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Studies on the Electron Transfer System. LIV. Isolation of the Unit of Electron Transfer*

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The procedure described for the fractionation of mitochondria leads to the isolation of a particle, which uniquely contains all of the fixed components of electron transfer; the enzymic activity increases concomitantly with the purification of the particle. The rates of oxidation of DPNH and of succinate have been increased by a factor of 2.5 and the concentration of cytochrome *a* by a factor of 2.2. The specific concentrations of the other components have been increased to about the same degree. The isolated particle (designated the elementary particle) appears to be a physical and functional aggregate of the four complexes known collectively to constitute the electron transfer system; the theoretical molecular weight (on a protein basis) is 1.4×10^6; that of the particle described was calculated to be 2.1×10^6. Further purification (by removal of structural protein, etc.) led to an estimated minimal molecular weight of 1.4×10^6 but also decreased the enzymic activity.

It has been appreciated for some time that the essential features of mitochondrial function are retained by submitochondrial particulate preparations—features such as electron transfer from substrate to oxygen and oxidative phosphorylation. The digitonin particle of Cooper and Lehninger (1956), the electron transfer particle described by Crane, Glenn, and Green (1956), and the particles of the Keilin-Hartree preparation (1947) are well-known examples of submitochondrial particles that retain many of the attributes of the intact mitochondrion. The problem has been not whether a functional unit smaller than the mitochondrion exists but rather what are the dimensions of this subunit and how is the mitochondrion built from such subunits.

When mitochondria are subjected to sonic irradiation they undergo a limited disruption as organized units and are fragmented into particles of much smaller dimensions. During the fragmentation of beef heart mitochondria some 20% of the total protein is released into the medium in soluble form whereas the remaining 80% is found associated with the water-insoluble particulate fragments (Linnane and Ziegler, 1958). The complete electron transfer chain and the apparatus for coupled phosphorylation are localized in the particles; in the soluble fraction are found auxiliary enzymes and enzyme complexes that are concerned with the citric acid cycle oxidation, fatty acid oxidation, and, in general, with the release of electrons from substrates for entry into the electron transfer chain (although significant amounts of the enzyme complexes remain with the particulate fraction). The fragmentation of mitochondria by sonic irradiation into a particulate structured fraction and a soluble fraction makes it clear that there cannot be a single mitochondrial subunit which is all-embracing, but rather that several fractions or subunits exist, each designed for a specific function. The particular subunit carrying out the special function with which we are concerned in the present communication is the one that contains the complete electron transfer chain (or chains)—the apparatus for the transfer of electrons from succinate and DPNH to molecular oxygen.

The electron transfer chain contains eleven known oxidation-reduction components, arranged in an orderly sequence based upon drops in potential; all these components participate in the transfer of electrons along the chain. If we assume that these components are present in repeating electron transfer chains and that all chains are identical single-branched chains each containing only one molecule (or a multiple thereof) of each component, it is possible to calculate the molecular or particle weight of a complete electron transfer chain. The flavoprotein associated with succinic–coenzyme *Q* reductase activity is an essential component of the electron transfer chain; there are many lines of evidence, which will be pointed out later, to support the thesis that there is only one molecule of this type of flavoprotein per chain. In the electron transfer parti-

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cle with phosphorylating properties (ETP_B)[1] the concentration of flavin, which is not extractable with acid (a relatively precise measure of the flavin associated with the succinic flavoprotein) is 0.28 mμmoles per mg of protein. This value leads to an estimate of about 3.6×10^6 as the particle weight (in terms of protein[2]) of this particular unit, which contains the complete electron transfer chain. There have been three recent developments, however, that point to a still smaller particle as the unit of electron transfer. First, the mitochondrion was shown to contain a colorless protein having a structural role; this protein accounted for at least 50% of the total protein of the mitochondrion (Green *et al.*, 1961a; Criddle *et al.*, 1961; Green *et al.*, 1961b). Second, Fernandez-Moran (1962a,b; 1963a,b) discovered by electron-microscopic examination of mitochondria that the membrane structures of the external envelope and of the internal cristae appear to be built up of paired arrays of particles, approximately 100 A in diameter, separated by a middle layer some 60 A in width. Since such paired arrays of particles could also be seen in preparations of ETP_H the possibility of a subunit with a particle weight considerably smaller than 3.6×10^6 had to be entertained. Third, the electron transfer chain was resolved into four complexes (Ziegler and Doeg, 1959; Hatefi *et al.*, 1960, 1961a; Griffiths and Wharton, 1961), each of which was isolated in highly purified particulate form. Under appropriate conditions, the four complexes are thought to combine in 1:1:1:1 stoichiometry to form a physical unit with a functionally complete electron transfer chain. This belief has been corroborated by the finding (Hatefi *et al.*, 1961b; Fowler and Hatefi, 1961; Hatefi *et al.*, 1962b; Fowler and Richardson, 1963) that three of the four complexes appear to combine in a 1:1:1 stoichiometry. The particle weight of a reconstituted unit (containing the four complexes in 1:1:1:1 stoichiometry) was calculated to be about 1.4×10^6 (on a protein basis), since the particle weight of each of the complexes had been established from data describing its composition and ultracentrifugation pattern. These three developments collectively suggested that in this isolated subunit (ETP_H) the integrated complex constituting the electron transfer chain was bonded to the structural protein—phospholipid network; if it were possible to separate the subunit from structural protein, the particle weight of such a unit would be about 1.4×10^6, calculated on a protein basis. Furthermore, the dimensions of the particles in preparations of the subunit from which structural protein had been stripped should approximate those of the particles seen by Fernandez-Moran in the membrane systems of both the mitochondrion and the ETP_H.

In the present communication, evidence will be presented which indicates that particles of the anticipated molecular size can, in fact, be isolated from mitochondria, and that these particles contain the complete electron transfer chain. This subunit of the mitochondrion which contains the complete electron transfer chain has been designated the elementary particle (EP) (Fernandez-Moran *et al.*, 1963a,b; Fernandez-Moran, 1962a,b). Thus, the elementary particle may be defined as an entity, pre-existing in mitochondria, which occurs in paired arrays within membrane systems and functions in the transfer of electrons from both succinate and DPNH to oxygen.

[1] The abbreviation ETP_H is used for the electron transfer particle prepared from heavy beef heart mitochondria.

[2] The molecular weights that are determined on a protein basis are minimal molecular weights based on the assumption that protein contributes the entire molecular weight and that a specified number of prosthetic groups are associated with each protein.

Experimental Procedure

Materials and Methods

Flavin Determinations.—Flavins were determined by a modification of the methods described by Green, Mii, and Kohout (1955) and by King, Howard, Wilson, and Li (1962). One sample was used to determine both acid-extractable and acid-nonextractable flavin and another sample was used to determine the total flavin. The total flavin content, found experimentally, in the two samples should be the same under proper assay conditions.

In our adaptation of the two methods, 40 mg of protein was utilized in a volume of 2.0 ml when both acid-extractable and acid-nonextractable flavins were estimated in the same sample. When only the total flavin was determined, 20 mg of protein was used in the same volume.

A solution of the sample containing 40 mg of protein was brought to a concentration of 7.5% with respect to trichloroacetic acid by slow addition of the acid while the solution was shaken on a Vortex mixer. After standing for 15 minutes, the suspension was centrifuged for 10 minutes at 20,000 rpm (No. 40 rotor, Spinco Model L ultracentrifuge). The supernatant fluid containing the acid-extractable flavin was decanted into a 10-ml test tube and was extracted seven times with anhydrous diethyl ether. The trichloroacetic acid was thus extracted to a level which required only a small amount of normal KOH to neutralize the aqueous solution. A measured volume (usually 1.0 ml) of the aqueous solution was subsequently made 0.1 M with respect to Tris-HCl, *p*H 7.5, prior to spectrophotometric analysis.

The residue from the acid extraction was first washed with 10 ml of absolute ethanol, followed by a second wash with 10 ml of distilled water. The washed residue (protein) was suspended in 0.1 M phosphate buffer, *p*H 7.4, and homogenized before the protein concentration was determined. The final volume was adjusted to 2.0 ml before 20 mg of (1–300) trypsin was added to release the acid-nonextractable (peptide-bound) flavin. This suspension was incubated at 38° for 30 minutes; it was then cooled and treated with acid as outlined above.

The estimation of the total flavin was accomplished by treating the sample exactly as described above after the washed residue was suspended in phosphate buffer.

The extracted flavins were estimated with a Beckman DU spectrophotometer. Absorbance readings (A) were taken at 450 mμ, 530 mμ, and 600 mμ in the oxidized, reduced, and reoxidized states. Only the difference between reoxidized and reduced states was used to calculate the concentration of flavin. The reducing agent was sodium dithionite. The following formula was used to obtain the corrected change in absorbance necessary for calculating the flavin concentration. The

$$\text{Corrected } \Delta A = [(A_{430m\mu} \text{ reoxidized minus } A_{450m\mu} \text{ reduced}) \text{ minus } (A_{530m\mu} \text{ reoxidized minus } A_{530m\mu} \text{ reduced})]$$

reading at 600 mμ is an index of the change in turbidity upon reduction. Rayleigh's law (turbidity caused by small particles increases as the reciprocal of the fourth power of the wavelength) may be applied if the change in absorbance is not large. The absorbance index for flavin was taken to be 10.3 cm^2 per mmole.

Protein Determination.—For routine analyses a biuret method for determining protein concentration

was used (Gornall *et al.*, 1949); the standard used was crystalline bovine serum albumin. This method was shown to be in good agreement with the dry weight method introduced by Slater (1949). The biuret method estimated about 5% more protein than did the dry weight method.

Estimation of the Cytochromes.—Cytochrome *a* was determined by the spectrophotometric method outlined by Yonetani (1959); an extinction coefficient of 16.5 cm^2/mmole was used for cytochrome *a*. The change in absorbance (spectrum of the reduced cytochrome versus water) from 605 mμ to 630 mμ was recorded with a Beckman DK-2 recording spectrophotometer. When mitochondria were analyzed, 5 mg of protein per ml was found to be an appropriate concentration provided the solution was first buffered with Tris-HCl (0.1 M, *p*H 8.0) and clarified with detergents (0.5 mg of cholate and 1.0 mg of deoxycholate per mg of protein). When the cytochrome *a* concentration of the elementary particle preparation was determined, the protein concentration of the specimen solution was 3 mg/ml. The elementary particle preparation was also buffered with Tris-HCl but only deoxycholate was required to clarify the suspension. All reductions were made with sodium dithionite.

For the estimation of cytochrome *b* the spectrophotometric method of Zaugg and Rieske (1962) was modified to include the other cytochromes. This method depends on the reduction of cytochrome *b* with dithionite after cytochromes *a*, *c*, and c_1 have been reduced with ascorbate in both the sample and reference cells of the Beckman DK-2 spectrophotometer. Cytochrome *b* is only slowly reduced with ascorbate at the temperature used (0°). A difference spectrum is recorded and an extinction coefficient $\Delta\epsilon$ (563 mμ minus 577 mμ reduced) equal to 28.5 cm^2/mmole was found appropriate for calculating the concentration of cytochrome *b* in the preparations of mitochondria and elementary particles after clarification with detergents.

The cytochrome c_1 concentration was estimated by oxidizing the system completely at 0° with a small amount of ferricyanide before reducing it with solid potassium ascorbate. The absorbancy index applied to the difference spectrum used was 17.1 cm^2/mmole (reduced minus oxidized at 554 mμ) (Green *et al.*, 1959). Cytochrome *a* seems not to interfere but cytochrome *c* cannot be distinguished from cytochrome c_1 by this method. Thus, both cytochromes were estimated together and then the cytochrome *c* concentration was subtracted from the combined value to yield the concentration of cytochrome c_1. Cytochrome c_1 cannot be estimated after cytochrome *c* has been extracted because this procedure leads to an insoluble residue which contains the remainder of the cytochromes in addition to other proteins.

Cytochrome *c* (or a cytochrome *c*-like material) was extracted from both mitochondria and elementary particles by the following procedure. Mitochondria in a solution buffered with Tris-HCl, *p*H 8.0, were treated with bile salts (0.60 mg of cholate and 0.70 mg of deoxycholate per mg of protein) before ammonium sulfate was added to 10% saturation at 0°. The final protein concentration was 60 mg/ml. Two ml of absolute ethanol was added to 2 ml of the mitochondrial suspension, mixing being accomplished on a Vortex test tube mixer. Immediately after mixing, the preparation was placed in a 38° water bath for 5 minutes. Next, it was incubated at −20° for 10 minutes before it was centrifuged for 5 minutes in an International Clinical centrifuge (rotor No. 809) at maximal speed. A known volume (usually 1.8 ml) of the supernatant fluid was made 0.1 M with respect to Tris-HCl, *p*H 7.5 (usually 0.2 ml of M Tris). A spectrum was run as soon as possible thereafter in a Beckman DK-2 recording spectrophotometer. The concentration of cytochrome *c* was determined by difference (reduced minus oxidized) at the peak wavelength (550 mμ); an extinction coefficient of 18.5 cm^2/mmole was used (Margoliash, 1954). This extraction and recording was performed rapidly because cytochrome *c* appears to be destroyed or denatured by prolonged contact with the extraction medium. The elementary particle preparation was treated in the manner described for the extraction of cytochrome *c* from mitochondria. However, the final concentration of detergents was 0.6 and 0.7 mg per mg of protein for cholate and deoxycholate, respectively, because the preparations of the elementary particle contained a residual amount of each of these two bile acids.

Determination of Iron.—Total iron was determined as the ferrous-bathophenanthroline complex (Smith *et al.*, 1952) after the sample was wet-ashed with concentrated H_2SO_4 and H_2O_2. Heme iron was estimated by summation of the iron content of the heme of the various cytochromes. Nonheme iron was obtained by difference (total iron minus heme iron).

Copper Determination.—Copper was estimated as the cuprous-neocuproine complex by the method of Smith and McGurdy (1952).

Extraction and Determination of Phospholipids.—Phospholipids were extracted with chloroform-methanol at room temperature in an atmosphere of nitrogen to minimize oxidation (Fleischer *et al.*, 1961; Rouser *et al.*, 1961; Folch *et al.*, 1957). Samples of the extract were digested with perchloric acid preliminary to the determination of phosphorus by the method recommended by Chen, Toribara, and Warner (1956). The phosphorus content was used as an index of the amount of phospholipid.

Estimation of Bile Salts.—The concentrations of both cholate and deoxycholate were determined by preparing the elementary particle in presence of the C^{14}-labeled compounds (Fleischer *et al.*, personal communication).

Extraction and Estimation of Coenzyme Q.—The concentration of coenzyme Q was estimated by the method described by Hatefi (1963). Coenzyme Q is extracted from the preparation with cyclohexane; the residue after removal of the solvent is taken up in ethanol. A difference in absorbancy (oxidized minus reduced) at 275mμ is used to estimate the concentration of coenzyme Q. Reduction is accomplished with KBH_4.

Concentration of Ammonium Sulfate.—Ammonium sulfate was determined chemically in the elementary particle only. After digestion with perchloric acid, the sulfate ion was determined by precipitation as the barium salt (Boyars, 1948).

Electron Transfer Activities.—DPNH oxidase activity was measured polarographically in the oxygen electrode (Gilson Medical Electronics Oxygraph). The oxygen content of 2.0 ml of a suspension containing all factors necessary for maximal activity was calculated to be 0.83 μatom of oxygen at 37 ± 1°. The order of addition of materials to the jacketed reaction vessel was: (a) 1.8 ml of 0.02 M phosphate buffer containing 1×10^{-4} M EDTA, *p*H 7.4; (b) 0.1 ml of enzyme at a protein concentration of 0.5 mg/ml; (c) 0.08 ml of 5% DPNH (Sigma); and (d) 0.02 ml of 1% cytochrome *c* (Sigma, type III).

Succinic oxidase activity was measured in a similar manner except for the addition of 0.01 ml of a 5×10^{-3} M solution of coenzyme Q in absolute ethanol and the use of 0.08 ml of 0.1 M succinate (instead of DPNH) as substrate. Activities are expressed as μatoms of oxygen consumed per mg of protein per minute.

TABLE I

PER CENT RECOVERY OF THE HEME COMPONENTS OF ELECTRON TRANSFER IN THE VARIOUS FRACTIONS OF THE PREPARATION OF THE ELEMENTARY PARTICLE

Data listed are mean values (average of five independent observations) with the standard error of each mean.

Fraction	Total Protein (%)	Each Cytochrome (%)		
		a	*b*	$c + c_1$
Mitochondria (sonicated)	100	100	100	100
KCl-washed mitochondria	90 ± 2	100	100	40 ± 2
First (mainly structural protein) residue	48 ± 2	10 ± 2	12 ± 3	<5
First (greenish-red) supernatant fluid	42 + 2	91 ± 1	89 ± 2	40 ± 2
Floating (residue) fraction	38 ± 2	91 ± 1	89 ± 2	30 ± 2
Infranatant (soluble protein) solution	5 ± 1	0	<2	10 ± 2
Elementary particle fraction	32 ± 3	77 ± 1	76 ± 3	26 ± 1
Final supernatant fluid	<10	14 ± 2	13 ± 3	<5

Isolation of the Elementary Particle

Preparation of Mitochondria.—Beef heart mitochondria (Crane *et al.*, 1956), suspended in 0.25 M sucrose at an approximate protein concentration of 65 mg/ml, were diluted to a protein concentration of 45 mg/ml with 0.9% KCl. This suspension was frozen and was subsequently thawed shortly before it was centrifuged for 20 minutes at 20,000 rpm (No. 30 rotor, Spinco Model L Ultracentrifuge). The residue was suspended in 10 volumes of 0.9% KCl; this mixture was homogenized before it was centrifuged at 20,000 rpm (Spinco, No. 30 rotor) for 20 minutes. The residue was collected in 0.66 M sucrose (final sucrose concentration about 0.30 M) and the protein concentration was adjusted with the sucrose solution to 30 mg/ml.

Removal of Structural and Soluble Proteins.—The KCl-washed mitochondria were treated as follows: (a) The mitochondria were adjusted to *p*H 8.0 with Tris-HCl buffer (0.02 M); (b) 0.3 mg of potassium cholate (as a 20% solution) per mg of protein was added slowly; (c) 0.3 mg of potassium deoxycholate (as a 10% solution) per mg of protein was added slowly; (d) Sufficient saturated ammonium sulfate (0–5°) was added slowly to bring the suspension to 33% saturation; (e) this turbid suspension was centrifuged at 30,000 rpm (Spinco, No. 30 rotor) for 15 minutes. The grayish-white residue (mainly structural protein) was discarded and the greenish-red supernatant fluid was brought to 50% saturation by the slow addition of saturated ammonium sulfate (a greenish-red sticky precipitate commenced to form at about 40% saturation). When this suspension was centrifuged at 20,000 rpm (Spinco, No. 30 rotor) for 5 minutes a floating greenish-red residue was collected (after the almost colorless infranatant fluid was decanted). This greenish-red residue, designated the *floating fraction*, has a composition (in terms of the components of the electron transfer chain) almost identical with that of the elementary particle; this fraction was dissolved in 0.25 M sucrose.

Dilution and Reconcentration of the Protein in the Floating Fraction.—The solution of the floating fraction was diluted with 0.25 M sucrose to a concentration of 0.5 mg of protein per ml and was allowed to stand for 30 minutes. Almost immediately after dilution the suspension became turbid; the 30-minute interval served to increase the recovery of protein. The turbid suspension was centrifuged at 30,000 rpm (Spinco, No. 30 rotor) for 4 hours. The relatively clear colorless supernatant fluid was decanted and the greenish-red residue was taken up in a buffered (0.05 M Tris-HCl, *p*H 7.5) solution of 0.25 M sucrose. This suspended residue was dispersed by sonication; it will be designated the suspension of the elementary particle.

All manipulations and solutions used (except cholate and deoxycholate) were at 0–5° unless otherwise stated. All centrifugation times include acceleration but not deceleration time and all centrifugations were carried out in the Spinco Model L ultracentrifuge unless otherwise stated.

RESULTS

Purification.—The stepwise purification of the components concerned with electron transfer has been summarized in Table I. It is evident that only small amounts of the cytochromes were lost during the isolation procedure. Some variation in the percentage of the cytochromes recovered may be attributed to analytic error introduced by turbidity, especially in those fractions that contain a high amount of salt and insoluble protein. The variations observed among fractions that can be brought into solution by the addition of reagents was much less, e.g., the floating fraction or the elementary particle preparation. The data in this table also indicate that a large proportion of the cytochrome $c + c_1$ fraction was removed by treatment with KCl. It will be shown later that this loss was due entirely to extraction of cytochrome *c* although a significant amount of this cytochrome remained in the mitochondrial residue. The values listed are averaged percentages (with their standard errors) of five typical preparations.

Composition of the Elementary Particle.—In Table II are summarized the concentrations of the components

TABLE II

COMPONENTS OF THE PREPARATION OF THE ELEMENTARY PARTICLE

Component	Concentration of Component/mg Protein
Total flavin	0.91 mμmoles
Acid-extractable	0.48 mμmoles
Acid-nonextractable	0.43 mμmoles
Total cytochromes	5.05 mμmoles
Cytochrome *a*	2.90 mμmoles
Cytochrome *b*	1.51 mμmoles
Cytochrome c_1	0.48 mμmole
Cytochrome *c*	0.16 mμmole
Total iron	12.20 mμmoles
Heme iron	5.05 mμmoles
Nonheme iron	7.15 mμmoles
Copper	3.32 mμmoles
Coenzyme Q	5.32 mμmoles
Phospholipid	0.83 mg
Bile salts	0.174 mg
Cholate	0.012 mg
Deoxycholate	0.162 mg
Sulfate ion	0.01 mg

concerned with electron transfer in addition to those of the reagents used in the fractionation. The fact that the acid-extractable flavin is 8.6% greater than the acid-nonextractable flavin indicates either that there was a carry-over of a significant amount of flavin associated with residual primary dehydrogenases or that the ratio of the flavin associated with DPNH oxidation to that associated with succinate oxidation is greater than 1:1. The copper-to-cytochrome *a* ratio approaches 1; the ratio of acid-nonextractable flavin to nonheme iron is approximately 17, whereas the ratio of acid-nonextractable flavin to coenzyme Q is about 12. The percentage of phospholipid (45%) in this particle is much greater than it is in mitochondria (30%).

Dilution of the floating fraction to 0.5 mg of protein per ml does not alter the specific concentrations of the electron transfer components or those of the phospholipids but it does release the sulfate and most of the bile salts (especially cholate) into solution. The concentration of bile salts in the floating fraction is 1.23 mg per mg of protein; in the elementary particle preparation it is only 0.17 mg per mg of protein. However, the amount of cholate removed is much greater than that of deoxycholate. About 75% of the deoxycholate is removed by a 120-fold dilution whereas approximately 98% of the cholate is removed. An independent study has been undertaken to determine the importance of this phenomenon in fractionation procedures (Fleischer *et al.*, 1963).

In the respiratory chain there are fixed components such as f_S (flavoprotein associated with succinate oxidation and estimated in terms of the acid-nonextractable flavin), f_D (flavoprotein associated with DPNH oxidation and estimated in terms of the acid-extractable flavin), and the three cytochromes (*a*, *b*, and c_1). These are all relatively tightly bound to the particle in contrast to the so-called mobile components, e.g., cytochrome *c* and coenzyme Q, both of which are extractable with relative ease. During the isolation of the elementary particle approximately 85% of the cytochrome *c* is extracted whereas some 65% of the coenzyme Q is retained within the particle.

Comparison of Concentration of Components Among Mitochondria, ETP_H, and the Elementary Particle.—The specific concentrations of the cytochromes (*a*, *b*, and c_1) and of the two flavoproteins (f_S and f_D) are compared in the starting mitochondrial preparation, in the ETP_H, and in the elementary particle (Table III). The analytical procedures used were those described in an earlier section. The mitochondrion and the ETP_H contain forms of acid-extractable flavin other than that associated with the flavoprotein implicated in DPNH oxidation; thus an overestimation of f_D relative to f_S in both these particles is expected. Cytochrome *c* occurs in the three particles although a relatively small amount is present in the isolated elementary particle. The other two particles (the sonicated mitochondrion and the ETP_H) require additional cytochrome *c* for maximal oxidation rates. The ETP_H has only 65% of the expected amount of cytochrome *c* based on the premise that the mitochondrion has a full complement. Doubtless the amount of cytochrome *c* shown in the table is a minimal value because the cytochrome is slowly destroyed by the procedure used in extraction.

So far as the three cytochromes and two flavoproteins provide a measure of the composition of the electron transfer chain, it appears that in the transition from the mitochondrion to the elementary particle the relative composition of the components of the electron transfer chain remained unchanged. The concentration of each of the five components per mg of protein increased with purification but the relative proportions were unaltered by the isolation procedures described. However, when the procedure for isolation of the elementary particle was made more drastic than that used routinely, certain components were concentrated to a greater extent than was the case when the elementary particle was prepared as described above. Concomitantly, other components were reduced in concentration or eliminated entirely. In other words, the chain was no longer intact, parts being concentrated and parts being lost. In the selection of a procedure for isolation of the elementary particle both the preservation of the stoichiometry among the functional components found in the mitochondrion (or in the EPT_H) and the preservation of activity were the yardsticks of suitability.

The greatest difficulty was experienced in retaining DPNH-oxidase activity. When the particle was damaged, the damage seemed to have occurred principally in the DPNH–coenzyme Q reductase portion of the branched chain (if there are not two independent chains for oxidation of DPNH and succinate), whereas succinic oxidase activity was retained. However, some damage was sustained by the reduced coenzyme Q—cytochrome *c* reductase complex because separation of cytochromes *b* and c_1 occurred under certain altered conditions involving heating, differential centrifugation, and sucrose concentration. The fact that, under these same conditions, little succinic oxidase activity was lost is compatible with the hypothesis that there are two separate chains; however, it must be remembered that the bc_1 portion of the chain does not limit electron transfer. Based upon the evidence at hand it seems likely that the DPNH–coenzyme Q reductase complex is the complex most readily detachable from the electron transfer chain. The elementary particle prepared by the standard procedure contained the full complement of acid-extractable flavin (f_D) relative to the other components; however, particles prepared at higher temperatures were usually deficient in the flavin associated with DPNH-oxidase activity. The amount lost depended upon the degree of heating.

When prepared by the standard procedure the elementary particle contained 45% (dry weight) of phospholipid; the damaged forms (prepared at higher temperatures) contained as much as 65%. The structural protein fraction in these particles (prepared at 35° to 38°) contained little phospholipid relative to the considerable amounts found in particles prepared by the standard procedure.

TABLE III

COMPARATIVE CONCENTRATIONS OF COMPONENTS PER MG PROTEIN IN MITOCHONDRIA, ETP_H, AND ELEMENTARY PARTICLES

Component	Particle Type and Concentration of Component (mμmoles/mg protein)		
	Mitochondria	ETP_H	EP[a]
Acid-extractable flavin (f_D + other acid-extractable flavins)	0.46	0.38	0.48
Acid-nonextractable flavin (f_S)	0.20	0.28	0.43
Cytochrome *a*	1.31	1.62	2.90
Cytochrome *b*	0.68	0.85	1.51
Cytochrome c_1	0.21	0.27	0.48
Cytochrome *c*	0.45	0.36	0.16

[a] Elementary particle.

TABLE IV

COMPARISON OF ENZYMIC ACTIVITIES

Activity Measured	Specific Activities[a]		
	Mitochondria (Sonicated)	ETP_H	EP[b]
DPNH oxidase[c]	3.3	4.2	8.4
Succinic oxidase[d]	1.5	1.8	3.6

[a] The specific activity is defined as the number of μatoms of oxygen consumed per minute per mg of enzymic protein when tested at 37 ± 1°. [b] The concentration of the elementary particle in the assay mixture was 50 μg of protein per ml. [c] The concentration of DPNH in the assay mixture was 5×10^{-3} M. [d] The concentration of succinate was 4×10^{-3} M and of coenzyme Q 5×10^{-4} M. Cytochrome *c* was added to both assay mixtures (200 μg/ml).

Enzymic Activities of the Elementary Particle.—The two measures of integrated electron-transfer activity are succinic oxidase and DPNH oxidase activities. Table IV contains a comparison of these two specific activities in preparations of mitochondria, electron transfer particles (ETP_H), and elementary particles. The increment in specific activity paralleled the increment in the specific concentration of the components concerned with electron transfer (Table II) in the transition from mitochondria to electron transfer particles (ETP_H) to elementary particles. As shown in the table the isolation of the elementary particles led to an (approximate) 2.5-fold increase in specific activity and to (about) a 2.2-fold increase in the concentration of cytochrome *a*. Thus a high positive correlation was retained throughout the isolation procedure between the increase in enzymic activity and the confinement of the components of electron transfer to a particular fraction. As mentioned previously, when higher temperatures (above 0–5°) were used for preparing the elementary particles, DPNH oxidase activity was in large measure destroyed whereas succinic-oxidase activity was increased as a result of an increase in the specific concentration of the components associated with electron transfer from succinate to oxygen. This apparent increase may be the consequence of removal of residual structural protein and of loss of some of the protein concerned with DPNH oxidase activity.

In Table V are listed the specific activities of succinic oxidase and DPNH oxidase with varying concentrations of substrate. Antimycin inhibits both DPNH and succinic oxidase activities about 95% (92–96%). The residual succinic oxidase activity (no cytochrome *c* or coenzyme Q added) was 20% of maximal, but 90% of maximal succinic oxidase activity (optimal amount of cytochrome *c* added) was retained in the absence of added coenzyme Q. From a Lineweaver-Burk plot of the data shown for DPNH oxidase activity (and other oxygraphic observations), it becomes clear that the primary oxidation or dehydrogenation of DPNH (substrate) is not the step that determines the rate of transfer of electrons from DPNH to oxygen.

The dependence of the oxidation rates of both enzyme complexes on the concentration of cytochrome *c* is illustrated in Table VI. The residual oxidase activities observed were approximately those to be expected in the presence of the amounts of cytochrome *c* determined by extraction. Approximately 20% of the total cytochrome *c* remained with the elementary particle and about 29% of the DPNH oxidase and 33% of the succinic oxidase activities show up in absence of added cytochrome *c*. To obtain maximal activity a large excess of cytochrome *c* was required; more was required for oxidation of succinate than for oxidation of DPNH. On a "molecular" basis about 200 molecules of cytochrome *c* were required for the maximal activity of each elementary particle when DPNH was the substrate, and more than 400 were required when succinate was the substrate.

TABLE V

RELATIONSHIP BETWEEN THE CONCENTRATION OF SUBSTRATE (DPNH OR SUCCINATE) AND THE SPECIFIC ACTIVITY OF THE ELEMENTARY PARTICLE[a]

Substrate	Concentration of Substrate (Molar)	Specific Activity (μatoms of oxygen/mg protein/min)
DPNH	5×10^{-5}	6.72
	1×10^{-4}	8.14
	5×10^{-4}	8.80
	5×10^{-3}	8.74
Succinate	1.5×10^{-5}	2.72
	3.0×10^{-5}	3.32
	6.0×10^{-5}	4.34
	7.5×10^{-5}	4.96
	1.0×10^{-4}	4.88

[a] The concentrations of the elementary particle and of the other reactants (except those varied as noted) were the same as described in the legend of Table IV.

Although added coenzyme Q augmented succinic oxidase activity, especially the residual activity, it inhibited the DPNH oxidase system. The inhibition may be correlated with the amount of coenzyme Q added. The coenzyme Q was added as a suspension in absolute ethanol but it was shown that the inhibition observed did not result from the addition of ethanol at the concentrations used.

From data of the kind shown, we have concluded that integrated electron transfer, as measured by DPNH and succinic oxidase activities, is associated exclusively with the elementary particle; increases in the degrees of purification of the particle and of integrated activities run parallel. Our results indicate that the presence of the major part of the structural protein is not essential for either oxidase activity.

The elementary particle preparation was refractionated with bile salts in an attempt to remove more structural protein (some 30–40% of the total protein),

TABLE VI

DEPENDENCE OF ACTIVITIES OF THE ELEMENTARY PARTICLE ON THE CONCENTRATION OF CYTOCHROME *c*[a]

Substrate	Amount of Cytochrome *c* Added (μg/ml)	Specific Activity (μatoms oxygen/mg protein/min)
DPNH	0	2.62
	5	3.94
	10	5.80
	20	7.30
	50	8.62
	100	9.18
	200	9.14
Succinate	0	1.92
	10	2.46
	20	3.00
	50	3.56
	100	4.30
	200	5.80
	300	5.80

[a] All of the conditions were those described in the legend of Table IV except the concentration of cytochrome *c*, which was varied as noted.

but this additional step in the purification did not lead to a concomitant increase in activity.

Reconstitution of the Elementary Particle During Isolation.—When the floating fraction was diluted with 0.25 M sucrose to a final protein concentration of 0.5 mg/ml, all complexes which had become dissociated recombined to form a single unit. In general, at protein concentrations below 10 mg/ml the particles constituting the floating pellet behave as single homogeneous units as judged by their behavior in the ultracentrifuge and in the assay of activity. At protein concentrations above 20 mg/ml the behavior of this fraction as a single unit is in doubt. When the floating fraction was centrifuged at a protein concentration of 60 mg/ml, an excess of cytochrome *a* was observed in the pellet. When centrifugation was continued until the supernatant fluid had a protein concentration of less than 5 mg/ml, no cytochrome *a* was detected in the supernatant fluid.

Bovine serum albumin neither hindered nor enhanced the "reconstitution" when present in the diluting solution (0.25 M sucrose) at a concentration of 10 mg/ml; nor did it affect the enzymic activity in any way. This reagent, however, prevents recombination of complexes (Fowler *et al.*, 1963).

The dilution effect is an aspect of reconstruction of the electron transfer chain from the component complexes. The floating fraction contains considerable amounts (1.23 mg per mg of protein) of bile salts and ammonium sulfate (0.12 mg per mg of protein). The presence of high levels of both bile salts and ammonium sulfate prevents recombination of the complexes. In fact, the dissociation of the native elementary particle into its component complexes is brought about by these same reagents. When the dissociating agents are diluted beyond a certain critical concentration, then recombination takes place readily. Under the conditions used for isolating the elementary particle the minimal dilution for reconstitution is exceeded and, equally important, the protein (recombined complexes) is then concentrated by centrifugation.

Molecular Weight of the Elementary Particle.—From the data presented in Table II the molecular (particle) weight can be computed. The cytochrome *a* content is perhaps the most reliable experimental value on which to base the calculation. If one assumes six units of cytochrome *a* per molecular weight unit of the elementary particle, the minimal molecular weight, in terms of protein, comes to a value of 2.1×10^6.

The four complexes that make up the elementary particle have been isolated in a high degree of purity; calculations based on chemical analyses indicate the following molecular weights: succinic-coenzyme Q reductase, 230,000 (Ziegler and Doeg, 1959, 1962); DPNH–coenzyme Q reductase, 530,000 (Hatefi *et al.*, 1960, 1961a, 1962a); reduced coenzyme Q–cytochrome *c* reductase 200,000 (Hatefi *et al.*, 1960, 1961a, 1962b); and reduced cytochrome *c* oxidase 430,000 (Ambe and Venkataraman, 1959; Criddle and Bock, 1959; Criddle *et al.*, 1962). Three of these four complexes have been shown (Hatefi *et al.*, 1961b; Hatefi and Fowler, 1961; Hatefi *et al.*, 1962c; Fowler and Richardson, 1963), to recombine to form a single unit having an approximate 1:1:1 stoichiometry; and if we assume that the fourth complex also combines in the same stoichiometric ratio, the resulting reconstituted units would have a minimal calculated molecular weight of 1.4×10^6 (protein basis). This value suggests that one-third of the molecular weight previously calculated for the isolated elementary particle consisted of protein which is not required for integrated electron transfer activity. It was mentioned earlier that 30–40% of the protein in the elementary particle preparation could be removed by a second fractionation with bile salts and ammonium sulfate, and that the protein removed had some characteristics of structural protein. If it should prove possible to remove this structural protein and still retain the integrated enzymic activities then the molecular weight would be very close to that obtained by summation of the molecular weights of the purified complexes, i.e., 1.4×10^6.

Correlative studies have been made in the SW39-L rotor of the Spinco Model L ultracentrifuge to determine whether inhomogeneity with respect to particle size could be referred to inhomogeneity with respect to chemical composition. The slowest and the fastest sedimenting components (as well as the intermediate fractions) in the preparation of elementary particles were separated by centrifugation and each was analyzed with respect to its flavin and cytochrome composition. Significant differences were not observed among any of the fractions having different sedimentation patterns. Thus the inhomogeneity of the elementary particle preparation appears not to be an expression of chemical differences among these particles with respect to the oxidation-reduction components of the chain. There is a whole range of particle sizes in the elementary particle preparations. The variable elements may be (1) the lipid content, (2) the bile salt content, and (3) the degree of aggregation of the units after reconstitution.

Discussion

Three lines of evidence lead to the conclusion that mitochondria contain a subunit of molecular (particle) weight about 1.4×10^6 with a complete electron transfer chain for the oxidation of succinate and DPNH by molecular oxygen. First, the primary dehydrogenase complexes and auxiliary enzymes (released as soluble proteins when mitochondria are disrupted by sonication) and the structural proteins together account for over 70% of the total protein of the mitochondrion; this conclusion is documented by the data of Table I plus the fact that an additional 30–40% of the protein of the elementary particle can be removed without loss of the oxidoreductive components albeit without retention of full activity. Thus, the proteins associated with the electron transfer chain cannot account for more than 30% of the total mitochondrial protein. On this basis it is theoretically possible to isolate an elementary particle with oxidoreductive components about 3.1 times more concentrated than in the mitochondrion and oxidase activities correspondingly greater. However, it has not yet been possible to achieve this degree of purification and at the same time to satisfy the criterion of parallel concentration of components and activity. We have succeeded only to the extent of realizing three-quarters of the expected purification without loss of activity. Second, if we assume that the four complexes of the electron transfer chain (isolated independently) at the highest stage of purity can recombine to form a particle that contains these four complexes in 1:1:1:1 stoichiometry (this assumption is not without justification as three of the four complexes have been shown to recombine with this approximate stoichiometry), the molecular weight is 1.4×10^6. Third, the mitochondrion can be resolved into three fractions: (a) an insoluble structural protein fraction that sediments readily, (b) a soluble protein fraction, and (c) an insoluble fraction that floats under appropriate conditions. The components associated with electron transfer and with the oxidase activities of the mitochondrion are found in the floating fraction. The

particles that are obtained after dilution of this floating fraction with sucrose solutions can be refractionated to obtain a particle with components of electron transfer purified to the extent that the cytochrome *a* content of the particle is 4.2 mμmoles per mg of protein with proportional increases in the other components. If we assume 6 units of cytochrome *a* per particle, then the particle weight would be 1.4×10^6. These three lines of evidence indicate that the unit of the electron transfer chain has a molecular weight of 1.4×10^6 if we accept the single branched-chain hypothesis of electron transfer (not all evidence points to this conclusion) rather than a hypothesis which stipulates two independent chains, one for DPNH oxidation and one for succinate oxidation. Since the number of molecules in a complete chain can be inferred from the stoichiometry of the components in the individual complexes, e.g., one molecule of f_S in each molecule of succinic–coenzyme Q reductase (Ziegler and Doeg, 1959, 1962), the possibility of the molecular weight being a multiple of 1.4×10^6 is unlikely.

Preparations of the elementary particle can be made either by isolating the four complexes individually, separating each one from structural protein (Ziegler and Doeg, 1959; Hatefi *et al.*, 1960, 1961b, 1962a,b; Griffiths and Wharton, 1961), and permitting them to recombine (Hatefi *et al.*, 1961b; Fowler and Hatefi, 1961; Fowler and Richardson, 1963) or by isolating them collectively as described in the present communication. In the former admittedly involved procedure and by the latter much simpler procedure the enzymic activity appears to be fully preserved.

We have mentioned earlier that our data do not entirely corroborate the possibility of a single branched chain for electron transfer but the presence of only one residue of cytochrome c_1 for each residue of f_S and f_D strongly supports this hypothesis. Other experimental evidence favoring such an hypothesis is afforded by (1) the interaction between succinate and DPN that is catalyzed by ATP (Low *et al.*, 1961; Azzone *et al.*, 1960; Chance *et al.*, 1960; Klingenberg, 1960); (2) the reconstitution experiments showing the stoichiometric relationship between DPNH–coenzyme Q reductase, succinic coenzyme Q reductase and reduced coenzyme Q cytochrome *c* reductase.

The elementary particle, as defined, is the unit of electron transfer from succinate or DPNH to molecular oxygen. For transfer neither structural protein nor the primary dehydrogenase complexes and their auxiliary enzymes are essential; only the four complexes of the structured electron transfer chain make up the elementary particle. In the mitochondrion electron transfer and synthesis of ATP are coupled. The use of bile salts in the preparation of the elementary particle has made it difficult to evaluate its coupling capacity.

Although each complex of the elementary particle has a large amount of phospholipid associated with it, which is required for integrated electron transfer (Fleischer *et al.*, 1961), the entire structural protein–phospholipid network to which the elementary particle is attached in mitochondrial membranes is apparently not essential for this function since the two entities are separable without loss of electron transfer, though structure may be greatly altered.

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Reactions of Cupric Ion with Lysine Vasopressin and Acetyllysine Vasopressin*

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Lysine vasopressin was purified by ion exchange chromatography and gel filtration to provide a peptide of adequate purity for quantitative physical chemical studies. The three *pK*'s of the peptide were determined by spectrophotometric and potentiometric titrations. Spectral analyses of cupric ion–lysine vasopressin solutions revealed that a 1:1 complex is formed with an absorption maximum at 525 mμ. Similar measurements with copper–acetyllysine vasopressin solutions demonstrate no evidence of complex formation of the type exhibited by copper–lysine vasopressin. Titration of the 1:1 copper–lysine vasopressin and spectral analyses provide data to support the hypothesis that the complex forms by interaction of the cupric ion at the free terminal α-amino acid group of the peptide followed by subsequent loss of three hydrogen atoms from neighboring peptide bonds. A tetradentate structure consistent with this interpretation is presented. The apparent molecular extinction coefficient, stability constant, and free energy of formation of the complex have been estimated. The use of solutions of copper–lysine vasopressin under conditions expected to maintain maximum complex formation resulted in no significant alteration of the effect of the hormone in the toad bladder assay.

There is considerable evidence indicating that cupric ions react with peptides in solution by interaction at the free terminal amino end with subsequent release of protons from the peptide nitrogen atoms. Dobbie and Kermack (1955a) described the formation of the 1:1 copper-glycylglycine complex as a reaction of cupric ion at the *N*-terminal amino group of the peptide followed by chelation at the peptide nitrogen atom to produce the loss of a peptide proton and subsequent loss of a proton from a coordinated water molecule. This interpretation of the reaction was supported by work of Datta and Rabin (1956) and by studies of Koltun *et al.* (1960). When the 1:1 copper-triglycine complex was titrated by Dobbie and Kermack (1955b), they observed that three equivalents of alkali per copper atom were taken up in the *p*H range 4–8. During the addition of the first two equivalents of alkali the color of the solution deepened from a pale greenish blue to a very dark blue, but thereafter the color of the solution changed through violet to a deep purple, while the third equivalent of alkali was added. This corresponded to shift in the wavelength of the maximum absorption of the solution from about 650 mμ to about 550 mμ. They suggested that this color change is produced by the formation of a tridentate complex in which the copper is attached to the free terminal amino group and the nitrogen atoms at two peptide bonds.

Martin *et al.* (1960) reported that tetraglycine reacts in a 1:1 molar mixture with cupric ions to release three protons in addition to the proton from the terminal amino group, and their data indicated that ionization of peptide hydrogens had occurred. Koltun *et al.* (1963) have also studied the reaction of tetraglycine with cupric ions, and these workers found that reaction of 1 mole of cupric ion with 1 mole of tetraglycine and 4 moles of base produced a complex with a wavelength maximum of 520 mμ and extinction coefficient of 145 $mole^{-1}$ cm^{-1}. The uptake of 4 moles of base by this complex occurred approximately between *p*H 4 and 10.5, and the dissociating protons were identified as those of the terminal α-amino group and three peptide bond nitrogens.

Campbell *et al.* (1960) described the spectral characteristics of the 1:1 copper–lysine vasopressin complex and the 1:1 copper-oxytocin complex, and Breslow (1961) reported data concerning the reaction of cupric ion with oxytocin and its derivatives. The work of Breslow suggested that cupric ion reacted with oxytocin to complex with the terminal α-amino group and to produce subsequent ionization of protons from neighboring peptide bonds. The evidence presented in this paper indicates that the cupric ion reacts with lysine-vasopressin to from a 1:1 complex by interaction at the terminal α-amino group with concomitant release of three protons from peptide nitrogen atoms.

Experimental

Purification of Lysine Vasopressin (LVP).[1]—An acetone powder of whole hog pituitary glands from which corticotropin had been removed by treatment with oxycellulose was kindly supplied by Dr. J. B. Lesh of Armour Pharmaceutical Company. The preparation of this powder has been described previously (Porath and Schally, 1962). The material was subjected to chromatography on carboxymethylcellulose and rechromatographed on the same exchanger using the techniques described by Schally *et al.* (1960a) and Schally *et al.* (1960b). The lysine vasopressin was then purified by Sephadex gel filtration in a manner similar to that of Porath and Schally (1962). A column, 3.2 × 68 cm, was packed with G-25 Sepha-

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[1] Abbreviation used in this paper: LVP, lysine vasopressin.