# On the Structure Arising from the Interaction of Phospholipid Micelles with Headpiece-Stalk Sectors Detached from the Mitochondrial Cristal Membrane*

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ABSTRACT: Electron microscopic evidence will be presented that phospholipid micelles in water have two structurally different regions, the peripheral edges and the interiors. A model at the molecular level will be proposed which describes the two structurally different regions. This model is based on the two possible orientations of phospholipid molecules in a bilayer. In addition, evidence will be presented that the detachable headpiece-stalk sectors of the repeating units of the mitochondrial cristal membrane can interact with bilayers having phospholipid molecules in only one of the two possible orientations.

The nature of this proposed interaction is correlated with the interaction of headpiece-stalk sectors with base pieces in the mitochondrial cristal membrane.

*A Description of Electron Micrographs of Micelles of Beef Heart Mitochondrial Phospholipid Negatively Stained with Phosphotungstic Acid.* Figure 1A is an electron micrograph of beef heart mitochondrial phospholipid, negatively stained with phosphotungstic acid by the method of Brenner and Horne (1959). The micellar dispersion in water was prepared by the method of Fleischer *et al.* (1962). Extensive structured areas are surrounded by dark unstructured areas. The outer edges (arrow A) of the structured areas usually exhibit a single white line, 40–45 Å in width. There are lengths of "spaghetti" (arrow B), which also have a single white line 40–45 Å in thickness. These single white lines will be referred to as "singlets."

By contrast, the interiors of the structured areas (arrow C) contain "doublets," that is, *pairs* of white lines, each single line of which is 20–23 Å in width. Thus, a doublet has a total width of 53–61 Å. This range of widths was calculated by determining first the sum of the smallest measured dimensions for each of the component parts of the doublet (*i.e.*, 20 Å × 2 + 13 Å = 53 Å), and then the sum of the largest dimensions for each of the component parts (*i.e.*, 23 Å × 2 + 15 Å = 61 Å). Actual measurements give values from 55 to 60 Å, and we will refer to the doublet as 55–60 Å thick. The doublets are separated from each other by very dark regions which may vary in thickness from an observed minimum of 15 Å up to much greater distances.

Figure 1B is an electron micrograph of a highly magnified region of a peripheral edge (arrow on the left) and of some spaghetti (arrow on the right pointing to a single strand). Both exhibit single white lines, *i.e.*, singlets, 40 Å in thickness. The insert is a diagrammatic representation of the singlet.

Figure 1C is an electron micrograph of a highly magnified interior region with an arrow pointing to one of the doublets. The insert represents the doublet pattern diagrammatically.

*A Description of Electron Micrographs of the Negatively Stained Structures Formed by the Interaction between Headpiece-Stalk Sectors and Micellar Phospholipid.* The description of the electron microscopic appearance of negatively stained beef heart mitochondrial phospholipid applies equally well to Asolectin extracted from soybeans, as well as to other structures. Asolectin which has been first exposed to headpiece-stalk sectors isolated from the repeating units of the mitochondrial cristal membrane by the method of Kopaczyk *et al.* (1968a,b) and, subsequently stained with phosphotungstic acid, is shown in Figure 2. While it is difficult to interpret the details of the interiors of these structures, the fact that headpiece-stalk sectors are associated with the edges is unmistakable. The headpiece-stalk sectors are attached to edges which are 55–60 Å in thickness. This thickness differs from the 40–45 Å thickness of the singlet. It does, however, correspond exactly with the 55–60 Å thickness of the doublet.

The insert on the right of Figure 2 is a highly magnified electron micrograph of the structure pointed to by the arrow in Figure 2. Headpiece-stalk sectors are attached to a doublet edge complete with a faint midline, and 55–60 Å thick. The left insert in Figure 2 is a diagrammatic representation of this same structure.

Figure 3A is an electron micrograph of what appear to be short segments of doublets with headpiece-stalk sectors attached on *both* sides. Here again, using the structure on the right, the clearer of the two structures, the doublets measure 55–60 Å in thickness, with each individual white line measuring approximately 20 Å in thickness, and the midline measuring approximately 15 Å in thickness. The insert in Figure 3A is a diagrammatic representation of the doublet with headpiece-stalk sectors on both sides. The dimensions of these doublets are almost identical with the dimensions of the doublets. Such structures are quite infrequent in the electron micrographs studied.

Occasionally, closed structures such as that shown in Figure

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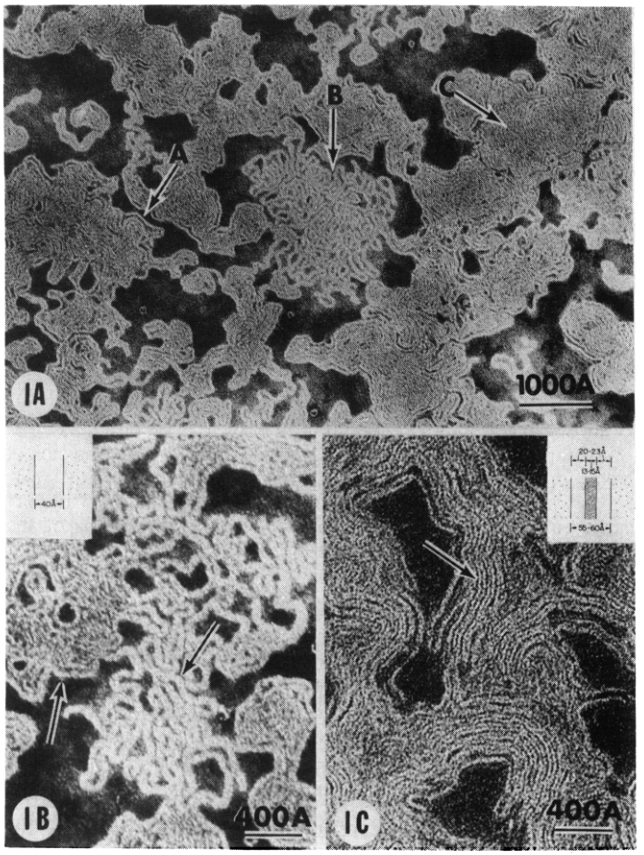


FIGURE 1: Electron micrograph of mitochondrial phospholipid negatively stained with phosphotungstic acid (Kopaczyk *et al.*, 1968a; Bloor, 1943). Part A: arrow A, peripheral "singlet" edge, 40 Å in thickness; arrow B, "spaghetti," 40 Å in thickness; arrow C, interior "doublet," 55–60 Å in overall thickness (two white lines each 20 Å in thickness plus a 15-Å thick midline). Part B: left arrow, peripheral "singlet" edge, 40 Å in thickness; right arrow, single strand of the spaghetti, "singlet" 40 Å in thickness; insert, diagrammatic representation of the 40-Å "singlet." Part C: arrow, a doublet, 55–60 Å in thickness, with two white lines each 20 Å in thickness with a 15-Å thick midline; insert, diagrammatic representation of the doublet with dimensions indicated.

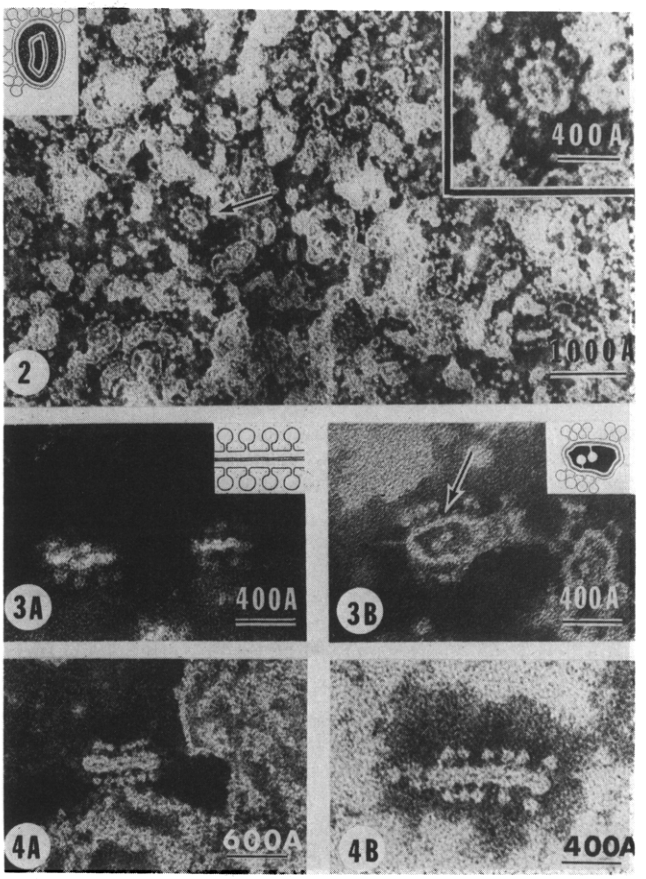


FIGURE 2: Electron micrograph of mitochrondrial headpiece-stalk sectors associated with asolectin negatively stained with phosphotungstic acid. Arrow points to structures of headpiece-stalk sectors associated with asolectin. Right insert, high magnification electron micrograph of the structure pointed to by the upper arrow. Headpiece-stalk sectors are attached to a doublet line with two white lines, each 20 Å in thickness separated by a 15-Å midline. Left insert, diagrammatic representation of the structure pointed to by the arrow.

FIGURE 3: Electron micrographs (A). Of fragments of doublet asolectin with mitochrondrial headpiece-stalk sectors attached on both sides, negatively stained with phosphotungstic acid. Right insert, diagrammatic representation of the doublet with headpiece-stalk sectors attached on both sides. (B) Of structure of asolectin associated with mitochondrial headpiece-stalk sectors. Arrow, headpiece-stalk sectors on both sides of a vesicular doublet. Two headpiece-stalk sectors are enclosed within the vesicle. Insert, diagrammatic representation of the vesicle with headpiece-stalk sectors attached both externally and internally.

FIGURE 4: Electron micrographs. (A) Of lyophilized mitochondria negatively stained with phosphotungstic acid. Vesicular structure of a doublet with headpiece-stalk sectors attached around the periphery of the structure. (B) Of phospholipase-treated mitochondria negatively stained with phosphotungstic acid. Vesicular structure of a 60-Å thick continuum with headpiece-stalk sectors attached around the periphery.

3B are seen. Headpiece-stalk sectors are attached not only on the outside edges of the structure, but on the inside as well. There is a doublet at the edges with dimensions ranging from a high of 60 Å thick, down to a low of 47 Å thick. The arrow points to a region where the doublet pattern is fairly clear.

In lyophilized preparations of whole mitochondria (Jolly *et al.*, 1969) subsequently stained with phosphotungstic acid, there are frequent examples of the structure seen in Figure 4A. Here we can see a small, closed, double-laminated structure, having a total width of 140 Å at its narrowest point. It has two "walls," each one of which is 55–60 Å thick. Each wall possesses a doublet with a 15-Å midline. Headpiece-stalk sectors are attached around almost the entire periphery.

Structures completely analogous to those seen with lyophilized mitochondria are also seen in electron micrographs of mitochondria which have been treated with phospholipase C (J. Asai, 1968, unpublished data), as shown in Figure 4B.

These last two described structures result from a complex perturbation of the mitochondria. We suggest that the perturbation releases membrane phospholipid, as well as detaches headpiece-stalk sectors from the cristal membrane, and subsequent reassociation of detached headpiece-stalk sectors occurs with the released phospholipid.

Figure 5 is a diagrammatic representation of the structure shown in Figure 4A. It could just as well be used to represent the structures in Figures 2, 3B, or 4B.

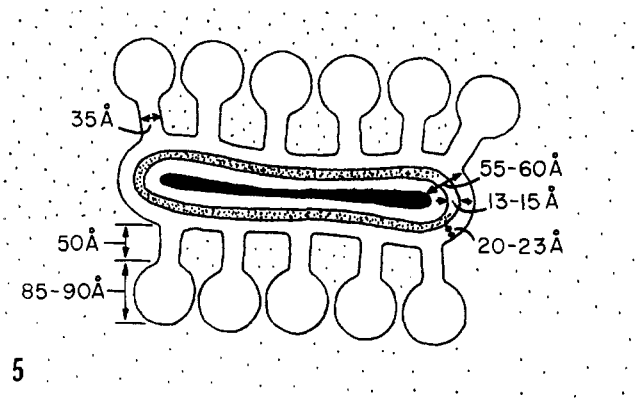


FIGURE 5: Diagrammatic representation of a vesicular doublet structure with mitochondrial headpiece-stalk sectors attached around the periphery. Dimensions of the doublet and of the headpiece-stalk sectors are given.

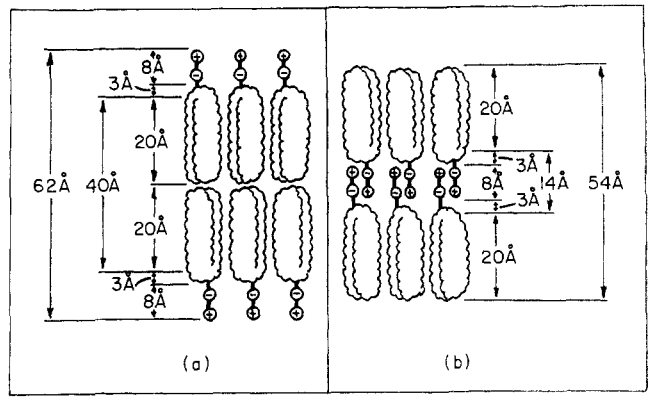


FIGURE 6: Diagrammatic representation of the two modes of association of the phospholipid molecules within a bilayer. (a) The singlet bilayer orientation. Fatty acid chains in apposition with polar "heads" external. (b) The doublet bilayer orientation. Polar "heads" charge paired with fatty acid chains external. Dimensions of the various regions and overall sizes of the bilayers given.

*Alternative Orientations of the Phospholipid Molecules within Phospholipid Bilayers.* Several questions immediately suggest themselves from the above descriptions. For example, what is the relationship between what we see in electron micrographs of phosphotungstic acid stained phospholipid and the structure of the original phospholipid micelle in aqueous medium? What are the possible orientations of the constituent phospholipid molecules within these phosphotungstic acid stained structures, and can one or more of these arrangements be reconciled with what we see in the electron micrographs? What relationship do these possible orientations of the constituent phospholipid molecules within the phosphotungstic acid stained structures have to the orientation of the phospholipid molecules in the original micelle? What is the relationship between the singlet pattern seen at the peripheral edges or in the spaghetti, and the doublet pattern seen in the interiors of these phosphotungstic acid stained structures? What is the nature of the interaction of the headpiece-stalk sectors with phospholipid that leads to the observed structure? What is the explanation of the fact that, in the absence of headpiece-stalk sectors, phospholipid appears in electron micrographs of phosphotungstic acid stained specimens to have one pattern at its peripheral edges, namely the singlet, while in the presence of headpiece-stalk sectors the edges appear to have an alternative pattern, namely, a doublet which is ordinarily found only in the interiors of phosphotungstic acid stained phospholipid? We believe that the orientation of the constituent phospholipid molecules within the phosphotungstic acid stained structures provides the answer to all the questions raised above.

Before considering the orientation of the constituent phospholipid molecules, let us consider the first question raised above, *i.e.*, what is the relationship between what we see in electron micrographs of phosphotungstic acid stained phospholipid and the structure of the original micelle in aqueous medium. Whatever the details, one incontrovertible conclusion must be drawn from the electron micrographs of the phosphotungstic acid stained structures, namely, *that not all regions of the original micelle are identical.* The fact that we see *two* distinct and *different* patterns, the singlet and the doublet, establishes this conclusion. In addition, the localization of the two patterns in electron micrographs, *i.e.*, the singlets seen only at the edges or in the spaghetti, and the doublet seen only in the interiors, and never *vice versa*, strongly suggests that the peripheral regions and interior regions of the original micelles untreated by phosphotungstic acid are nonidentical. We propose that this nonidentity of the peripheries and interiors reflects a difference in the orientation of the phospholipid molecules on the periphery of the original micelle from the orientation of the phospholipid molecules within the interior of the original micelle. This view does not invoke nor deny a segregation or selection of particular molecular species on the peripheries as compared with the interiors as a necessary requirement, but is concerned solely with the orientations of phospholipid molecules in three-dimensional space.

The orientations of the phospholipid molecules in the phosphotungstic acid stained structures can be deduced from Figure 1A and thus related back to their orientations in the original micelle. It is generally agreed, and there is ample experimental evidence for the fact, that phospholipid molecules can align themselves to form bimolecular arrays (Green and Fleischer, 1964) and can do so in one of two possible ways, as shown diagrammatically in Figure 6. Of these two possibilities, it is only the arrangement given in Figure 6a which can account for the singlet pattern seen upon phosphotungstic acid staining, and it is only the arrangement given in Figure 6b which can account for the doublet pattern seen upon phosphotungstic acid staining.

Measurements made on a Corey–Pauling–Koulton (CPK) atomic model (Koulton, 1965) of a typical phospholipid molecule show that a phosphoethanolamine polar "head" is approximately 8 Å in length, a glycerol moiety about 3 Å across, and a $C_{18}$ fatty acid chain about 20 Å in length when fully extended. A photograph of a CPK space-filling model of a phospholipid molecule is shown in Figure 7, with the dimensions outlined above indicated. Thus, using the dimensions measured from the molecular model, phospholipid molecules associated in a bilayer according to the arrangement given in Figure 6a, would be expected to interact with phosphotungstic acid in such a way as to show up in electron micrographs as a single white line, *i.e.*, a singlet with a maximal thickness of 40 Å, assuming no interdigitation of the fully extended fatty acid chains. This single white line, resulting from the relative in-

ability of the quite polar phosphotungstic acid to penetrate into the hydrophobic regions of the fatty acid chains (Lucy and Glouert, 1964), would have dark lines or regions of variable thickness on both sides, where the phosphotungstic acid can penetrate into polar regions of the phospholipid molecules (the polar heads and the glycerol moieties with their high oxygen contents), as well as into the surrounding aqueous medium. No invariant midline would be expected. The edges of the phosphotungstic acid stained structures seen in Figure 1A, as well as the spaghetti, are completely consistent dimensionally with the arrangement of the phospholipid molecules in the singlet.

By contrast, the electron microscopic appearance of phospholipid molecules associated according to the arrangement given in Figure 6b can completely account for the doublet pattern seen in Figure 1A (arrow C) and in Figure 1C, since the dimensions of the atomic model are consistent with the observed thicknesses of the various lines within the doublet. Negative staining of the structures in Figure 6b would result in *two white lines*, each about 20 Å in thickness (assuming that the fatty acid chains are fully extended), as well as in an invariant dark *midline* between the two white lines. The midline could result from the presence of phosphotungstic acid in the region of the polar heads of the phospholipid molecules, as well as in the relatively polar regions of the two glycerol moieties with their high oxygen contents. The midlines would have a calculated thickness of 14 Å, assuming that the two 8-Å polar heads charge pair by lying in directions of opposite sense, and thus interdigitate one with another. The intermolecular charge pairing of one phosphate oxygen with a quaternary nitrogen should be a quite stable association. Since two such salt bonds would be possible in this arrangement of interdigitated polar heads, the interaction would be very strong indeed, especially when screened from water by the fatty acid chains.[1] The interiors of the phosphotungstic acid stained structures seen in Figure 1C are completely consistent dimensionally with the arrangement of the phospholipid molecules in the doublet.

The phosphotungstic acid monomer is approximately 15 Å across at its widest point, as measured from CPK atomic models. Thus, the midline, which upon close examination of greatly enlarged electron micrographs seems to be a discontinuous line of dark dots, could be due to the packing of phosphotungstic acid molecules between the polar heads of the phospholipid molecules, resulting in a single layer of phosphotungstic acid molecules sandwiched between the two half-bilayers of the doublet.

We are now in a position to answer the first question raised in this section, *i.e.*, what is the relationship between what we see in electron micrographs of phosphotungstic acid stained phospholipid and the structure of the original micelle in aqueous medium. It is our thesis that the singlet and the doublet, respectively, bear a 1:1 relationship with the peripheries and

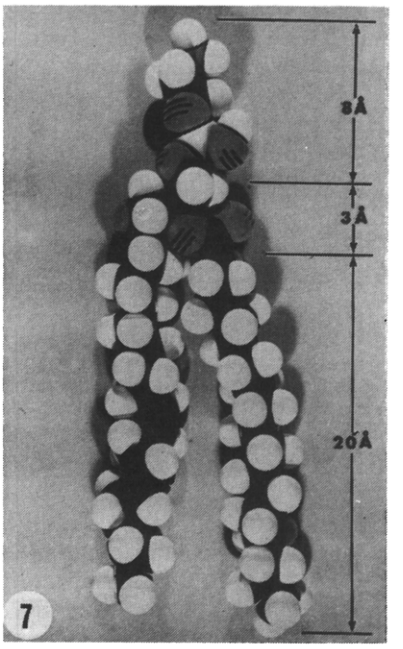


FIGURE 7: Photograph of a Corey–Pauling–Koulton space-filling model of a typical phospholipid molecule with $C_{18}$ fatty acid chains fully extended and phosphoethanolamine polar "head." Dimensions of the various parts of the molecule are given.

interiors of phospholipid micelles in aqueous medium. We propose that the micellar periphery is made up of a singlet bilayer, while the micellar interior is made up of doublet bilayers. According to this model, the peripheries of the original micelles, which are in intimate contact with the surrounding aqueous environment, have the polar groups of the constituent phospholipid molecules of the singlet bilayer oriented into the aqueous phase, a necessary requirement for any model of phospholipid micelle structure. The interiors of the micelles, unlike the peripheries, are not in intimate contact with the aqueous environment, but rather occupy a region more akin to the interior of an oil droplet, and accordingly, would have the nonpolar groups of the constituent phospholipid molecules of the doublet bilayer oriented externally, again a necessary requirement for any model of phospholipid micelle structure proposed.

One serious difficulty in visualizing a micelle having an interior composed of doublet bilayers (with nonpolar fatty acid chains oriented outward), and a periphery composed of a singlet bilayer with the exact opposite arrangement (with polar groups outward into the aqueous environment) seems immediately apparent. The interface at which these two oppositely oriented bilayers meet would give rise to a polar–nonpolar interaction. This unlikely interface is shown in Figure 8. In addition, it is difficult to visualize any but a discontinuous transition in molecular orientation in going from the outside of the micelle into the interior. We propose that the bilayers of the structure can arrange themselves to undergo a completely smooth transition from the orientation on the periphery to the orientation in the interior by the tactic shown diagrammatically in Figure 9.

By this tactic the peripheral bilayer exposes polar groups to the external environment of water at all points. The peripheral bilayer associates its polar groups with the polar groups of

[1] Mitochondria contain two phospholipids which are charge paired (lecithin and phosphatidylethanolamine) and one phospholipid which is negatively charged (cardiolipin). The former account for 80% of the total and the latter for the rest (Fleischer *et al.*, 1961). Cardiolipin would fit into the postulated pairing arrangement for interdigitating polar heads if the phosphate groups chelate divalent metal ions, such as $Mg^{2+}$ or $Ca^{2+}$. Each individual divalent metal ion could be chelated by two phosphate groups, one phosphate group from one cardiolipin molecule, the other phosphate group from the other. The divalent metal ion would act like a "bridge" across the midline of the doublet.

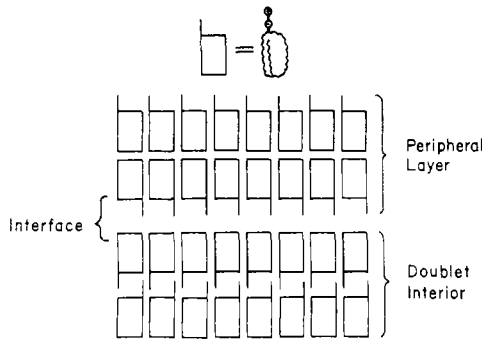


FIGURE 8: Diagrammatic representation of the interface between a singlet bilayer (peripheral layer) and a doublet bilayer (doublet interior). Note apposition of polar heads from the peripheral layer with the hydrophobic fatty acid chains from the doublet interior.

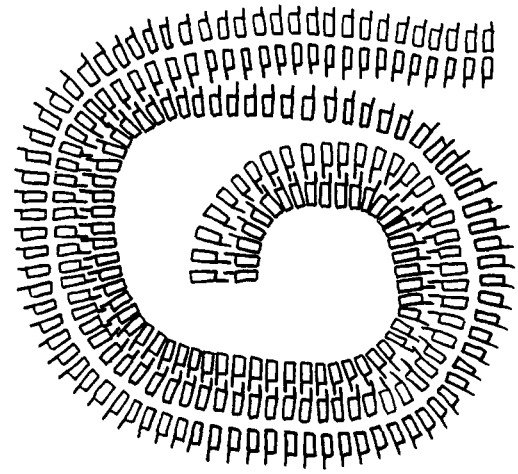

FIGURE 9: Diagrammatic representation of the smooth transition of the peripheral singlet layer (with polar groups exterior and fatty acid chains in apposition) to the doublet orientation in the interior (with polar groups charge paired and fatty acid chains external).

the molecules lying adjacent to it but toward the interior of the micelle, resulting in effect in a fusion of the singlet with a doublet. This rationalization is consistent with the observations of Saunders (1966) which led him to suggest that lecithin micelles formed by sonication of lecithin in an aqueous medium have an essentially anhydrous laminar form which is stabilized by electrostatic interactions between phosphate and choline groups.

Another problem arises in the interpretation of the doublet pattern seen in the interiors. If one doublet bilayer lies adjacent to another, the nonpolar fatty acid chains from one bilayer should be in apposition to the fatty acid chains of the other bilayer. This apposition should result in a completely nonpolar region between bilayers. How is it possible, as is required by our explanation, for phosphotungstic acid to invade such a region to give a dark line between adjacent doublet bilayers? One possible explanation is that when micelles are dried during preparation for electron microscopy, fissures could occur along the lines of cleavage between apposed doublets, and phosphotungstic acid could then fill the fissures. This suggestion seems reasonable when it is realized that phosphotungstic acid is not absolutely polar, but has nonpolar regions where some of its weakly acidic hydroxyl groups are undissociated.

*The Hydrophobic Character of the Interaction of Headpiece-Stalk Sectors from the Mitochondrial Cristal Membrane with Phospholipid.* The variety of structures described and illustrated earlier, in which headpiece-stalk sectors of the mitochondrial cristal membrane associate with phospholipid, are here proposed to result from a hydrophobic interaction of the stalk with the bilayer of phospholipid. This hydrophobic association is proposed to be a phenomenon common to all of the structures described.

The hydrophobic character of the end of the stalk which associates with the basepieces in the cristal membrane has been deduced from the studies of Kopaczyk *et al.* (1968a) in which the repeating units of the cristal membrane can be stripped of headpiece-stalk sectors by exposure to bile salts and ammonium sulfate, reagents which weaken the predominantly hydrophobic interactions linking the headpiece-stalk sectors to the base pieces. Therefore, it is not surprising that isolated headpiece-stalk sectors exhibit a "generalized" capacity to associate with hydrophobic surfaces other than those in the base pieces. The association of headpiece-stalk sectors with phospholipid must be of just this kind, that is, an association of the nonpolar fatty acid chains of the phospholipid molecules with the hydrophobic end of the stalk.

Such a hydrophobic association of the stalk with micellar phospholipid can occur only if the phospholipid molecules have their nonpolar groups available for association. Therefore, it must be the bilayer in the doublet orientation, represented diagrammatically in Figure 6B, which can interact with the hydrophobic region of the stalk. It is thus deduced that of the two possible orientations of the molecules in the phospholipid bilayer, it is uniquely the one with polar groups charge paired and with nonpolar groups external with which the headpiece-stalk sectors should associate. This orientation of the bilayer, namely, the doublet, is always observed in the electron microscopic examination of structures formed by the interaction of headpiece-stalk sectors with phospholipid.

*The Inversion of the Phospholipid Bilayer.* The micelle surface is currently believed to be covered with polar groups extending into the polar aqueous medium. Such a polar surface would not be expected to interact with the hydrophobic region of the stalk. It is therefore deduced that headpiece-stalk sectors must be capable of inducing an "inversion" of the phospholipid molecules on the surface of the micelle. This inversion is proposed to occur as the result of the interaction of the bilayers on the surfaces of two phospholipid micelles as they touch. In Figure 10 we see only one example of the numerous contacts seen between one micelle and its closest neighbors. Figure 11 is a diagrammatic representation of the interaction which is proposed to lead to inversion.

As indicated in Figure 11, the outer polar regions of two separate but contiguous phospholipid micelles can intercalate their polar groups to give rise to what is essentially a doublet "tail." This newly formed doublet tail is rather unlikely to exist for very long under normal circumstances, since it would have two hydrophobic surfaces exposed to the aqueous phase. In addition, a hydrophobic layer on the surface of the micelle would result, which would be equally unlikely to remain long exposed to water. However, if an agent capable of interacting with the hydrophobic surfaces is present, a coating of the hydrophobic surfaces by that agent would result and

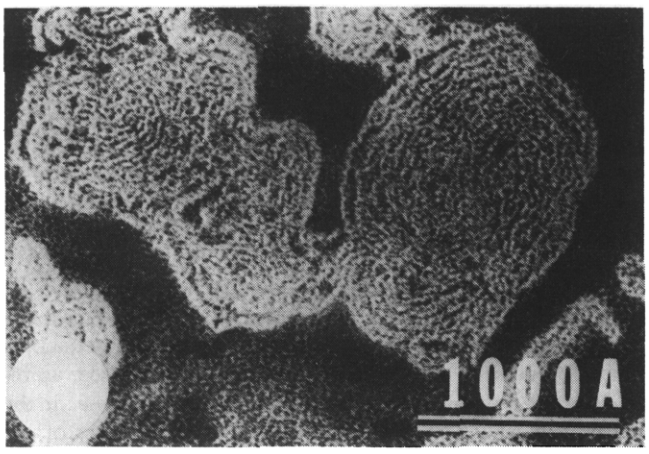


FIGURE 10: Electron micrograph of mitochondrial phospholipid negatively stained with phosphotungstic acid. Note the touching of two micelles at their peripheries.

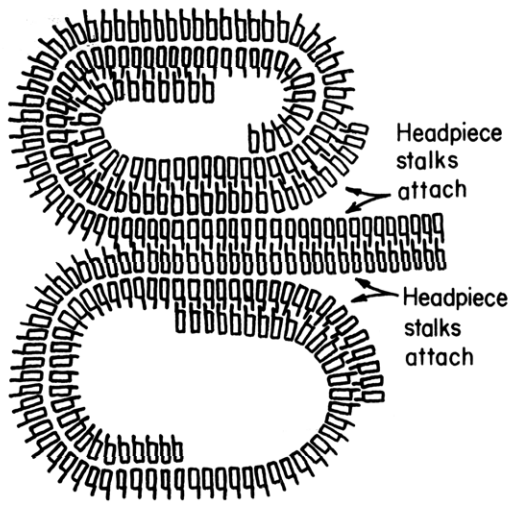


FIGURE 11: Diagrammatic representation of the intercalation of polar heads of phospholipid molecules on the periphery of two contiguous phospholipid micelles. Note the formation of a doublet "tail" with hydrophobic fatty acid chains external. Also, note the remaining half-bilayer on the surface of the micelle, hydrophobic fatty acid chains exposed to the exterior.

tend to stabilize these rather unlikely structures, especially if that agent has another quite hydrophilic end to expose to the aqueous medium. The headpiece-stalk is ideal as such an agent. Since the headpiece ordinarily extends into an aqueous medium when the headpiece-stalk sector is in its normal environment in the cristal membrane, it is probably quite polar. Thus, if headpiece-stalk sectors are present in the same medium with the phospholipid micelles, they would be expected to associate with all the hydrophobic surfaces generated by the pairing tactic. In fact, the headpiece-stalk sectors would coat not only the outer hydrophobic surfaces of the micelles to give rise to the structures seen in Figure 2, but it was predicted, and subsequently found, that they would coat *both* surfaces of the doublet tail to give rise to structures such as those seen in Figure 3A,B. All of the proposals offered above are consistent with the related observation that micelles in association with cytochrome *c* can exist in either water or heptane (Das *et al.*, 1962), one form of which would require polar groups external, the other form of which would require hydrophobic groups external.

Thus, all of the structures seen as the result of the exposure of micellar phospholipid to headpiece-stalk sectors result from a phenomenon common to them all, namely, the interaction o, the hydrophobic region known to exist in the stalk with the hydrophobic fatty acid chains of the phospholipid bilayer which have become exposed as the result of inversion. The inverted phospholipid bilayer "mimics" the base pieces of the mitochondrial cristal membrane by providing a hydrophobic surface for the interaction with the stalk.

A certain amount of variability has been observed in the dimensions of the doublet to which headpiece-stalk sectors are attached. For example, in Figure 3A, the doublet in the segment on the left, which is admittedly not a clearly seen structure, measures approximately 70 Å across. Also, in Figure 3B, the edge to which headpiece stalks are attached varies from a low of 47 Å to a high of 60 Å across. The 55–60-Å dimension cited above for the thickness of the doublet in mitochondrial phospholipid unassociated with protein, was calculated from a large number of measurements from vast fields of structures. In general, the most reliable measurements can be made on electron micrographs of phospholipid unassociated with protein, in which case the patterns are extraordinarily regular since the phospholipid is essentially crystalline in character. In the case of phospholipid associated with protein, the micrographs are necessarily less well defined because of the greater complexity of the system. Even here, however, a very large number of measurements have been made from vast fields of structures such as those shown in Figure 2. In micrographs from Asolectin associated with headpiece-stalk sectors, the pattern and dimensions calculated from a very large number of measurements of doublets coincide with the pattern and dimensions of doublets from mitochondrial phospholipid unassociated with protein. The observed variability could be due to a variety of causes. In those cases in which the dimensions exceed 60 Å, the only cause which seems immediately plausible is the visualization of a structure seen during the dissolution of the doublet. For those structures in which the dimension is less than 55 Å, one possible cause could be that the fatty acid chains, which are probably not fully extended in the original micelle, do not become everywhere fully extended when exposed to phosphotungstic acid. This explanation leads to the prediction that both the pattern of the doublet and the invariant 15-Å measurement of the midline should be independent of the thicknesses of the two white lines and of the doublet as a whole. This is precisely what is observed.

In addition to the direct visual electron microscopic evidence, one of the present authors (E. F. Korman, unpublished results) has recently obtained chemical evidence which is consistent with the presence of the doublet at the peripheries of the micelles when headpiece-stalk sectors are attached. Micelles prepared from total mitochondrial phospholipid can combine with reduced cytochrome *c* to give an insoluble micelle–cytochrome *c* "association" which can be easily removed from solution by centrifugation. This association is attributed to the interaction of a cluster of positive charges present in the reduced cytochrome *c* molecule with clusters of negative charges on the surface of the mitochondrial phospholipid micelles, due

to the presence there of cardiolipin. Thus, it has been demonstrated that mitochondrial phospholipid micelles can remove reduced cytochrome *c* from solution, as measured by the loss in optical density at 550 mμ, in a fashion which is linear with phospholipid concentration. The ability of mitochondrial phospholipid micelles to remove cytochrome *c* from solution is completely lost when the micelles have first been incubated with headpiece-stalk sectors from the mitochondrial cristal membrane. Headpiece stalks themselves have only a slight capacity to associated with cytochrome *c*, even at very high ratios of headpiece-stalk protein to cytochrome *c* protein.

These facts are consistent with the idea that the cluster of positive charges in the reduced cytochrome *c* molecule are unable to interact with the clusters of negative charges on the surface of the phospholipid micelle when headpiece-stalk sectors are attached to the micelle. This inability to interact could be due to either: (1) steric hindrance caused by the presence of headpiece-stalk sectors, which prevents approach of the cytochrome *c* molecules to the existing clusters of negative charges on the micelle surface. (The surface of the micelle could, thus, still retain its original structure); or, (2) a disappearance of the clusters of negative charges from the micelle surface due to a change in the structure of that surface. The first of these possibilities has been shown not to be the case. This fact has been established by studying the interaction between cytochrome *c* and electron transport particle. Electron transport particle is a vesicular submitochondrial particle which contains a full complement of headpiece-stalk sectors oriented outward on its surface. The distribution of head-stalk sectors on the surfaces of both electron transport particle and micelles is equally dense. Electron transport particle has the usual complement of cristal membrane phospholipid with polar groups extended outward into the aqueous medium. If the presence of headpiece-stalk sectors sterically hinders interaction of cytochrome *c* with clusters of negative charges on micelles to which those headpiece-stalk sectors are attached, one would expect the headpiece-stalk sectors on the outer surface of electron transport particles similarly to hinder the interaction of cytochrome *c* with the phospholipid on the electron transport particle surface. However, cytochrome *c* binds to electron transport particle in a fashion which is linear with electron transport particle concentration and in amounts which are relatively massive (approximately 20–30 times) compared with the amounts normally found in cristal membranes. The bound cytochrome *c* has been shown to be tightly associated with electron transport particle since it cannot be easily removed by merely washing with buffer. It can, however, be removed from electron transport particle essentially quantitatively by extraction with high salt (0.1 M KCl), not at all like the cytochrome *c* associated with the electron transfer chain which cannot be extracted from electron transport particle with high salt. This high salt dissociability of cytochrome *c* from electron transport particle is consistent with an association between cytochrome *c* and cardiolipin on the electron transport particle surface. Evidence that it is cardiolipin with which cytochrome *c* associates on the electron transport particle surface is the fact that after extraction with 90% acetone electron transport particle essentially loses none of its ability to associate with cytochrome *c*. Extraction of membranes with 90% acetone has been shown (Fleischer *et al.*, 1961) to remove phospholipids other than cardiolipin essentially quantitatively. However, if electron transport particle is extracted with chloroform–methanol (2:1, v v), a reagent which is known to extract cardiolipin quantitatively, electron transport particle almost completely loses its ability to associate with cytochrome *c*.

From the evidence cited above, headpiece-stalk sectors do not constitute a steric problem to the association between cytochrome *c* and clusters of negative charges on a surface, when those charges are *actually present*. The inability of cytochrome *c* to bind to micelles associated with headpiece-stalk sectors must be due to the *absence* of clusters of negative charges on the surface of the micelle, and not due merely to the inaccessibility of such charges. We conclude from these experimental findings that when headpiece-stalk sectors attach, a change occurs in the structure of the micelle surface, as reflected in the absence of clusters of negative charges, *i.e.*, in the orientation of the molecules of the peripheral bilayer of the micelles. Coupled with the evidence from electron microscopy, we conclude that the change in orientation is from the singlet bilayer to the doublet bilayer.

The association of headpiece-stalk sectors to phospholipid micelles having an inverted doublet bilayer surface poses a problem which must be resolved. To clarify this problem, we must first discuss certain aspects of the mitochondrial cristal membrane. In the cristal membrane, headpiece-stalk sectors associate with base pieces *via* the stalk, the stalk having a diameter of approximately 35 Å, which corresponds to an approximate circular cross-sectional area of 1000 Å$^2$. Thus, only about 10% of the total surface area of the surface region to which a stalk attaches is covered by that stalk. This means that about 90% of that area is exposed to the polar aqueous environment. Since the headpiece-stalk sectors are distributed with about an equivalent frequency on the surface of phospholipid micelles as on the surface of the cristal membrane, again about 90% of the surface of the micelle will be exposed to the aqueous medium, *i.e.*, with the nonpolar groups of the doublet external. Such an arrangement is seemingly thermodynamically unfavorable. However, it has been cited above that headpieces are themselves quite polar in character, since they extend into the polar aqueous environment when attached to either the cristal membrane or to the surface of phospholipid micelles. A simple calculation of the surface area of a single headpiece gives an area of approximately 25,000 Å$^2$. This surface area is thus approximately 2.5 times the 10,000 Å$^2$ area of the surface on the membrane or the micelle with which each headpiece-stalk sector is associated. Thus, a balance of hydrophobic and hydrophilic interactions is present, and a predominance of the hydrophilic character associated with the headpiece could lead to the stability of the structures we see.

## Summarizing Discussion

The electron microscopic evidence presented in this communication and the deductions made from that evidence can be summarized as follows: (1) There are two different patterns of white and dark lines seen in electron micrographs of negatively stained micelles of mitochondrial phospholipid and of other phospholipids: (a) the "singlet," consisting of a single white line 40 Å in thickness, seen only at peripheries and in "spaghetti;" (b) the "doublet," consisting of two white lines arranged in pairs, each single line of which is 20 Å in thickness and having a 15-Å thick dark midline, seen in ordinarily in interiors. (2) There are two possible modalities for the arrangement of the phospholipid molecules in a bilayer: (a) nonpolar

fatty acid chains in apposition. This modality would result in the appearance of a single white line 40 Å in thickness in electron micrographs of negatively stained specimens to be expected; this expected result corresponds exactly with the observed singlet seen in electron microscopy; (b) polar groups in charge-paired apposition. This modality would result in the appearance of a pair of white lines, each 20 Å thick, with a dark midline 15 Å thick, in electron micrographs of negatively stained specimens to be expected. This expected result corresponds exactly with the observed doublet seen in electron microscopy. (3) The periphery of the micelle, which is known to be polar, has a singlet bilayer. The interior of the micelle, which is known to be nonpolar, has doublet bilayers. (4) Headpiece-stalk sectors from the mitochondrial cristal membrane attach to micellar phospholipid, and do so only on doublet regions. (5) The attachment of headpiece-stalk sectors to micellar phospholipid doublet regions is a variant of the physiological hydrophobic attachment of these sectors to the base pieces of the tripartite repeating units of the mitochondrial cristal membrane. The surface of the bilayer with doublet character to which headpiece-stalk sectors attach is composed of the nonpolar fatty acid chains of the phospholipid molecules. This hydrophobic surface mimics the hydrophobic sites in the base pieces at which the hydrophobic ends of the stalks associate. (6) The headpiece-stalk sectors induce an inversion of the polar periphery of the phospholipid micelle by stabilizing the nonpolar doublet structure. This stabilization is achieved by hydrophobic binding.

These observations, and the deductions made from them, provide a rational basis for interpreting phenomena which hitherto have been inexplicable. One such phenomenon is the fact that phospholipid micelles associated with cytochrome *c* in water can be extracted into heptane by addition of ethanol to a final concentration of 20% (Green and Fleischer, 1964; Das *et al.*, 1962), which carries the implication that there must be an inversion of the molecules of phospholipid within the bilayers of the micelle. An inversion of orientation involving thousands of phospholipid molecules in a micelle would involve large energy requirements and is not readily accounted for in terms of a simple mechanism. If, however, there is no true inversion at all, but a pairing of two half-bilayers, each from a different micelle, as illustrated in Figure 11, leading to a new orientation of the molecules in the newly formed bilayer, an orderly transition can be visualized without invoking reorientations of 180° of the phospholipid molecules within the bilayers.

Another hitherto unexplainable phenomenon can now be rationalized. Phospholipid micelles in water are known to increase in size during prolonged storage, and this size augmentation continues to the point of visible precipitation (Green and Fleischer, 1964), despite the fact that micelles should be very stable structures in water. The postulate that this precipitation is due to coalescence of micelles is untenable because of electrostatic repulsion of micelles due to their external charges. An actual incorporation of micellar material from one micelle into another is required. This incorporation can be visualized if there is a pairing of two half-bilayers from two different micelles, the new bilayer being stabilized by a wrapping mechanism. The greater the micellar size, the greater the stability of the overall system because of the decrease in the ratio of surface area to volume.

One further phenomenon made understandable by the

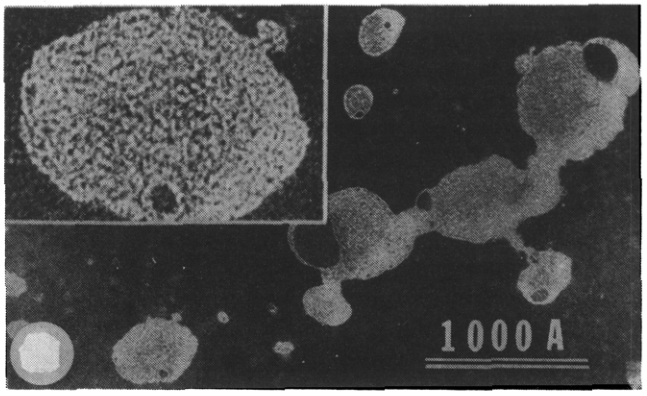


FIGURE 12: Electron micrograph of β-hydroxybutyrate dehydrogenase associated with asolectin, negatively stained with phosphotungstic acid. Insert, high magnification indicating peppering of protein units over the entire surface of the phospholipid micelle.

explanations given above is the observation that the hydrophobic interaction of micellar phospholipid with solvent-extracted mitochondria involves the transfer of *all species* of the phospholipid molecules in the micelle to protein when the micelle is presented to the solvent-extracted mitochondria (Fleischer *et al.*, 1962). There is no selection of certain individual species of phospholipid molecules with exclusion of other species during the interaction. It may be that the micelle is "fed" into the mitochondrion in continuous ribbons or sheets and each ribbon or sheet contains the phospholipid molecules in an orientation appropriate for hydrophobic interaction with the mitochondrial protein. The studies presented here are consistent with the notion that doublet bilayers can be payed out from pairs of touching micelles when a favorable acceptor system is present which can interact with the doublet on both sides of the bilayer. Thus, a doublet bilayer of phospholipid can result from the tailing tactic, followed by association and stabilization of the doublet by attachment of solvent-extracted mitochondria, thus resulting in the eventual total unwinding of the micelle. One half-bilayer of the doublet tail could associate with one mitochondrion, the other half-bilayer with another. This procedure would result in mitochondria to which phospholipid had hydrophobically reassociated with the protein and in which the polar groups of the reassociated phospholipid are oriented externally into the aqueous medium.

The proposals made in this communication also allow us to make predictions about the two possible ways in which protein can associate with micellar phospholipid. These two ways are hydrophobically and ionically. We have already discussed in detail our proposals for the method of hydrophobic interaction, with the headpiece-stalk sectors from the mitochondrial cristal membrane being a typical example of a hydrophobic protein which undergoes such an interaction, and in which the doublet form of the phospholipid bilayer is involved. By contrast, an ionic interaction between phospholipid and protein would perforce be limited to the singlet form of the bilayer on the periphery of the micelle. It would be expected that in an electrostatic interaction of a protein with a phospholipid micelle, the original micelle could remain unchanged in size but would be completely covered with the protein. In negatively stained electron micrographs of

such reacted micelles, the laminar arrangements would no longer be seen. Precisely this result has been observed in the interaction of the β-hydroxybutyrate dehydrogenase with micellar mitochondrial phospholipid, as seen in Figure 12 (Green *et al.*, 1967). Since it is the periphery of the micelle which is covered with the dehydrogenase, it follows that the interaction of the protein with the micelle is probably electrostatic in character.

In conclusion, we would like to reemphasize the fact that we have offered a series of proposals to explain a wide variety of observations by a relatively few simple concepts. In particular, we make the salient point that the ability of the doublet bilayer of phospholipid to provide a hydrophobic surface essentially functionally equivalent to that presented by a hydrophobic region on a face of the base pieces accounts for the fact that a doublet bilayer of phospholipid can combine with headpiece-stalk sectors in the same way as do base-pieces. This substitution of bilayer for base pieces does not necessarily mean that phospholipid itself is involved in the interaction of these sectors with base pieces. The ability to act as a substitute merely establishes that both bilayer and base pieces provide hydrophobic surfaces which can interact with the headpiece-stalk sectors.

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