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Physical Characteristics of Proteins of the Electron Transfer System and Interpretation of the Structure of the Mitochondrion*

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A protein has been isolated in large amounts from beef heart mitochondria which satisfies various criteria for a structural protein. The homogeneity of the isolated structural protein has been established by ultracentrifugal, electrophoretic, and end-group analysis. At neutral *p*H the structural protein forms a water-insoluble polymeric aggregate, but at *p*H 11, or in the presence of anionic detergents, a monomeric form of the protein can be demonstrated (m.w. 2×10^4–3×10^4). Cytochromes *a*, *b*, and c_1 show analogous behavior with respect to the polymer-monomer transition. Structural protein forms one-to-one water-soluble complexes with each of the cytochromes, which are soluble in aqueous media at *p*H 7; the identity of these complexes has been rigorously established. The hydrophobic bond is the predominant type responsible both for the polymerization phenomenon and for complex formation between the monomeric species of the structural protein and of the cytochromes. Structural protein is capable of binding phospholipid; this property is shared by the three cytochromes. The interactions between structural protein and cytochromes and between structural protein and lipid have considerable relevance to the problem of mitochondrial organization.

For many years it has been recognized that the oxidation of substrates of the citric acid cycle by molecular oxygen depends on the structural integrity of a catalytic unit constituting a multienzyme system (Green, 1956–1957). Several observations have led to the postulate that the transfer of electrons involved in this oxidation takes place within an array of oxidation-reduction enzymes that are organized not only in an enzymatic sense but also in a structural sense. The very fact of an organized system of enzymes suggests a complex in which specific chemical interactions at the molecular level stabilize the molecular array. The study of mitochondrial proteins can, therefore, yield information on both the structure of the organized enzyme system and the nature of the specific forces involved in stabilizing the system.

Inherent in the concept of the mitochondrion as an organized and structured system of enzymes has been the assumption that all the component parts (proteins, lipids, and coenzymes) fulfill some role relevant to the function of the mitochondrion (Green, 1956–1957). It was only when the program of systematic isolation of all the protein components of the mitochondrion had neared completion and when a rough balance sheet could be prepared that it became possible to put this assumption to the test. The amounts of oxidation reduction protein components in the mitochondrion were insufficient by a very large margin (they constituted no more than 25%) to account for the total protein of the mitochondrion (Green *et al.*, 1961b). This conclusion was reinforced by our observations that in the isolation of cytochromes *a*, *b*, and c_1 the principal contaminant was a colorless protein without any oxidation-reduction groups and that this protein formed such tight complexes with each of the cytochromes

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that in the final stages of the isolation procedure the cytochromes appeared to be homogeneous even though the concentration of heme per mg of protein could be increased by further purification. These circumstances led us to the assumption that a colorless protein was implicated in the structural alignment of the respiratory assemblies. Such a protein was finally isolated in relatively high yield from beef heart mitochondria (Green *et al.*, 1961b); the present communication deals with its properties.

The mitochondrion can be conceived of as a structural mosaic of three entities: (1) a particulate electron transfer chain; (2) an insoluble structural protein network; and (3) a group of detachable (readily solubilized) dehydrogenase systems and auxiliary enzymes which, respectively, function in generating DPNH from a variety of oxidizable substrates and in catalyzing ATP-dependent syntheses (Green, 1961). By appropriate procedures the mitochondrion can readily be resolved into these three entities.

When mitochondria are exposed to sonic irradiation the primary dehydrogenase complexes and auxiliary enzymes are almost quantitatively detached from the structured particulate residue (Linnane and Ziegler, 1958). These become soluble and can readily be separated from the particulate fraction, containing the electron transport chain still linked to the structural protein, into what is called the electron transfer particle.

The electron transfer particle can be broken down further and each of the cytochromes (*a*, *b*, *c*, c_1), flavoprotein dehydrogenases (f_S, f_D), and the structural protein can be isolated in a purified form (Green, 1961). The isolated forms of these proteins have been shown in this paper to be able to interact markedly both with themselves, to form higher polymers, and with each other, to form specific stoichiometric complexes (Criddle *et al.*, 1961; Green *et al.*, 1961a).

In this study attempts have been made to define more precisely the various problems concerned with mitochondrial structure in terms of the following questions: (1) How are the proteins of the mitochondrion ordered into stable molecular arrays? (2) What is the nature of the forces stabilizing the interaction? (3) What concept of mitochondrial organization can be deduced on the basis of the capabilities of the various proteins of the mitochondrion to interact with each other and with the other components of the mitochondrion? In this study, the analytical ultracentrifuge has been employed as a tool for quantitative determination of various soluble protein complexes and of their specific association or dissociation. Specific protein-protein interactions can be detected by the stoichiometry of components in complexes that give a single sedimenting boundary, and also by determining equilibrium molecular weights that are indicative of strong stoichiometric interaction. Interactions that lead to precipitates or to heterogeneous non-stoichiometric complexes may reflect important properties of the biological system but cannot be described in the simple, quantitative terms that describe soluble complexes. Even within these limitations this study has demonstrated a variety of specific reactions that cast light on the role of structural protein in mitochondrial organization.

Experimental

Materials.—The various cytochromes were prepared by the following procedures: Cytochrome *a* by the method of Griffiths and Wharton (1961); cytochrome *b* by the method of Bomstein *et al.* (1961). Cytochrome *c* was obtained from the Sigma Chemical Company (Type III).

Structural Protein.—When beef heart mitochondria[1] (in a 0.25 M sucrose suspension containing 20 mg of protein per ml) are treated at 0° with deoxycholate (2 mg per mg of protein), cholate (1 mg per mg of protein), and 0.75 mg of sodium dodecyl sulfate (SDS) per mg of protein, an essentially clear "solution" is obtained (Green *et al.*, 1961b). After clarification at 40,000 × *g* to remove a brown-green residue (protein free), enough solid $Na_2S_2O_4$ is added to reduce the cytochromes; then the solution is brought to 12% saturation with respect to ammonium sulfate (*p*H kept at 7.0, and temperature at 0–5°). The copious white precipitate is sedimented and washed thoroughly with 0.25 M sucrose. The washed residue, suspended in 0.25 M sucrose, is then treated with butanol (20% by volume) in the presence of 20% saturated ammonium sulfate and deoxycholate (1 mg per mg of protein) to remove lipid. The extracted precipitate is washed in 0.25 M sucrose and is then extracted with 10 volumes of 75% methanol at 50° ten to twenty times to remove deoxycholate. The yield is about 33% of the total protein in mitochondria and is 55% of the particulate protein (total protein minus readily extractable protein).

The supernatant fluid, after removal of the structural protein, contains the four cytochromes. At higher ammonium sulfate concentrations (20 to 30%) these are precipitated at a purity level some three to four times greater than that of the same components in the mitochondrion. The cytochromes do not show spectral evidence of structural modification.

Structural protein can also be prepared from particulate fractions of the electron transfer chain, or from preparations of purified cytochromes, by similar procedures. Our initial evidence that this material fulfills the role of a structural protein was based on the demonstration that what appeared to be the same protein was isolated from preparations of cytochromes *b*, c_1, and *a*. The preparations made from these cytochromes, which occupy different positions in the electron transfer sequence, were found to have similar characteris-

[1] We are indebted to Oscar Mayer of Madison, Wis., for the gift of fresh beef hearts.

tics with respect to sedimentation, molecular weight, electrophoretic migration, and solubility.

Other Reagents.—Myoglobin was obtained from the Pentex Laboratories. Sodium dodecyl sulfate, obtained from Eastman Kodak, was recrystallized from ethanol. The bile salts, products of Matheson, were recrystallized from aqueous ethanol before use.

Analytical Procedures.—Protein was determined by the biuret procedure of Gornall *et al.* (1949) or by direct weighing of the anhydrous residue after removal of lipid, bile salts, and inorganic salts. The respective procedures for removal of deoxycholate and of lipid from preparations of the structural protein have been described above. The concentration of lipid in the various cytochrome preparations and in the preparation of structural protein was determined by estimation of the total phosphorus (on the assumption that one mg of mitochondrial phospholipid is equivalent to 0.038 mg of phosphorus). These analyses were conducted on dialyzed preparations, free from inorganic phosphate. The method of Chen *et al.* (1956) was used for the determination of total phosphorus.

Experimental Procedures.—All of the sedimentation experiments described in the remainder of this paper were carried out in a Model E Spinco analytical ultracentrifuge (phase plate schlieren optics and either the standard 12-mm cells or matched 12-mm cells with a prismatic window). Molecular weight measurements were made by Ehrenberg's (1957) method of approach to sedimentation equilibrium or by determining both the sedimentation coefficient and the diffusion coefficient. Electrophoresis and diffusion experiments were carried out in a Spinco Model H electrophoresis apparatus with the Tiselius type of cell.

Partial specific volumes were measured by the falling drop method of Linderstrøm-Lang and Lanz (1938) in a linear-density gradient column, formed by a mixture of water-saturated *n*-chlorobutane and chlorobenzene, or were estimated from the data on the amino acid composition of the protein.

As molecular weight determinations were usually made in the presence of detergents it was necessary to correct for bound detergent. At the protein–detergent ratio of about 10:1 (w/w), which was utilized in many of these experiments, one may assume that all of the detergent was bound by the protein (Schachman, 1960). This assumption makes it possible to correct both the partial specific volume of the protein and the molecular weight for the amount of bound detergent. The correction for $\bar{v}$ is made by evaluating the relative amounts of protein and sodium dodecyl sulfate in the complex and assuming additivity of the respective partial specific volumes. The molecular weight, computed by substituting for $\bar{v}$ the value determined by measurement of the protein-bile salt complex (Schachman, 1960), is the molecular weight of the protein plus bound detergent if the assumption of complete binding of the detergent is correct. The weight of the detergent must be subtracted to obtain the molecular weight of the protein. All of the molecular weights referred to in this paper have been corrected in this manner for detergent.

Carboxyl-Terminal Amino Acid Determination.—Carboxyl-terminal analysis was carried out by subjecting structural protein to hydrolysis by carboxypeptidase. This proteolytic enzyme, which acts by cleaving successively the carboxyl-terminal amino acids from the end of the peptide chain, was dissolved in *p*H 8.5 NaOH to a final concentration of 1 mg per ml. The enzyme (0.8 ml of a 0.1% solution) was added to 8 ml of a solution of structural protein containing 5 mg of protein per ml. This solution was prepared by dissolving structural protein in dilute NaOH (*p*H 10.5) containing 0.05 per cent sodium dodecyl sulfate. The solution was dialyzed against NaOH, *p*H 9.5, for 12 hours, and then was incubated at 25°. After 2, 4, and 6 hours, 2.5-ml aliquots were removed from the reaction mixture and the reaction was stopped by the addition of a drop of concentrated HCl. This acidification also resulted in precipitation of the structural protein which could then be removed by low-speed centrifugation. After the *p*H of the samples was readjusted to 8.5 with $NaHCO_3$, 0.5 ml of a 1% solution of 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanol was added, together with a glass bead, and the mixture was shaken for 1 hour at room temperature to yield the dinitrophenyl derivatives of the amino acids remaining in solution (Fraenkel-Conrat *et al.*, 1955). After the *p*H of the samples was lowered to 1.5 with concentrated HCl, the solutions were extracted three times with 3-ml portions of water-saturated, peroxide-free ether. All of the non-basic DNP-amino acids derivatives were extracted into the ether layer. The combined ether extracts from the acidified amino acid solutions were then evaporated to dryness. The residue was dissolved in 0.05 ml of acetone and the solution was chromatographed on paper according to the method of Fraenkel-Conrat *et al.* (1955). The water phase was checked separately for the presence of DNP-arginine and DNP-histidine.

Amino-Terminal Amino Acid Determination.—Two methods were utilized for the determination of the amino-terminal amino acids of structural protein. Apart from some details in the preparation of the samples and in the times for hydrolysis of protein to amino acids, the Sanger 1-fluoro-2,4-dinitrobenzene method was used as outlined by Fraenkel-Conrat *et al.* (1955). The Edman phenylisothiocyanate method for the cleavage of amino-terminal amino acids was also followed as outlined by Erikson and Sjöquist (1960). The solvent for chromatography consisted of heptane, ethylene chloride, and 75% formic acid in the volume ratio 30:60:5 respectively.

Several methods of preparing the structural

protein samples for amino-terminal determinations were tested since the usual techniques for solubilization were ineffective. The extreme insolubility of structural protein under the conditions commonly used for amino-terminal analysis limited the extent of the labeling reaction to such a degree that chromatography of the final products revealed no amino-terminal amino acids. The inaccessibility of the amino-terminal amino acids to the labeling reagents could be the consequence of any of several difficulties. First, the binding between components in the insoluble protein residues could be sufficiently stable, and the arrangement of molecules could be such, that access of the labeling reagent to the amino-terminal groups is physically prohibited. Second, the configuration of each individual molecule could give rise to the same screening effect; the tail would then be buried in the interior of the protein. Third, the presence of other reagents, such as the detergent that is bound to the protein molecule, might block access of the labeling reagent to the terminal group. To obviate each of these possibilities, the following techniques were used in the preparation of the samples: (1) Structural protein was dissolved in a solution of high detergent concentration to minimize protein-protein interaction. (2) Structural protein was dissolved in 67% acetic acid to break up all internal structure; excess detergent was then added and the resulting solution was neutralized. (3) Structural protein was dissolved in 8 M urea, *p*H 9, with 0.02% sodium dodecyl sulfate added to break up internal structure. (4) Structural protein was extracted with warm 70% methanol to remove detergent and was then dissolved in 9 M urea brought to *p*H 10.5 with sodium hydroxide. The structural protein that was solubilized by these procedures was always partially precipitated as the 1-fluoro-2,4-dinitrobenzene reagent was added to the solution.

Results

Physical Characteristics of Cytochrome c_1.—The first of the cytochromes to be studied in detail was cytochrome c_1. The isolation of this protein in a water-soluble, lipid-free form, uncontaminated by the presence of other cytochromes, was first accomplished by Green *et al.* (1959) by treatment with butanol, sodium dodecyl sulfate, and cholate and by repeated fractionation with ammonium sulfate. The heme-iron ratio of 1:1 found in the product indicated that all of the iron was bound in the heme.

The molecular weight of cytochrome c_1 was determined by evaluating the sedimentation coefficient (S), diffusion constant (D), and partial specific volume ($\bar{v}$) of the protein. The molecular weight (M) was calculated by substituting the values for S, D, and $\bar{v}$ in the familiar Svedberg equation ($M = RTS/D(1-\bar{v}p)$ (Svedberg and Pederson, 1940).

On the basis of a value for $S_{20,w}$ of 12.05, a value for $D_{20,w}$ of 3.31×10^{-7} cm^2 per second, and a value for $\bar{v}$ of 0.762 ml per g, the calculated value for M is 360,000 grams per mole. Since the value for M, calculated by the Svedberg equation, is close to six times the minimal molecular weight of cytochrome c_1, calculated from the heme content, it follows that there are approximately six heme groups per molecule. Experiments in which both sedimentation velocity and electrophoretic mobility were measured showed the absence of any major contaminating material.

Since six heme groups per molecule were indicated, attempts were made to break this molecule down further into what was hoped would be a monomeric cytochrome c_1. The results of these attempts are shown in Table I.

Table I

The Stability of the Hexamer of Cytochrome c_1, in Presence of Various Reagents

Additions to the Aqueous Solution	S	m.w.
0.05 M Thioglycolate (*p*H 7.5)	12	360,000
1% Triton X-100	11	
Alkali to *p*H 10.5	10.2	350,000
As above after neutralization by dialysis to *p*H 7	12	350,000
Acid to *p*H 5.5	11.5	
6 M Urea	6.8	340,000
As above after dialysis against Tris buffer, *p*H 7.5	11.5	
0.0005 M sodium dodecyl sulfate	3.9	51,000
As above after dialysis against Tris buffer, *p*H 7.5	12	360,000
0.05 M Thioglycolate + 0.01% Triton X-100 (*p*H 7.8)	3.9	51,000

It can be seen that treatment with acid at *p*H 5.5, a point at which the molecule undergoes denaturation, or with base at *p*H 11, resulted in a change in frictional properties of the molecule without concomitant alteration of molecular weight. Treatment with 6 M urea, thioglycolate, or Triton X-100 (a non-ionic detergent) was also ineffective in reducing the molecular weight. It was found, however, that thioglycolate, in conjunction with a non-ionic detergent, or low concentrations of an anionic detergent alone (0.0005 M sodium dodecyl sulfate), brought about depolymerization. These observations suggested that the disruption of hydrophobic interactions (by sodium dodecyl sulfate) and intensification of charge repulsion (sulfhydryl anions) were the driving forces in the depolymerization.

The resulting product had only one heme per molecule of molecular weight 51,000 as determined by Ehrenberg's modification of the Archibald method of approach to sedimentation equilibrium. Figure 1 shows a comparison between the sedimentation properties of the monomeric and hexameric forms of cytochrome c_1. The depolymerization of the hexamer by sodium dodecyl sulfate was readily reversed by dialysis of the c_1, dissolved in 0.0005 M sodium dodecyl sulfate,

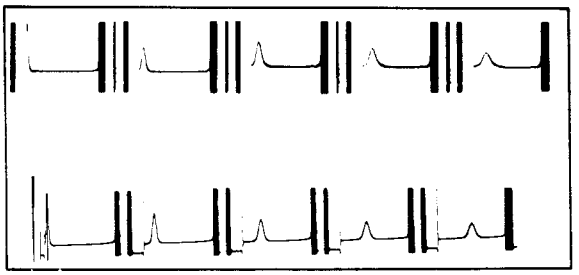

FIG. 1.—Comparison of sedimentation patterns of cytochrome c_1, monomer (upper half) and hexamer (lower half). The buffer system (*p*H 7.4) was 1×10^{-3} M in Tris (chloride) and 5×10^{-2} M in NaCl. The hexamer was converted to the monomeric form by treatment with 5×10^{-4} M sodium dodecyl sulfate for 12 hours before sedimentation analysis. The centrifugal speed was 59,780 rpm and the interval between photographs was 8 minutes.

against Tris chloride buffer, *p*H 7.5 (Criddle *et al.*, 1961). Monomer production could be brought essentially to completion within four hours after exposure of the hexamer to 0.0005 M sodium dodecyl sulfate. After a shorter time (1 hour) both the hexamer and the monomer could be observed in the centrifuge but there was no evidence of appreciable amounts of aggregates of intermediate size.

To explain these observations, we have postulated sites of lipophilic nature on the cytochrome c_1 monomer, which account for the production of stable polymers during the isolation procedure. As has been mentioned, sodium dodecyl sulfate, which reversibly breaks the hexamer down into monomers, was also used in the preparation of cytochrome c_1. As a result of this, we cannot assign any physiologic significance to the isolation of the cytochrome in the hexameric form. It is probable that the chemistry and geometry of the associating sites on the monomer are the factors that determine that the most stable polymer configuration is that of a hexamer.

Sodium dodecyl sulfate is able to break down the hexamer by competing with the cytochrome c_1 molecules for the individual hydrophobic binding sites. It appears that in addition to the competition by the detergent, another effect, that of charge repulsion, is also essential for depolymerizing the hexamer. For this reason the non-ionic detergents (for example, triton X-100) are ineffective whereas anionic detergents are successful. When thioglycolate, which is capable of introducing a charged group into a few key positions in the molecule, was used in conjunction with a non-ionic detergent, we did, however, obtain the monomeric species.

Characteristics of Cytochrome a.—Most of our studies on cytochrome *a* (cytochrome oxidase) were carried out with samples prepared by the method of Ambe and Venkataraman (1959), although modifications of this procedure give rise to preparations of cytochrome oxidase with the same general characteristics. The most notable

FIG. 2.—Sedimentation pattern of cytochrome *a* in its polymeric forms. The buffer used (*p*H 8.5) was 1×10^{-3} M in Tris (acetate) with NaCl added to bring the ionic strength to 0.05. Photographs were taken at 8, 24, and 40 minutes after sedimentation at 59,780 rpm.

property of the cytochrome oxidase obtained by these procedures is the tendency for the enzyme to aggregate into higher polymers. With this cytochrome, however, no polymer of specific size analogous to the c_1 polymer could be isolated. Instead, sedimentation analysis revealed several discrete peaks of differing sedimentation coefficients, probably corresponding to monomer, dimer, trimer, etc., as shown in Figure 2. That these peaks correspond to polymers, and not to molecular species devoid of cytochrome *a*, was shown from partition-cell experiments in which all of the sedimenting components had the characteristic spectrum of heme *a*. Furthermore, experiments with free electrophoresis indicated that all of the components had the same charge-to-mass ratio (Criddle and Bock, 1959).

Attempts at depolymerization of these aggregates by means of thioglycolate, deoxycholate, 6 M urea, or Triton X-100 met with little success. However, as in the case of the cytochrome c_1, low concentrations of sodium dodecyl sulfate caused an immediate breakdown of the higher polymers into a predominantly monomeric species, although some higher polymers were still present. Further addition of sodium dodecyl sulfate increased the concentration of the monomers at the expense of the higher polymers. The molecular weight of the monomer obtained by sodium dodecyl sulfate treatment was found to be approximately 72,000, corresponding to one heme group, one copper atom, and one iron atom per molecule.

Recently, Takemori *et al.* (1960) have been able to obtain a purified (but relatively inactive) cytochrome *a* preparation using the detergent "Emasol." They were able to isolate a specific pentamer of cytochrome oxidase analogous to the specific hexamer of cytochrome c_1. This pentamer apparently had 5 heme groups, 5 copper atoms, and 5 iron atoms per molecule. This complex was probably formed during the isolation of the cytochrome *a* in a manner similar to that in which the hexamer of cytochrome c_1 was formed.

Polymer-Monomer Relations of Cytochrome b.—Cytochrome *b* has been isolated only in the form of a complex having a large molecular weight. This complex also could be broken down with lipophilic reagents. In this case, the cationic detergent cetyldiethylmethyl ammonium bromide was most effective for the disaggregation of the com-

plex into a particle of m.w. 28,000 grams per mole (Goldberger *et al.* 1961).

Cytochrome c.—Cytochrome *c* was the first of the cytochromes to be isolated from beef heart in a purified form (Keilin and Hartree 1938; Theorell and Åkeson, 1941). In contrast to the other cytochromes, cytochrome *c* is readily extractable from tissues into aqueous solutions, shows no marked tendency to aggregate, and is quite stable. Crystalline preparations of cytochrome *c* have been obtained from a variety of sources, including fish muscle, beef heart, swine heart, and bakers yeast (Bodo, 1955; Hagihara, 1956).

The particle weight of cytochrome *c* has been determined to be about 13,000 in agreement with the minimal molecular weight calculated from an iron content of 0.43% (Theorell and Åkeson, 1941). Hence, there is only one iron atom present per molecule of heme protein.

These observed differences between the behavior of cytochrome *c* and that of the other cytochromes in solution will be referred to later and will be significant in the interpretation of our findings on the structure of the electron transfer chain of the mitochondrion.

Mitochondrial Structural Protein.—The proteins that are known to participate in the oxidation-reduction reactions of the electron transfer particle (*i.e.*, succinic dehydrogenase, DPNH dehydrogenase, and cytochromes *a*, *b*, c_1, and *c*) have all been isolated in a fairly homogeneous state and their minimal molecular weights have been determined. From these data, in conjunction with the known concentrations of the prosthetic groups of these six proteins, one may calculate how much of the total protein of the electron transport particle is accounted for by the proteins having oxidation-reduction properties. Such a calculation (*cf.* Table II) shows that the six known oxidation-reduction proteins account for only about 21% of the total protein of the electron transport particle. It is obvious from these figures that there must be a large amount of protein yet unaccounted for in the electron transport particle. This fact led to the search for, and the study of, another protein that is basic to mitochondrial structure.

In the isolation of cytochromes *a*, *b*, and c_1 it is relatively easy to obtain a separation of any one cytochrome from the others early in the preparation. The main component that must be removed for further purification is a colorless protein (or group of proteins) that is bound tenaciously to the individual cytochromes. These observations have led us to postulate that the mitochondrion contains structural protein that itself lacks oxidation-reduction components, but is intimately associated both with the oxidation-reduction proteins of the electron transfer chain and with lipids (Green *et al.*, 1961b).

Physical Properties of Structural Protein.—Structural protein is almost completely insoluble in water at neutral *p*H values, but, like the structural proteins from many viruses, ribosomes, etc., it is readily soluble in aqueous media containing reagents that induce major charge repulsions between the molecules or that attack hydrophobic bonds (Anderer, 1959). The most effective reagents for dispersing the interacting subunits of the structural protein are those used in bringing about the depolymerization of the cytochrome aggregates, *i.e.*, anionic detergents. Table III lists the solubility of structural protein in various media along with the results of sedimentation and molecular weight measurements made on the resultant soluble species. The accuracy of these latter measurements is subject to some question, as will be discussed later, but for the present they indicate an order of magnitude and show that apparently the same species of protein is obtained by each of the solubilization techniques.

As indicated in Table III, anionic detergents and extremes of *p*H are most effective in the solubilization of structural protein. Eight M urea, which by itself fails to bring about solubilization of structural protein, aids in its solubilization in other systems, probably by breaking up the secondary structure of the protein molecules. Non-ionic detergent solutions and organic solvents proved to be ineffective in dissolving structural protein.

TABLE II

PERCENTAGE OF THE TOTAL PROTEIN OF ELECTRON TRANSPORT PARTICLES (ETP) ACCOUNTABLE IN TERMS OF THE COMPONENT OXIDATION-REDUCTION PROTEINS

Protein	In ETP[a] (1)	In the Isolated[b] Component (2)	% of Total Protein[c] in ETP (3)
	μ moles/g		
Succinic dehydrogenase	0.23	4.3	5.4
DPNH dehydrogenase	0.27	14.3	1.9
Cytochrome *a*	1.53	13.8	11.1
Cytochrome *b*	0.72	40.0	1.8
Cytochrome c_1	0.23[d]	27.2	0.9
Cytochrome *c*	0.23[d]	83.3	0.3
		Total	21.4

[a] The concentrations listed for ETP are taken from the data of Fernandez-Moran, Blair, Oda, and Green (in preparation). [b] References for the values listed are as follows: succinic dehydrogenase (Ziegler and Doeg, 1959; Singer *et al.*, 1957); DPNH dehydrogenase (Ziegler *et al.*, 1959); cytochrome *a* (Griffiths and Wharton, 1961); cytochrome *b* (Goldberger *et al.*, 1961); cytochrome c_1 (Bomstein *et al.*, 1961); cytochrome *c* (Theorell and Åkeson, 1941). [c] The values shown in column 3 were calculated by the following formula:

$$\frac{\text{concentration in ETP}}{\text{concentration in the isolated components}} \times 100$$

[d] Cytochromes *c* and c_1 were estimated together, but in ETP the two are present in approximately equal amount.

TABLE III
SOLUBILITY AND MOLECULAR WEIGHT OF STRUCTURAL PROTEIN IN VARIOUS AQUEOUS MEDIA

Additions	Solubility[a]	S	m.w.
Alkali to *p*H 11	+	—	—
Sodium dodecyl sulfate (0.1%); *p*H 11	++	2.1	2.2×10^4–2.8×10^4
Sodium dodecyl sulfate (0.1%); *p*H 7.8	+	—	—
Sodium dodecyl sulfate (0.3%); *p*H 7.8	++	1.8	—
Acetic acid (67%)	++	2.2	2.3×10^4–2.8×10^4
Urea (8 M); *p*H 7.8	−	—	
Urea (8 M); sodium dodecyl sulfate (0.1%); *p*H 7.8	++	2.2	2.0×10^4–3.0×10^4
Thioglycolate (3 M); *p*H 8.5	−		
Guanidinium chloride (4 M), *p*H 7.8	−		
Triton X-100 (1%); *p*H 7.8	−		
Pyridine (50%)	−		

[a] The range of solubility is as follows: ++ denotes solubility >0.5%; + denotes solubility <0.5%; − denotes no measurable solubility.

Sedimentation Studies and Molecular Weight Determination of Structural Protein.—The determination of the molecular weight of mitochondrial structural protein is complicated by the strong tendency of the protein to aggregate to higher polymers. Furthermore, those same conditions that bring about solubilization of the protein also introduce uncertainties into the molecular weight determination. It is desirable, for example, to determine molecular weights by the centrifugal method at a *p*H somewhere near the isoelectric point of a protein to minimize nonideality and particle interactions. The only known way to solubilize structural protein near neutral *p*H values, however, is to add large quantities of anionic detergent (0.3%). Detergents at such high concentrations then introduce other uncertainties into the determination of molecular weight because they are bound, to an undetermined extent, by the protein. First, this results in a change in the apparent molecular weight of the protein; second, it causes a build-up of charge on the protein, of sign corresponding to that of the detergent, and thus leads to nonideality; third, it probably disrupts the organized internal structure of the protein subunits themselves through rupture of hydrophobic bonds and repulsion of charges. The presence of an electrolyte, added to protein solutions for centrifugal study, has the effect of "swamping out" much of the nonideality due to charge interaction. With mitochondrial structural protein solutions, however, the addition of an electrolyte, in a concentration adequate to swamp out the charge effect, results in an increase in ionic strength and also has the effect of strengthening the hydrophobic interactions between the subunits to an extent such that the protein is precipitated by these salts, except at elevated *p*H values or in concentrated detergent solutions.

As a result of these difficulties, an attempt was made to find the "mildest" conditions possible for the solubilization of structural protein for physical study. Although these conditions seem extremely harsh, from the standpoint of the protein chemist, it should be remembered that structural proteins from all sources appear to be stable. The conditions arrived at (largely by trial and error) were 0.1% sodium dodecyl sulfate, *p*H 10.5, and $\Gamma/2$, brought to 0.1 with NaCl for a 1% solution of protein. Also, it should be remembered that, harsh as these conditions may be, they are effective in solubilizing the protein; evidence will be presented later indicating that the protein, so treated, can still carry out at least some aspects of its original function.

In Figure 3 is shown a typical sedimentation pattern of mitochondrial structural protein, solubilized as described above. It can be seen that a single, symmetrical migrating boundary was sedimented throughout the length of the cell. The sedimentation coefficients of a large number of different preparations of structural protein, dissolved in this manner, were found to fall within two ranges, approximately $^4/_5$ of the samples having coefficients between about 1.9 and 2.2 *S* and the other $^1/_5$ between 2.6 and 2.8 *S*; this disparity indicated a lack of complete reproducibility of the preparation.

Molecular weight determinations verified the two types of preparations. Those with sedimentation coefficients in the lower range had molecular weights of about 22,000 and those in the higher range had molecular weights of about 45,000. This immediately suggests the presence of a dimer in those preparations showing the higher molecular weight. Increase in the concentration of detergent, or of the *p*H of the solution, does

FIG. 3.—Sedimentation of structural protein depolymerized with 0.2% sodium dodecyl sulfate. The measurement was made at 20° in a medium that was 1×10^{-3} M in phosphate, 1×10^{-1} M in NaCl, and 0.2% in sodium dodecyl sulfate; the final *p*H was adjusted to 8.5. The centrifugal speed was 59,780 rpm and the interval between photographs was 16 minutes.

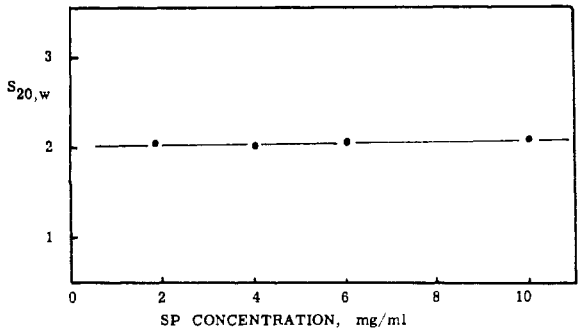

FIG. 4.—The sedimentation coefficient of structural protein as a function of the concentration of structural protein. The conditions of the sedimentation analysis were the same as those described in the legend of Figure 3.

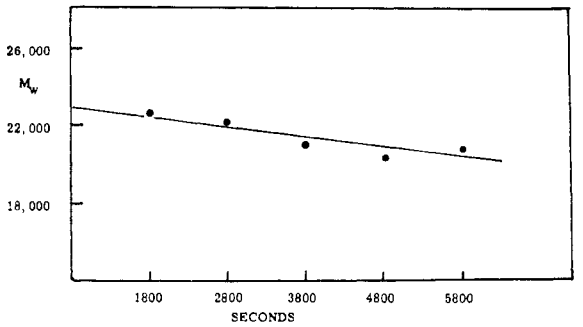

FIG. 5.—Apparent weight average molecular weight of structural protein at the meniscus as a function of time in an approach to sedimentation equilibrium analysis.

not shift the balance in favor of the monomeric species. A tentative correlation between increasing storage time of the mitochondria used as the starting material and increase in the probability of obtaining the species of higher molecular weight exists, but this is indefinite.

Molecular weights of the structural protein preparations were all determined by the Ehrenberg method, which yields the weight average molecular weight of the protein solution at the meniscus of the cell. Since the weight ratio of protein to sodium dodecyl sulfate was kept at about 10:1 in these experiments, it may be assumed that all of the detergent was bound by the protein and that it was, therefore, permissible to correct the experimentally determined weight for the contribution made by the detergent.

Because structural protein has such a marked tendency to aggregate, it was suspected that, even in the solutions used for these studies, sufficient reversible dimerization could take place during sedimentation to affect the apparent molecular weight of the monomer. Two methods were used to determine whether this was the case. If the dimerization were rapidly reversible, a plot of S versus concentration should show an increase with increasing concentration. Figure 4 shows that this was the case only to a slight extent. When m.w. is determined by the Ehrenberg method, a plot is made of the ratio S/D (which is proportional to the molecular weight at the meniscus) vs. time. From plots of this type, it is possible once more to detect inhomogeneity of the sample because the aggregates of higher m.w. pull away from the meniscus more rapidly than does the monomer (Criddle and Bock, 1959). Thus, the weight average molecular weight at the meniscus decreases with time. A constantly decreasing slope is indicative either of a wide distribution of particle sizes or of a system undergoing rapidly reversible equilibrium; if a marked change in slope is noted, the system probably contains two or more distinct molecular species. Figure 5 shows a plot of S/D vs. time for a fresh structural protein preparation. This Figure indicates that a slight decrease in m.w. at the meniscus occurs with time and that the apparent molecular weight of the monomeric form (corrected for detergent binding) is approximately 20,000 to 25,000; the exact value is difficult to determine because the slope of the plot indicates the presence of some polymeric forms.

Further evidence that polymerization occurs in structural protein preparations was obtained by repeating this m.w. study on the same sample after it had stood at 4° for 4 weeks. In this case the initial weight average molecular weight had increased and the slope of S/D vs. time had become steeper. The evidence indicates that there are two types of aggregation taking place, one a rapidly reversible polymerization and the other a slower, more nearly irreversible polymer formation.

These molecular weight determinations point out the uncertainties introduced by the solvent systems and by the protein itself. Therefore, another procedure was sought in which these problems might be eliminated. The very tenacious binding properties of the structural protein afforded the solution. The significance of the binding properties of structural protein will be discussed in detail later; presently we consider only the results of the binding of the protein, myoglobin, by structural protein. It is possible to form a 1:1 complex of structural protein with myoglobin simply by adding an equimolar quantity of myoglobin to a solution of structural protein in 0.1% sodium dodecyl sulfate at *p*H 10.5, lowering the *p*H of the resulting solution to approximately 8.0, and dialyzing it for 3 days against Tris chloride buffer, *p*H 8, to remove any dialyzable detergent. The sedimentation patterns of the myoglobin–structural protein complex prepared in this manner is shown in Figure 6. It can be seen that a single, symmetrical, sedimenting boundary was obtained for the complex and that the myoglobin color migrated with the boundary. The sedimentation coefficient of the complex, corrected to standard conditions, was 2.6 S.

In the form of this myoglobin complex it was possible to solubilize structural protein at neutral *p*H, in an aqueous medium that was relatively

low in detergent, with no apparent tendency toward the formation of higher polymers. This procedure resolves the major uncertainties present in previous molecular weight determinations. As the molecular weight of myoglobin is well known, it was only necessary to determine the m.w. of the complex and to subtract the weight of myoglobin to obtain the m.w. of structural protein. The experimental procedures by which the molecular weight of the complex was determined were those previously described. The fact that the curve showing S/D as a function of time had zero slope is an indicator of uniformity. The S/D observed corresponded to a molecular weight of 40,000 g/M. Since 17,500 g/M is contributed by myoglobin (Polson, 1937) the molecular weight for structural protein is approximately 22,000—a value in good agreement with those measured directly.

When two equivalents of myoglobin were added to one equivalent of the dimeric form of structural protein (m.w. 44,000–45,000) under the conditions described above, once again a single migrating species was obtained upon centrifugation, with a sedimentation coefficient of 3.1 S in this case. The m.w. of this species was 76,000. This result supports the conclusion that the structural protein preparations of high molecular weight contain structural protein in the dimeric form.

Role of Structural Protein in the Mitochondrion.—While we have been using the term structural protein up to this point to describe this protein that occurs so abundantly in mitochondria, no evidence has been presented to support the thesis that a structural role is the major function of the protein. It is, generally speaking, a difficult task to prove that a protein does, in fact, function in a structural capacity. When dealing with enzymes, we can establish a role for an isolated enzyme simply by demonstrating its catalytic effect on a chemical reaction; there is no analogous single property that may be utilized to demonstrate a structural role.

If we were to assign a role in mitochondrial structure, analogous to that of structural protein in other known systems, we could say that its purpose is to interact both with itself and with the functional constituents of the complex in order to increase the order and stability of the unit (*cf.* structural protein of viruses and of ribosomes). In this case, the structural protein in the mitochondrion should be able to interact specifically with the proteins engaged in electron transfer, with the mitochondrial lipid components, and also with other molecules of its own kind. In order to demonstrate that this protein is, in fact, a structural protein, one should be able to present evidence for all of these interactions. That the protein interacts with itself is obvious from the extreme insolubility of the material under physiologic conditions. Evidence for its interaction with other mitochondrial constituents will be covered in detail for each individual mitochondrial component studied.

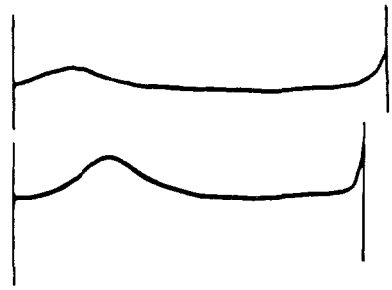

FIG. 6.—Sedimentation of myoglobin (upper half) and of a 1:1 molar complex of myoglobin with structural protein (lower half). The centrifugal speed was 59,780 rpm and the time at which photographs were taken was 64 minutes. The buffer (*p*H 8.0) was 1×10^{-2} M in Tris (chloride) and 1×10^{-1} M in NaCl.

Interaction of Structural Protein with Cytochrome c_1.—During the course of isolation and purification of cytochrome c_1, it was shown that c_1 could be separated from the other cytochromes and from flavoproteins with relative ease to yield a product that behaved electrophoretically and centrifugally as a single species. Further purification, which increased the heme-to-protein ratio, could be obtained, however, to yield what still appeared to be a homogeneous molecular species (Bomstein *et al.*, 1961). This apparent inconsistency can be explained on the basis that in the initial preparation c_1 exists as part of a complex containing structural protein. Each step that increases the purity of the cytochrome should then result in the removal of some of the structural protein. Support for this explanation has, indeed, been obtained from sedimentation studies on the fractions released during purification of cytochrome c_1, which show the proteins to have the same characteristics as structural protein.

More specific evidence for the interaction of structural protein with cytochrome c_1 has been obtained from "reconstitution" experiments. Structural protein was depolymerized at *p*H 10.5 in the presence of 0.1% sodium dodecyl sulfate; the protein solution was then dialyzed, first against 0.05 M NaCl; then, to remove excess detergent, it was dialyzed for 2 days against a solution of 0.05 M NaCl brought to *p*H 10.0 with sodium hydroxide. The dialysate was completely clear; the protein had a sedimentation coefficient of 2.1 S. Next, the hexameric form of c_1 (sedimentation coefficient 12 S) was dissolved in 0.025 M Tris acetate buffer, *p*H 7.6. Upon mixing roughly equimolar quantities of these two proteins, the structural protein, when complexed with c_1, remained in solution even though the free structural protein was completely insoluble at the resulting *p*H, 7.8. The original 2.1 S peak disappeared after mixing; the 12 S peak had almost vanished and a new 3.9 S peak was observed. Study of the sedimentation patterns showed that

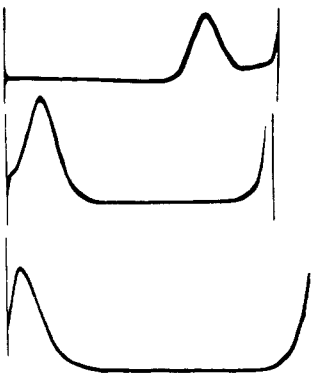

FIG. 7.—Demonstration by sedimentation analysis of an interaction between structural protein and cytochrome c_1. There are three sets of sedimentation patterns. These are, respectively (proceeding from top to bottom: (1) cytochrome c_1 hexamer; (2) a 1:1 molecular complex of structural protein and cytochrome c_1; and (3) structural protein. The experiments were carried out in Tris chloride buffer (*p*H 7.8) as described in the text. All pictures correspond to 56 minutes effective sedimentation at 59,780 rpm.

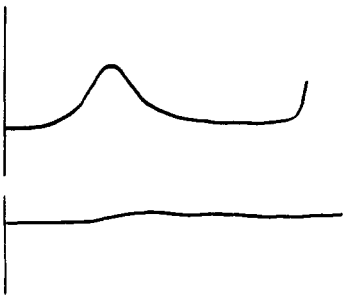

FIG. 8.—Sedimentation patterns of mixtures of cytochrome c_1 and structural protein. The mixture corresponding to the upper picture contained structural protein and c_1 in a 2:1 molar ratio; that corresponding to the lower picture contained structural protein and c_1 in a 1:2 molar ratio. In addition to the sedimenting species (of broad distribution) shown in the lower picture, a significant fraction of the complex under analysis had already sedimented to the bottom of the cell at the time the photograph was taken (48 minutes). Sedimentation was carried out at 59,780 rpm.

not quite all of the c_1 polymer of high molecular weight had been coverted into the complex. Further addition of another small quantity of structural protein to this mixture brought about the complete disappearance of the 12 *S* species and a slight increase in the sedimentation velocity of the hybrid complex (Fig. 7). The characteristic red color of the c_1 heme could be observed visually and was found to move both with the 12 *S* hexamer peak and with the newly formed peak. Moving boundary electrophoresis of the hybrid complex confirmed the presence of a single migrating species resulting from the formation of a protein complex.

It appears that the structural protein monomers are competing with the cytochrome c_1 subunits for those same protein binding sites that stabilize the hexamer. The result is a breakdown of the c_1 hexamer and the formation of a complex with molecular weight and sedimentation coefficient intermediate between those of the two components.

A determination of the stoichiometry of the binding of the structural protein to c_1 provides a further check on the molecular weights both of the c_1 and of the structural protein monomers. When a solution of structural protein, with an assumed m.w. of 22,000, is added to a solution of the cytochrome c_1 subunit (m.w. 55,000), a 1:1 complex is formed with sedimentation coefficient 3.9 *S* (*cf.* Fig. 7) and molecular weight 76,000 as determined by the approach to equilibrium method. This result provides additional evidence that the molecular weight of structural protein is 22,000 g per mole.

When structural protein (SP) was added to the c_1-SP complex in a 1:1 molar ratio once again a single migrating species was observed, with *S* 4.0, as shown in the upper portion of Fig. 8. The molecular weight of this complex was calculated to be 98,000, corresponding to one mole of the c_1 subunit complexed with two moles of structural protein. If, on the other hand, the c_1 subunit was added to the SP-c_1 complex in a 1:1 ratio a single complex was not formed; instead, the diffuse sedimenting boundaries shown in the lower portion of Figure 8 were observed. The preparation of a $(SP)_2$-c_1 complex was readily accomplished simply by adding more structural protein in stoichiometric amount. All attempts to prepare SP-$(c_1)n$ complexes, where *n* was greater than one, were unsuccessful.

It appears, therefore, that one molecule of structural protein can bind no more than one molecule of cytochrome c_1 in the form of a soluble complex. The composition of the $(SP)_2$-c_1 complex indicates either that c_1 can bind two molecules of structural protein, or that structural protein, when bound to c_1, can also bind an additional molecule of structural protein. The possible significance of this observation will be discussed later.

Interaction of Structural Protein with Cytochrome a.—The same type of binding experiments that were carried out on cytochrome c_1 were repeated for cytochrome *a*. However, the conditions obtaining for maximal binding were found to be somewhat different. The cytochrome *a* used was dispersed in the presence of 0.05% sodium dodecyl sulfate, *p*H 10. This solution contained the monomeric form predominantly, with a small proportion of the dimer (Criddle and Bock, 1959). Approximately 50% of the original cytochrome oxidase activity survived this treatment.

This cytochrome *a* preparation was added to an equimolar quantity of structural protein pre-

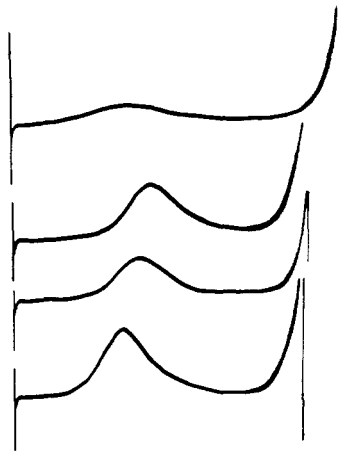

FIG. 9.—Sedimentation of complexes of cytochrome *a* with structural protein. The experiments were carried out in a buffer (*p*H 8) that was 10^{-2} M in Tris (acetate) and 5×10^{-2} M in NaCl. The photographs, from top to bottom, correspond to cytochrome *a* and three complexes in which the molar ratios of structural protein to cytochrome *a* were, respectively, 3:1, 2:1, and 1:1. The centrifugal speed was 59,780 rpm; pictures were taken at 174, 174, 168, and 168 minutes.

pared by the following procedure. Structural protein was solubilized in a buffer of 0.1% sodium dodecyl sulfate, 0.05 M NaCl, and 0.01 M phosphate, *p*H 10.5; the final protein concentration was brought to 10 mg per ml. This mixture was allowed to stand at room temperature for 24 hours and was then dialyzed against 0.01 M Tris acetate buffer, *p*H 8, containing NaCl at a concentration of 0.05 M.

Sedimentation and electrophoresis experiments once again indicated the formation of a complex between structural protein and cytochrome *a*. Figure 9 shows the sedimentation patterns of a 1:1 structural protein–cytochrome *a* complex. The complex moved with a single symmetrical boundary and the green color of the *a* was observed to move with the peak. Sedimenting boundaries characteristic of *a* and of structural protein were both absent from this solution.

Studies of the stoichiometry of the cytochrome *a*–structural protein interaction have also been made. The 1:1 addition of cytochrome *a* to structural protein in the experiment described above yields a complex of molecular weight 91,000. As the molecular weight of cytochrome *a* monomer has been found to be 70,000 to 72,000 (Ambe and Venkataraman, 1959), this leaves approximately 20,000 as the contribution made by structural protein, a value in good agreement with its determined molecular weight. Once again, complexes containing two and three molecules of structural protein for each molecule of cytochrome *a* were formed simply by changing the molar ratios of the two components in the mixtures, whereas mixtures containing two molecules of cytochrome *a* for each one of structural protein again gave heterogeneous

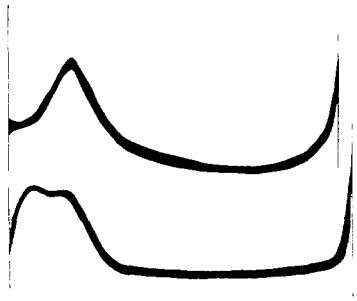

FIG. 10.—Sedimentation analysis of complexes formed by interaction of cytochrome *b* with structural protein. The single major peak visible in the top set of pictures shows the sedimentation pattern of a 1:1 molar complex of cytochrome *b* with structural protein. The lower set of pictures shows the pattern observed in an analogous experiment in which complex formation did not occur. The raised base lines resulted from high concentrations of detergent (sodium dodecyl sulfate). The photographs were taken after 32 minutes of effective sedimentation at 59,780 rpm.

products. Sedimentation patterns for these experiments are shown in Figure 9. The m.w. of the $(SP)_2$-*a* complex was found to be approximately 105,000.

Cytochrome b Complex Formation.—The formation of a structural protein–cytochrome *b* complex was much more difficult to demonstrate than was the formation of the other cytochrome complexes; it has been impossible to obtain this soluble complex in yields greater than about 50%. The remainder of the material forms large aggregates that can be spun out of solution at low speeds.

The conditions necessary for complex formation are as follows: (1) A suspension of cytochrome *b*, at a concentration of 5 mg per ml, is brought to *p*H 11 by addition of 1.0 N KOH. The precipitate (mostly sodium dodecyl sulfate) is removed by spinning in a clinical centrifuge. (2) A suspension of structural protein (5 mg per ml) in 0.05% sodium dodecyl sulfate is brought to *p*H 11 with KOH and the mixture is allowed to stand for at least 3 hours. (3) Equal volumes of the two solutions (1 and 2) are mixed and the resulting solution is allowed to stand 4 to 5 hours at room temperature. (4) The solution is brought to *p*H 8.0 very slowly by the addition of 1.0 M phosphate buffer, *p*H 7.4.

Even slight deviations from this procedure lead to failure of complex formation. The most critical step in the process is the slow neutralization. Typical sedimentation patterns of a structural protein–*b* complex and of a solution in which the two components failed to form a complex are shown in Figure 10. The fact that these solutions still contained a large quantity of sodium dodecyl sulfate (introduced by way of the *b* preparation) made the results somewhat uncertain. Comparison of the two examples shown in Figure 10 strengthens the belief that complex formation be-

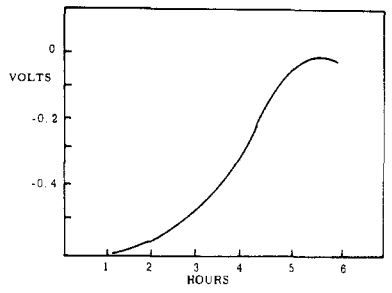

FIG. 11.—Oxidation-reduction potential of cytochrome *b* as a function of time of incubation after mixing with an equal weight of structural protein.

tween the two species was observed. No determination of the stoichiometry of binding in this system was attempted because of the poor yields.

Cytochrome *b* has long puzzled workers studying the mitochondrion in that its oxidation-reduction potential in the isolated state was approximately 0.34 volts lower than that calculated from measurements carried out on the cytochrome within the mitochondrion (Goldberger *et al.*, 1962). It appeared that at some step in the isolation and purification of this enzyme a change in its structure had taken place or that its active center was modified in a way that interdicted normal function.

To ascertain whether the formation of the structural protein complex would have any major effect on the enzymic properties of this protein, the oxidation-reduction potential of the structural protein–*b* complex was determined by Goldberger *et al.* (1962). It was found that in the form of the structural protein–*b* complex, cytochrome *b* had the same potential as obtains in the whole mitochondrion. This observation provides further evidence that complex formation occurs and that, when *b* is bound by structural protein, the resulting product resembles the form of cytochrome *b* native to the mitochondrion.

As was mentioned in the section on preparation of this complex, the reconstitution proceeds slowly over a period of 4 to 5 hours. The time course of this reaction was followed both by enzymic and centrifugal probes. The change in the oxidation-reduction potential with time is shown in Figure 11. The exact values of the potential are subject to some uncertainty, but the change of potential (which is of major concern here) is quite certain. The fact that sedimentation diagrams correlate well with these results indicates that, after an incubation period of one hour, essentially no complex formation is observed, whereas at the end of the 5-hour period, essentially all of the *b* is bound.

Interactions of Structural Protein with Lipid and Cytochrome c.—It was mentioned earlier that the only cytochrome readily isolated from mitochondria is cytochrome *c*. It appears that cytochrome *c* is not bound so firmly into the mitochondrion as are the other cytochromes; as a result, it can be readily extracted into dilute saline solutions (Green, 1956–1957).

In keeping with the ready extractability of cytochrome c, this protein was found to have little or no affinity for structural protein unless lipid was added to the system. This observation gives added support to our thesis that the protein that we have called structural protein has the capacity to interact either directly or indirectly (through lipid) with all of the major components of the electron transport particle.

Structural protein has a marked affinity for mitochondrial lipids, particularly in the form of preparations that have been comminuted to micellar dispersion. When this material is added to solubilized structural protein the lipid is bound. Several factors determine the amount of lipid bound per unit weight of protein: (1) *p*H, (2) time, (3) the chemical nature of the phospholipid, and (4) the state of dispersion of the phospholipid preparation. This aspect of the problem will be dealt with *in extenso* in another communication. It is significant, however, that there are sites on the structural protein that do show a definite affinity for this major class of components of the mitochondrion (*i.e.*, the lipids). By virtue of this property structural protein meets another criterion by which a structural role for the protein could be evaluated.

As the cytochrome *c*–phospholipid complex is lipid-soluble (Widmer and Crane, 1958), the structural protein–lipid complex will take up large quantities of this cytochrome. The phospholipids, which account for more than 90% of the total mitochondrial lipids (Green and Lester, 1959), interact with cytochrome *c* to form a complex that is insoluble in water but soluble in hydrocarbons.

Multiple Complex Formation.—When cytochrome c_1 was mixed with cytochrome *a* in roughly equimolar quantities at *p*H 10 (in dilute sodium hydroxide) there was no interaction between the cytochromes, as judged by the presence of a separate sedimenting boundary for each component in the mixture. When structural protein, solubilized as described previously, was added to this mixture, and the resulting solution was then dialyzed against Tris buffer, *p*H 7.6, for 12 hours, a small amount of an aggregate of high molecular weight formed; this could be spun out of the mixture in a low centrifugal field. The resulting supernatant fluid, when studied in the ultracentrifuge, showed a single sedimenting boundary. The molecular weight of this complex was determined to be approximately 160,000.

Spectral analysis of this complex at the respective absorption maxima of cytochrome c_1 (550 mμ) and of cytochrome *a* (605 mμ) indicated that the heme of cytochrome c_1 and that of cytochrome *a* were present in a 1:1 ratio. If minimal molecular weights are assumed (calculated from the heme contents of the two cytochrome preparations of highest purity and from the measured

molecular weight of structural protein), it may be concluded that the complex formed consisted of one molecule each of cytochrome c_1 and of cytochrome *a*, and two of structural protein.

Figure 12 summarizes the work on complex formation from mitochondrial components and indicates the central role of structural protein. It should be noted that satisfactory experimental data have not yet been obtained demonstrating the coupling of the SP-*b* complex with c_1 to form SP-*b*-c_1 as indicated by Figure 12. Each time this reaction was tried, a large insoluble aggregate was formed. We have not made an extensive survey to find conditions appropriate for this reaction. Other reactions indicated in Figure 12 have been documented with sufficient evidence to establish particle interaction satisfactorily.

As is illustrated in Figure 12, cytochrome *c* was bound by the particles only after the addition of mitochondrial lipids. This is in keeping with observations on whole mitochondria, but raises the question of the specificity of this interaction. All of the enzyme complexes described were formed by successive application of the procedures utilized for the formation of the individual structural protein–cytochrome complexes described in earlier sections.

Specificity of Binding by Mitochondrial Proteins.—For these binding experiments to have functional significance for the mitochondrion, it is necessary to determine whether the observed capacity of structural protein to form complexes is a general phenomenon or whether only specific components of the electron transfer chain of the mitochondrion are bound. Control experiments were done to test the affinity of structural protein for each of fourteen soluble, non-mitochondrial proteins. These included hemoglobin, bovine serum albumin, protamine, hexokinase, phosphatase, trypsin, subtilisin, β-casein, catalase, xanthine oxidase, glutamic acid decarboxylase, and lysine decarboxylase. In no case was any measurable amount of binding demonstrated. The observed binding of the cytochromes by structural protein can therefore be regarded as specific interactions. It was predicted that the protein, myoglobin, which functions in supplying oxygen to mitochondria and is known to be tightly bound to whole mitochondria, would also be bound by structural protein. The stoichiometric complex formed between structural protein and myoglobin served as the basis of one method for the determination of the molecular weight of structural protein, as described above.

Amino Acid Content of Structural Protein.—If mitochondrial structural protein is a single protein species, it would be expected that a single amino acid residue would appear at the amino terminus of the molecule and another at the carboxyl end of the chain. Should structural protein consist of a mixture of species (in contradistinction to an individual species with all molecules having the same sedimentation and electrophoretic constants) it would seem highly improbable that each of these species would also have the same terminal amino acids. Instead, it would be expected that a group of nonidentical proteins would give rise to several different amino-terminal and carboxyl-terminal amino acids. Thus, the identification of the terminal amino acids in a preparation of structural protein may be used as another test of the homogeneity of the preparation.

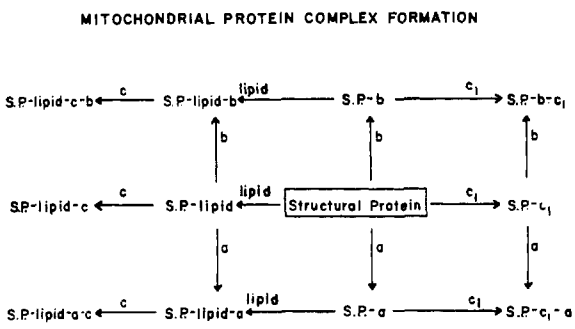

FIG. 12.—A schematic summary of the interaction between structural protein and other mitochondrial components.

Carboxyl-Terminal Amino Acids.—A tracing of the results obtained from a typical chromatographic examination of the products of 1-fluoro-2,4-dinitrobenzene labeling after treatment of the structural protein with carboxypeptidase is shown in Figure 13. The sample was a pooled ethereal extract of the acidified reaction mixture. Any carboxyl-terminal amino acid would be demonstrated under the conditions of the extraction procedure. The structural protein was treated with carboxypeptidase for 2, 4, and 6 hours respectively; the chromatographic patterns of the samples corresponding to these different times of incubation are shown in Figure 13. Both the 2 and 4 hour samples show migrating spots corresponding to dinitrophenol, dinitroaniline, and DNP-leucine. The 6-hour sample indicates the presence of each of these spots plus one additional spot, probably that corresponding to DNP-tyrosine.

These results indicate that carboxypeptidase first cleaves a leucine residue from the carboxyl-terminal end of structural protein. It appears that structural protein has predominantly one carboxyl-terminal amino acid. This evidence reinforces that of the physical studies and suggests that structural protein is predominantly one species.

Amino-Terminal Amino Acid.—In spite of numerous attempts to determine the amino-terminal amino acid of structural protein under a variety of conditions, and with several different techniques for the preparation of the sample, it was not possible to obtain positive evidence of a specific amino-terminal amino acid. In repeated experiments in which the 1-fluoro-2,4-dinitrobenzene procedure (Fraenkel-Conrat *et al.*, 1955) was

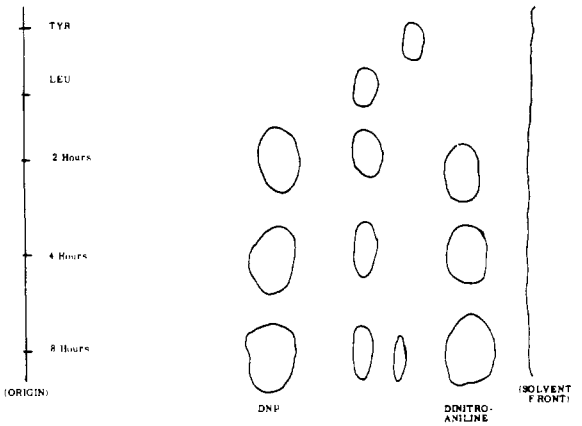

FIG. 13.—Comparison of the chromatographic behavior of the 1-fluoro-2,4-dinitrobenzene derivatives of reference amino acids with that of the analogous derivatives formed from structural protein. The figure was made up of tracings of the original chromatograms. The conditions for the chromatography are described in the text.

used the presence of terminal DNP-threonine was suggested, but the amounts observed were at the trace level. The Edman method (Erikson and Sjoquist, 1960) did not reveal the presence of any amino-terminal amino acids.

The usual conditions of hydrolysis utilized in Sanger's 1-fluoro-2,4-dinitrobenzene method (Fraenkel-Conrat *et al.*) (12 N HCl at 105° for 16 hours) were inadequate to hydrolyze structural protein completely to amino acids; a large amount of insoluble residue remained. More complete hydrolyses could be achieved by prolonging the time, but it could be anticipated that this would have an adverse effect on any labeled DNP derivative. Alterations in the procedure for preparation of the sample were ineffective in increasing either the amount of labeled amino acid or the degree of hydrolysis.

The failure to identify any amino-terminal amino acids by these procedures may indicate that this end of the protein is blocked by a chemical group, or by some steric configuration of the protein molecule or aggregate, which denies the labeling reagent ready access to the end of the chain. Also, the failure to find an amino-terminal amino acid by the Sanger method might be explained in terms of the complete or partial breakdown of some of the DNP–amino acid derivatives under the usual conditions of hydrolysis (*e.g.* the DNP derivatives of cystine, glycine, phenylalanine, tryosine, and lysine). Because of the extended times required to hydrolyze structural protein, any of these derivatives might be destroyed to such an extent that its detection would not be feasible. In the thiohydantoin method, however, the complete destruction of these particular amino acids is avoided. Thus, the inability to find any amino-terminal amino acid cannot be accounted for in terms of the breakdown of the more unstable derivatives.

Although the acid hydrolysis of structural protein was incomplete, sufficient reaction took place to produce a whole family of labeled amino acids (that would have appeared on the chromatograms) in the event that many different proteins were present, each with its characteristic amino-terminal amino acid. The absence of these products is another indication that structural protein is a single protein species rather than a class of proteins.

TABLE IV
AMINO ACID COMPOSITION OF STRUCTURAL PROTEIN[a]

Amino Acid	μmoles Amino Acid per 100 mg Protein[b]	Moles Amino Acid per Mole Structural Protein[c]
Aspartic acid	58	12.8
Threonine	37	8.1
Serine	37	8.1
Proline	20	4.4
Glutamic acid	55	12.1
Glycine	69	15.2
Alanine	74	16.3
Valine	48	10.5
Methionine	18	4.0
Isoleucine	44	9.7
Leucine	68	15.0
Tyrosine	28	6.2
Phenylalanine	39	8.6
Lysine	56	12.3
Histidine	11	2.4
Arginine	36	7.9
Amide-NH_2	90	9.7
Tryptophan[d]	29	6.5
Cystine (as cysteic acid)	68	10.3

[a] Results are based on a single analysis of a sample hydrolyzed 22 hours in 20% HCl under reflux. The automatic amino acid analyzer of the Beckman Spinco Company was used for the analysis, and the procedure of Spackman *et al.* (1958) was followed. [b] It is assumed in this calculation that 16 mg of N is equivalent to 100 mg of protein. Nitrogen was determined by the Dumas procedure. [c] The molecular weight of structural protein was assumed to be 22,000 g per mole in this calculation. [d] Tryptophan was determined spectrophotometrically by the method of Holiday and Ogston (1938).

Amino Acid Analysis.—An amino acid analysis of a structural protein preparation was carried out by Dr. T. Gerritsen of the Department of Pediatrics of the University of Wisconsin. The results of this analysis, summarized in Table IV, indicate that there is a relatively high content (about 41%) of amino acids having nonpolar side-chains (*e.g.* isoleucine, leucine, valine, phenylalanine, tyrosine, and methionine). The combined aspartate plus glutamate content is only 16%, a value that is slightly lower than average, based on comparison with corresponding values for representative proteins, compiled by Waugh (1954).

Discussion

From the studies of the individual mitochondrial proteins, a general picture emerges of how proteins may be organized into complex systems. Each of the component fixed cytochromes of electron transport particles has the capacity to interact (a) with other molecules of the same species and (b) even more strongly with the one component that seems to have the capacity to form complexes with all of the fixed proteins of the electron transport particle, namely, the structural protein. Studies of stoichiometry indicate that there is probably but one site on the structural protein molecule for binding cytochromes and that each molecule of structural protein can react with any one of the cytochrome molecules. This follows from the fact that a 1:1 molar addition of structural protein to the cytochrome monomers gives rise to a 1:1 complex with any of the cytochromes except cytochrome *c*, whereas mixtures of bound and unbound cytochromes are formed when the molar ratio of cytochrome to structural protein exceeds one. Such studies also indicate either that the cytochromes must be able to bind more than one molecule of structural protein or that structural protein, when bound to cytochromes, must be able to bind additional molecules of structural protein. The extreme insolubility of structural protein indicates that one molecule of structural protein binds at least two other molecules of its own kind. It would seem that if structural protein is functioning in the role assigned and is, indeed, the material holding the complex together in an ordered structure, in each molecule at least two sites for binding other structural protein molecules would be necessary in addition to one for binding a cytochrome molecule. This could result, then, in a protein complex stabilized by a "backbone" of structural protein.

The types and relative importance of the chemical bonds stabilizing the mitochondrial structure may be inferred from the reagents and solvents utilized to rupture these inter-protein bonds. It was noted earlier that the reagents classically used to isolate mitochondrial proteins were directed toward rupturing lipid-protein bonds, considered to be the stabilizing factors in this structured system. While it is still evident that lipid plays a major functional role in the mitochondrion, our studies demonstrating strong protein-protein interactions, based upon hydrophobic bonding, suggest that lipid-protein binding is quantitatively a less important determinant of mitochondrial structure. The "lipid reagents" used in isolation procedures could equally well exert their major influence on the structure by competing for the hydrophobic sites of interaction between the protein constituents.

Hydrophobic bonding between the proteins of the electron transfer chain arises as the result of the affinity of molecules for those of like nature. The high density of amino acids with non-polar side-chains, present in all proteins of the electron transfer chain, makes it reasonable to postulate extensive non-polar regions within or near the surface of the protein molecule. If the geometry of the molecules is such that hydrophobic regions of one molecule are complementary to those on the surface of a second molecule, an intermolecular interaction could occur that would lead to increased stability of the system. This type of binding between non-polar groups is stabilized mainly by the increased entropy of water molecules (Kauzmann, 1959). Non-polar groups, dissolved in water, decrease the entropy of the water associated with these groups. When these non-polar groups coalesce to form a non-polar region, the surface area of contact of this region with water is decreased and the entropy of the water is increased. This increase in entropy is thought to result from the extra freedom of movement of the water molecules acquired when they are no longer in the vicinity of the hydrophobic area (Kauzmann, 1959).

Other evidence supporting the conclusion that the major stabilizing interaction of electron transport particles are largely of a hydrophobic nature comes from the effects of solvents on these interactions. As was noted, anionic detergents are functionally the most effective agents for breaking up the complexes. These reagents have the capability of entering the non-polar regions that bind a complex together, of introducing a large number of like charges, and of bringing about a resultant charge repulsion and breakdown of the complex into its component parts. The twin facts (a) that anionic detergents are the most effective reagents for solubilizing mitochondrial components and (b) that these detergents have their main effect on hydrophobic interactions, suggest that mitochondrial integrity is stabilized largely by hydrophobic interactions. Another property expected of this type of bond is that an increase in the ionic strength of the solution should strengthen the bonds. This has already been demonstrated to be the case by the precipitation of structural protein and its complexes with very small increments in ionic strength. On the other hand, reagents such as non-ionic detergents, or some of the lower alcohols, should tend to weaken hydrophobic bonds when the dielectric constant of the solution is reduced and, indeed, these conditions are employed successfully in the procedures used to release active particles from the mitochondrion.

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Preparation and Biological Activity of Dihydroaminopterin*

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Dihydroaminopterin was synthesized by the reduction of purified aminopterin with sodium dithonite and isolated by fractional precipitation with acid. This compound is 34 to 92 times more potent than aminopterin as a growth inhibitor of *Streptococcus faecalis* and *Pediococcus cerevisiae* respectively. It has, however, the same activity as aminopterin against cultured sarcoma 180 cells and as an inhibitor of folic acid reductase from chicken liver or *Streptococcus faecalis*. Paper chromatographic analysis of a preparation of tetrahydroaminopterin revealed that the inhibitory activity of this compound is mainly due to the presence of some substance in the preparation of the former compound other than tetrahydroaminopterin itself. It is suggested that this substance is dihydroaminopterin.

The role of different tetrahydrofolate co-factors in several biosynthetic reactions has directed increasing attention to the synthesis of reduced analogues of folic acid (pteroylglutamic acid) as potential metabolic antagonists. Both synthetic and biological work, however, are complicated by the instability of these compounds. Weygand *et al.* (1951) reported that catalytic hydrogenation of aminopterin resulted in partial loss of inhibitory activity for *Pediococcus cerevisiae* and *Streptococcus faecalis*. Only the crude reaction mixtures were tested in these experiments and the product of the reaction was not isolated. Since that time more has been learned about the properties of tetrahydro and dihydrofolic acid and techniques have been developed for the isolation and stabilization

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