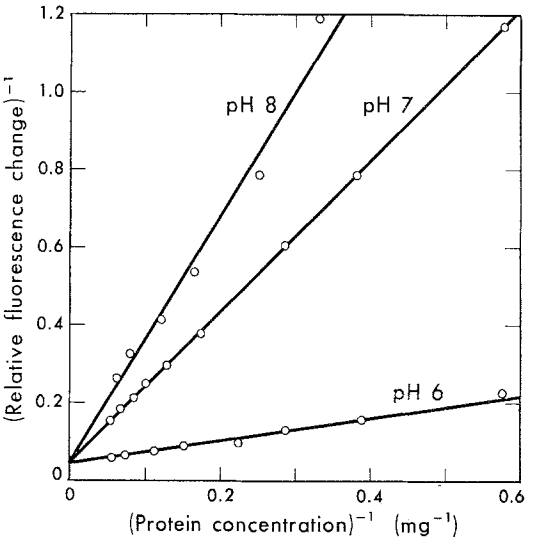

Figure 4. pH dependence of the binding of ANS to submitochondrial vesicles. Membranes were suspended to the protein concentrations indicated in distilled water containing ANS (7·8 μM) and antimycin A (270 nM). Excitation was between 290 nm and 404 nm and detection was made between 455 nm and 578 nm.

Circular Dichroism Studies

Both the spectral shape and the magnitude of the ultraviolet circular dichroism spectra of submitochondrial vesicles depend on pH (Fig. 5). At pH 10 the spectra have two negative

TABLE II. Effect of pH on the binding of ANS to submitochondrial particles

pH	N (n moles ANS/mg protein)
8·0	6·4 ± 3
7·0	20·0 ± 3
6·0	40·5 ± 4

Conditions of measurement were 1·84 mg protein in distilled water containing 270 nM antimycin A. ANS concentration was varied from 0 to 360 μM. For saturation binding, a protein concentration of 18·4 mg/ml was used. Values were derived according to Daniel and Weber.[14]

extremes, one at 222 nm and one at 210 nm. These values and the shape of the curve are characteristic of the α-helix conformation measured on model polypeptides and proteins.[15] The magnitude of the ellipticity, however, is reduced over that given by a model compound in 100% helix form. As the pH is lowered, a red shift of the 222 nm minimum together with a reduction in magnitude is observed (Fig. 5). It is especially noted that the 210 nm minimum does not exhibit any wavelength shift. These alterations continue down to pH 6, after which the 222 nm trough starts to shift back again to lower wavelengths (Fig. 6). Below approximately pH 4 the general shape of the spectra begins to alter, with the 210 nm trough shifting to 208 nm and predominating in magnitude over a 220 nm trough.

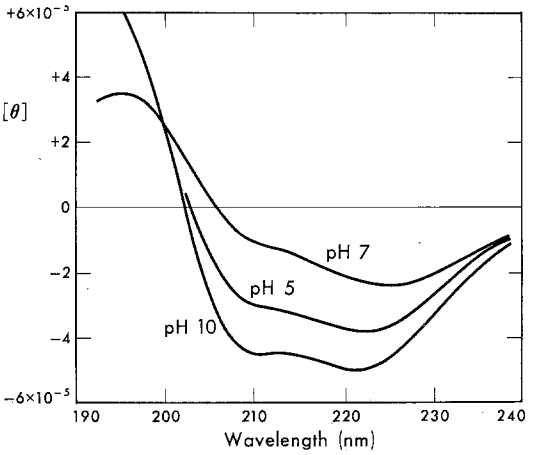

Figure 5. pH dependence of the mean residual ellipticity of submitochondrial vesicles. Circular dichroism measurements were made in water at 0·203 mg protein/ml using a path length of 1 mm.

Certain controls are required in order to draw conclusions from optical rotation spectra measured on particulate systems because of possible distortions from the light scattering exhibited by turbid samples.[16] Figure 7 illustrates the perturbation caused by light scattering on the dichroism measurements of submitochondrial vesicles. The wavelength position of the 222 nm minimum is followed with increasing sample concentration (i.e. increased light scattering). It can be seen that below a concentration of 0·6 mg protein/ml the position of the trough is constant but above this concentration shows an increasing red shift up to approximately 0·8 mg protein/ml. A semiempirical treatment of the perturbing effects seen in Fig. 7 has been given by Urry and Ji.[17] Below a concentration of 0·6 mg protein/ml the wavelength shift of the spectra from that of model compounds is constant and independent of vesicle concentration (Fig. 7). It is thus interpreted as being due to *intra*-particle scattering by the macromolecular components in the vesicle. In an

assembly such as a membrane, the molecules will act as individual scattering centers and, because of close packing, scattering distortion will be large and very sensitive to changes in the molecular arrangement of the scatterers. Above a concentration of 0·6 mg protein/ml another effect, due to the colligative properties of the molecules is also present. Scattering from one assembly effectively removes the incident beam from another. As can be seen in Fig. 7, the resulting scattering distortion in this range is strongly dependent on vesicle concentration until a concentration is reached where little signal reaches the detector. Based on these results the experiments presented in Figs. 5 and 6 were performed under conditions where the linear part of the concentration curve was obtained, i.e. below 0·6 mg protein/ml, where effects due to *inter*-particle scattering were absent.

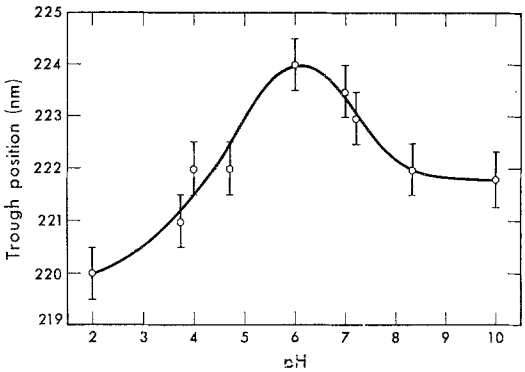

Figure 6. pH dependence of the trough minimum position of the circular dichroism of submitochondrial vesicles. Samples were suspended in distilled water at a protein concentration of approximately 0·3 mg/ml (see Fig. 7).

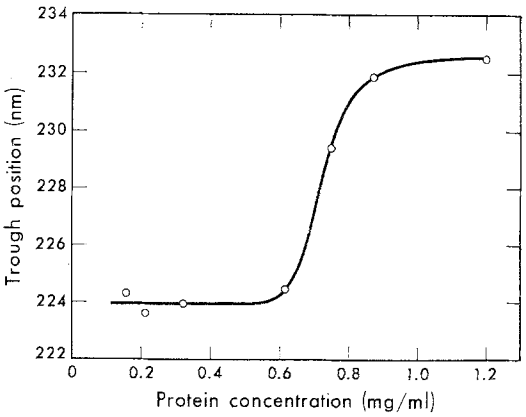

Figure 7. Concentration dependence of the position of the trough minimum in the circular dichroism of submitochondrial vesicles. Samples were measured in distilled water at pH 6·5 using a path length of 1 mm. Data in Fig. 6 were obtained in the linear portion of the curve below 0·6 mg protein/ml.

Infrared Studies

The shape and wavelength changes of the circular dichroism spectra below pH 4 suggest that changes other than quaternary structure are occurring in the macromolecular arrangement of the membrane proteins. Infrared studies were therefore used to characterize further the occurrence of secondary conformational changes.[18, 19] The infrared spectra of submitochondrial vesicles at different pH values is shown in Fig. 8. In the amide I region (1650 cm^{-1}) the peak at 1652 associated with the helical and random coil conformations is present at the four values of pH tested. This peak is, however, reduced in intensity below pH 4. A distinguishable shoulder at 1630 cm^{-1} is present in all the spectra but becomes more pronounced upon acidification to pH 2. This wavelength region has been associated with antiparallel chain β-structures[18] and has been found lacking in plasma membranes[20, 21] but present in mitochondrial material.[22] Thus the present results further show that below pH 4 the signal associated with this conformation becomes enhanced.

Discussion

A number of experimental findings have established that proton movements,[23–25] conformational,[26, 27] configurational,[6] and volume[28–30] changes are associated with

energy transfer in mitochondria. The present results describe in more detail the nature of the possible sequence of structural changes which can occur in mitochondrial membranes following artificially induced changes in H^+ activity. These are summarized in Fig. 9. The scheme indicates the two possible sequences of metabolically induced structural changes which correspond to the chemiosmotic hypothesis (pathway a) and to the chemical or conformational hypothesis (pathway b). In the latter, energy can be stored in the "bond energy" of a chemical intermediate or directly in the form of a change in microstructure of the membrane. Also shown are the scope and limitation of the techniques which have been used to probe structure. The nature of the changes in structure and the probes used are considered below.

pH-dependent Structural Changes

The results of packed volume measurements and the analysis of electron micrographs clearly establish that, under the conditions employed, an alteration in medium pH results in a change of vesicle size. These differences in size are also accompanied by other structural changes. An indication of this is given by the pH-dependent light scattering results. As shown in Fig. 9, light scattering changes can reflect alterations in structure at three levels, extending from microstructure to gross morphology. The contribution of pH-dependent conformational changes to the light scattering levels has previously been assessed using a membrane (structural) protein fraction where vesicles are absent.[12] The technique of light scattering, however, while indicating the presence of conformational changes in membrane components tells us little about their nature. Circular dichroism, infrared and fluorescent probes provide more specific information on conformation.

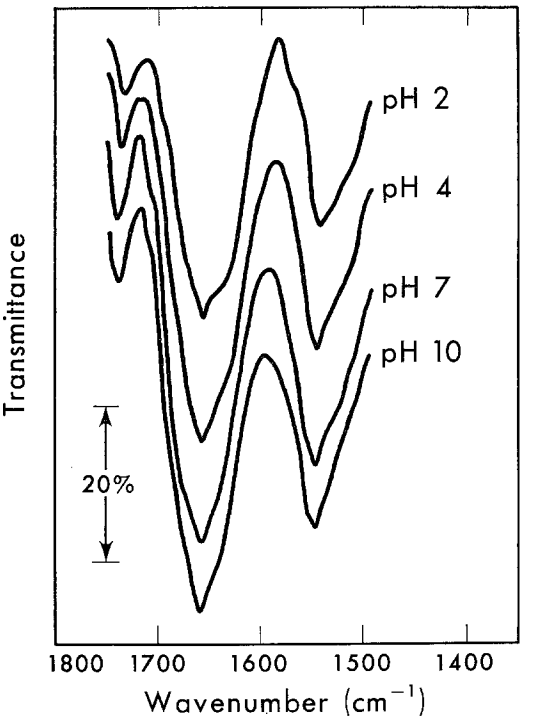

Figure 8. Infrared spectra of submitochondrial vesicles. Samples were frozen at different pH values, lyophilized and resuspended in mineral oil before measurement. Final protein concentration was approximately 5 mg/ml. The curves are displaced from each other by 10% on the transmittance scale for presentation.

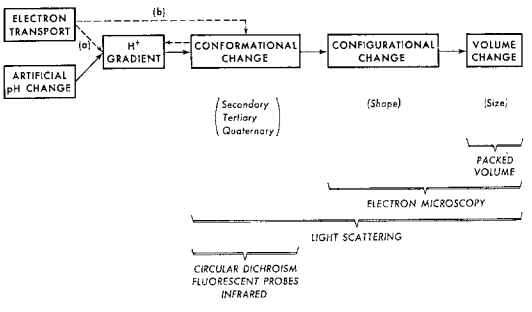

Figure 9. Proposed scheme indicating the causative sequence and nature of structural changes in mitochondrial membranes following metabolically or artificially induced changes in H^+ activity. Pathways corresponding to the chemiosmotic hypothesis (a) and the conformational hypothesis (b) are indicated. Also given are the scope and limitation of some techniques used to probe structure. For details see text.

In the ultraviolet, the main factor determining the optical activity of proteins is the conformation of the various peptide chromophores.[16, 31] At pH 10 the circular dichroism of submitochondrial vesicles gives a spectrum with shape and wavelength characteristics of a mixture of "unordered" and helical protein conformation. Down to pH 6, however, the n-π^- band shows an increasing red shift. Numerous ideas have been advanced to explain this type of shift to the red found in membrane preparations but none have been found to be fully satisfactory. The contribution of phospholipid optical activity seems to have been excluded by the use of lipid extracted mitochondria[26] and isolated protein preparations.[32] The present circular dichroism results exclude artifacts due to vesicle size and interparticle scattering[33] and also exclude displacements due to an increased n-π band-width[34] which would only affect the optical rotatory dispersion spectra. Two remaining explanations which have been advanced for mitochondrial membranes are the presence of β-conformation[22] and the scattering effect due to quaternary protein structures.[12] The presence of β-conformation in mitochondrial membranes has been detected using infrared by Wallach[22] and is confirmed in the present work. However, only below pH 4 does the contribution of this conformation begin to significantly increase, whereas the spectral shifts in the circular dichroism spectra above pH 4 are considerable. β-conformation may contribute to the optical activity of the vesicles over the entire pH range but cannot account for the changes which occur above pH 4. These are more reasonably explained in terms of an alteration in light scattering around the chromophores caused by pH-induced quaternary changes in protein structure. Such light scattering effects would result in the characteristic distortions seen in the optical spectra.[17, 35] Below pH 4 the changes in dichroism spectra begin to deviate from those predicted by this effect and now probably are a reflection of the changes in β-conformation detected by infrared.

The above interpretation is strengthened by the results from ANS experiments where pH-induced changes in fluorescence are found to be primarily due to altered binding characteristics, and not to changes in hydrophobic environment of the bound molecules. Quaternary structural changes in membrane proteins would involve far less rearrangement of nonpolar side groups than would changes in secondary structure, and would result in altered binding characteristics rather than an alteration in hydrophobic environment of the fluorescent probe.

Cause of the pH-dependent Structural Changes

Unlike mitochondria, submitochondrial vesicles do not appear to possess an osmotic barrier to sucrose or monovalent ions.[12, 36] In addition, an energy-dependent accumulation of osmotic species is precluded in the present experiments by the insensitivity of the volume changes to antimycin A. Thus, an osmotic mechanism of structural change cannot explain the present results.

A consequence of a change in environmental pH would be a change in net fixed charge on the membranes. This becomes apparent in flocculation effect in the range pH 4 to pH 6[12, 37] and also in the changed binding characteristics of ANS on the membrane. The affinity of the negatively charged ANS molecule for binding increases as the negative charge on the membrane is reduced by lowering the pH from 8 to 6. Rubalcava, de Muñoz, and Gitler[13] have previously shown that the addition of salts to hemoglobin-free erythrocyte membranes causes an enhanced fluorescence due to an increase in the number

of ANS binding sites. On the assumption that the determining factor of ANS binding was charge, this effect was attributed to the electrostatic shielding effect of the salt. A similar effect is seen in the present experiments. The question arises of how these alterations in fixed charge can lead to configurational and volume changes.

An increase in fixed charge on the interior of a vesicle could, by virtue of a Donnon equilibrium, lead to an increased volume. This possibility has recently been suggested for mitochondria by Blondin, Vail, and Green.[38] However, any contribution by this mechanism in the present experiments would be small since the volume changes are reversible over the pH range 6 to 9. The only way in which contraction could occur is for the elasticity of the membrane to be of sufficient magnitude to drive water out of the vesicle once the internal fixed charge is reduced. A membrane elasticity necessary to cause up to an eight-fold contraction in volume as the pH is suddenly lowered would be improbably high.[41]

Circular dichroism and infrared results indicate that quaternary changes in membrane proteins are occurring in the physiological pH range. This could result from an increase in fixed charge on the membrane. Electrostatic repulsion between fixed sites would increase as the pH is raised. The comparatively large structural effect upon adding divalent ions indicates that one of the major fixed sites responsible for the control of conformation by charge repulsion might be the phosphate group on the phospholipids. Bridging and shielding by Mg^{2+} and Ca^{2+} would reduce charge repulsion to a greater extent than monovalent ions, and closer packing of membrane components would occur. This would lead to the observed volume decrease via the pathway indicated in Fig. 9. The shape of the light scattering curve shows two major regions of structural change (with midpoints at pH 6.5 and pH 10·5) and suggests that the charge groups responsible for these structural changes have their pK at these values.

In summary, an alteration of pH in the range above pH 4 is thought, by changing the ionization of fixed charges on the membrane, to lead to an alteration in electrostatic repulsive forces. This promotes aggregation changes in membrane components, thus altering the membrane surface area and causing a change in vesicle size.

Relationship to Bioenergetic Mechanisms

From the previous argument, the consequence of an H^+ gradient across a membrane would lead to a configurational change. In the physiological range, the more acidic side would "contract" and the more alkaline side "expand". Thus the membrane would bend with its concavity to the acid side. If H^+ gradients, or indeed charge separation across a biological membrane, are basic to any bioenergetic mechanism, then these structural considerations must be applied. For example, an H^+ gradient in a complex system of membranes such as found in heart mitochondria would result in membrane twisting[6] when examined at the configurational level by electron microscopy. A discharge of the gradient, by any method, would lead to a discharge of the twisted configuration. In other aspects of membrane function, pinocytosis for example, the generation of H^+ gradients by ATP hydrolysis[30, 40] could provide a mechanism of controlling membrane movements.

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