CYTOCHEMICAL STUDIES OF MITOCHONDRIA

II. Enzymes Associated with a Mitochondrial Membrane Fraction*

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(Received for publication, June 22, 1956)

In the preceding paper (2) it was shown that a fraction consisting of mitochondrial membranes can be separated from isolated liver mitochondria treated with deoxycholate. In this paper certain chemical and biochemical properties of this fraction are described.

It has long been thought that the mitochondrial membrane, or, as it was once called (3–5), "cortex" or "shell", is composed of lipide or lipoprotein material (3–5). Some biochemical properties of this membrane have also been predicted (*cf.* review 6). Recently, from results of studies with the Keilin-Hartree (7) heart muscle preparation, which is essentially a concentration of the succinoxidase complex, Cleland and Slater (8) have postulated that this preparation originates from sarcosomal (mitochondrial) material and that it consists of fragments of sarcosomal membranes. Also, Hogeboom and Schneider (9) found that sonic disintegration of rat liver mitochondria produced a fraction which still contained a large proportion of the succinoxidase, cytochrome oxidase, and DPNH-cytochrome *c* reductase activities of intact mitochondria, (but without much concentration of the activity (*cf.* below)). Examination with the electron microscope (10) showed that this fraction included vesicular elements (but *cf.* reference 2). The present study consists of a biochemical examination of the mitochondrial membrane fraction described morphologically under reference 2. The results experimentally verify the postulate alluded to above, and in brief, show that this membrane fraction has a higher phospholipide/protein value than intact mitochondria, and contains, in appreciably higher concentration, most of the succinoxidase and cytochrome oxidase activities of the original mitochondria.

Experimental

Rat liver mitochondria were isolated and treated in suspension with 0.3 per cent DOC as described previously (2). The treated mitochondrial suspension was fractionated as follows: spinning at 13,000 *g* for 10 minutes yielded a first fraction; its supernatant was then spun at 25,000 *g* for 20 minutes; the pellet obtained constituted the second fraction; the supernatant was further centrifuged at 105,000 *g* for 60 minutes and the sediment represented the third

* A part of this paper was presented at the Meeting of the American Society of Biological Chemists at Atlantic City, April 16, 1956 (1).

fraction. The characteristic morphological features of the successive pellets have been described in the preceding paper (2). In brief, the 13,000 *g* pellet contains relatively intact mitochondria, the 25,000 *g* pellet contains swollen and disintegrated mitochondria and unidentified granules of mitochondrial dimensions, while the 105,000 *g* pellet is the membrane-containing pellet described in detail in reference 2. For enzymatic study the pellets were resuspended in 0.44 M sucrose solution at the dilutions required for the various enzyme assays. None of the pellets was washed except the tightly packed 105,000 *g* pellet, and in this case, 0.44 M surcose solution was layered on top of the pellet, swirled a few times, and then decanted.

For chemical determinations the pellets were resuspended in 0.44 M sucrose solution, trichloracetic acid added to a final concentration of 10 per cent, and ribonucleic acid (RNA), phospholipide (PL), and fat-free protein extracted by the Schneider procedure (11). The orcinol reaction (12) was used to determine RNA; the phosphate of the phospholipide fraction was determined by the Fiske-SubbaRow method (13) after digesting the alcohol-ether extract with concentrated H_2SO_4 (14), while the protein N was determined by nesslerization (14).

Enzymatic activities were determined as follows: cytochrome oxidase by the spectrophotometric method of Cooperstein and Lazarow (15); succinoxidase by the manometric method of Schneider and Potter (16); DPNH-cytochrome *c* reductase by the spectrophotometric method of Hogeboom (17); and adenylate kinase by the ion-exchange method of Siekevitz and Potter (18). Conditions designed to test for oxidative phosphorylation are given in Table IV. Manometric determinations were performed at 30° with a 5 minutes equilibration period, while spectrophotometry was carried out at room temperature in a Beckman spectrophotometer (model Du). For every determination two concentrations of mitochondria or mitochondrial fraction were used to ascertain that the data came from the straight line portion of the curve of tissue concentration *vs.* activity. In the manometric and spectrophotometric determinations readings were taken at various time intervals, while the adenylate kinase determination was stopped after 15 minutes at 30°. In all cases the results were recalculated to 60 minutes from the straight line portion of the activity *vs.* time curves.

Chemicals used were deoxycholic acid (DOC) from Wilson Company, Chicago; cytochrome *c*, DPN (90 per cent) and hexokinase from Sigma Company, St. Louis; Na adenosine diphosphate (ADP) from Pabst Company, Milwaukee, and antimycin A from the Wisconsin Alumni Research Foundation, Madison. The substrates were commercial products of various companies. Reduced DPN was prepared by the method of Hogeboom and Schneider (19) and reduced cytochrome *c* by the method of Cooperstein and Lazarow (15).

RESULTS

Table I gives the results of a typical experiment in which the distribution of protein, RNA, and phospholipide was studied in mitochondria and the fractions derived from them. The mitochondrial values found per gram wet weight original tissue are lower than those reported earlier (*cf.* 20). The difference can be explained by the fact that in our case the mitochondria were carefully washed (2) to reduce contamination with microsomes and "fluffy layer" material, a procedure expected to give a relatively poor recovery of mitochondria from the liver homogenate. The phospholipide/protein value is similar to that reported by Swanson and Artom (21), while the value of the RNA/protein is approximately one-half that given previously (*cf.* 20). Since the electron micrographs of untreated mitochondria show contamination with microsomes despite the precautions taken (Fig. 7, reference 2) it is likely that even this small amount

of RNA is too high a figure for true mitochondrial RNA. It has been found previously (22) that after treatment with 0.3 per cent DOC, most of the RNA of the microsomes is still sedimentable at 105,000 *g*. Thus, since only 40 to 50 per cent of the RNA present in the mitochondrial pellet is sedimentable (*cf.* Table I), it is possible that only that RNA which goes into solution when mitochondria are treated with DOC is true mitochondrial RNA. For this reason it is thought that most of the RNA in the 105,000 *g* pellet is derived from contaminating microsomes. This view is supported by electron micrographs (2) of

TABLE I

Chemical Composition of Intact Mitochondria and of Fractions Obtained from Mitochondria Treated with 0.3 Per Cent Deoxycholate

The fractionation of the mitochondrial fragments and the extraction and determination of chemical components are described in the Experimental section. In this and succeeding Tables, the term *equivalent grams mitochondria* (*eq. gm. Mt.*) refers to the amount of mitochondria or fraction obtained from one gram wet weight rat liver. The amount of protein (Pr) was obtained by multiplying the protein N value by 6.25, while the amount of phospholipide (PL) was obtained by multiplying the phospholipide P value by 25 (20). Mt. = mitochondria, suspended in 0.44 M sucrose solution; 13,000 *g*, 25,000 *g*, 105,000 *g* = pellets obtained at the specified centrifugal fields after treatment with 0.3 per cent DOC and resuspended in 0.44 M sucrose; S = supernatant from 105,000 *g* pellet.

	Mg. Pr/ eq. gm. Mt.	Mg. RNA/ eq. gm. Mt.	Mg. PL/eq. gm. Mt.	Mg. RNA/mg. Pr	Mg. PL/mg. Pr
Mt.	9.50	0.068	2.12	0.007	0.22
13,000 *g*	0.06	<0.002	<0.02	—	—
25,000 *g*	1.19	0.010	0.70	0.008	0.59
105,000 *g*	0.56	0.023	0.33	0.041	0.59
S	7.87	0.033	1.45	0.004	0.18
Recovery	9.68	0.066	2.48	—	—

this pellet which show the presence of particles of the same dimensions as microsomal nucleoprotein particles (22). Microsomal phospholipide on the other hand, is nearly completely solubilized[1] by 0.3 per cent DOC (22). Thus the high concentration of phospholipide in the 105,000 *g* pellet is probably of mitochondrial origin and is not due to microsomal contamination. The 105,000 *g* fraction thus contains some 16 per cent of the mitochondrial phospholipide, only 6 per cent of the mitochondrial protein, and 40 per cent of the original RNA of which little is of mitochondrial origin. The intact mitochondria have 4.7 parts protein for one part phospholipide, while the 105,000 *g* pellet has 1.7 parts protein for one part phospholipide. Since most of the 105,000 *g* pellet is derived from mitochondrial membranes (2), the lipoprotein character of these structures is readily apparent (*cf.* reference 23). The significance of the

[1] The term solubilization as used in this paper does not necessarily mean that a true solution exists, but it refers instead to non-sedimentability under certain specified conditions.

high concentration of phospholipide in the 25,000 *g* pellet, which contains swollen and disintegrating mitochondria and unidentified granules, is not known.

Tables II, III, and IV give representative values obtained for the relative concentrations of mitochondrial enzymes in the mitochondrial subfractions which were isolated. It is clear that two of the enzymes or enzyme systems

TABLE II

Some Enzymatic Properties of Intact Mitochondria and of Fractions Obtained from Mitochondria Treated with 0.3 Per Cent DOC

The fractionation of DOC-treated mitochondria and the determination of enzymatic activities are described in the Experimental section. The amounts of cytochrome *c* oxidized or reduced were calculated as described (13, 14). The amount of AMP formed from ADP could be used as a measure of adenylate kinase activity since the preparations exhibited no inorganic phosphate production or net ATP synthesis (*cf.* reference 17). The symbols for the fractions are explained in Table I; Mt DOC = mitochondria suspended in 0.44 M sucrose solution containing 0.3 per cent DOC. Specific Activity = activity per mg. protein N.

	Succinoxidase		Cytochrome oxidase		DPNH-cytochrome *c* reductase		Adenylate kinase	
	$\mu l.O_2$/60 min./eq. gm. Mt.	Specific Activity	Δ log (ferrocytochrome *c*)/ min./eq. gm. Mt.	Specific Activity	μM cyt. c red./60 min./eq. gm. Mt.	Specific Activity	μM AMP/60 min./eq. gm. Mt.	Specific Activity
Mt.....	1320	868	233	153	492	337	90.0	59.2
Mt. DOC....	590	388	143	94	245	162	95.0	62.5
13,000 *g*.....	15	1500	0.6	66	0	0	0	0
25,000 *g*.....	229	1206	7	39	0	0	0	0
105,000 *g*....	498	5550	79	878	0	0	0	0
S.....	70	56	55	44	180	143	90.4	71.7
Recovery....	898*	—	177‡	—	360§	—	90.4	—

* Assuming 55 per cent inhibition by DOC in supernatant only.

‡ Assuming 39 per cent inhibition by DOC in supernatant only.

§ Assuming 50 per cent inhibition by DOC in supernatant only.

tested can be solubilized by DOC while two others remain bound to the fragments.

Previously, it had been found that adenylate kinase could be solubilized by sonic treatment of mitochondria (24, 25), but the per cent released was not given. Table II shows that 0.3 per cent DOC releases the entire enzymatic activity. This enzyme is also readily removed from mitochondria by milder means. For example, if mitochondria are placed in water for 10 minutes at 4°C. and then centrifuged at various speeds, 21 to 39 per cent of the protein N and approximately 75 per cent of the adenylate kinase are no longer sedimentable after 30 minutes at 50,000 *g*.[2] However, keeping mitochondria for as long as 60

[2] All the succinoxidase and α-ketoglutarate oxidase activities are sedimentable after this treatment.

TABLE III

Comparison of Some Chemical and Biochemical Properties of Several High-Speed Fractions Obtained from Mitochondria Disrupted with 0.3 Per Cent DOC

The fractionation of DOC-treated mitochondria is the same as described in the Experimental section, except that: (a) the supernatant from the 105,000 *g* centrifugation was subjected to 150,000 *g* for one hour in a bucket-type rotor to give a pellet similar in appearance to the 105,000 *g* pellet; (b) the supernatant from the 150,000 *g* centrifugation was subjected to 150,000 *g* for 6 hours to give a similar pellet and a final supernatant (F.S.). The determination of enzymatic activities was as described in the Experimental section.

	Mg. Pr./eq. gm. Mt.	Mg. RNA/eq. gm. Mt.	Mg. PL/eq. gm. Mt.	Mg. RNA/mg. Pr.	Mg. PL/mg. Pr.	Succinoxidase		Cytochrome oxidase	
						μl. O_2/60 min./eq. gm. Mt.	Specific Activity	Δ log (ferrocytochrome *c*)/min/eq. gm. Mt.	Specific Activity
105,000 *g*....	0.63	0.053	0.47	0.084	0.75	603*	6025*	57	570
150,000 *g*; one hour..	0.60	0.028	0.51	0.047	0.85	508‡	5300‡	37	386
150,000 *g*; six hours..	1.12	0.067	0.29	0.060	0.26	—	—	—	—
F.S.....	10.00	0.011	0.47	0.001	0.05	—	—	—	—

* Without exogeneous cytochrome *c*, corresponding figures are: 394, 3990.

‡ Without exogeneous cytochrome *c*, corresponding figures are: 388, 4000.

TABLE IV

Comparison of the Oxidation of Various Substrates by Intact Mitochondria, Mitochondria in DOC, and 105,000 g Fraction

The assay medium was an oxidative-phosphorylation mixture which contained the following: 0.01 M phosphate buffer, pH 7.4, 0.005 M $MgCl_2$, 3×10^{-4} M DPN, 1.5×10^{-5} M cyt. *c*, 0.001 M ADP, 0.1 ml. hexokinase, 0.015 M glucose, the amount of substrate given below, mitochondria (usually 200 to 400 eq. mg.) or 105,000 *g* fraction (usually 400 to 800 eq. mg.), and 0.44 M sucrose solution to give a final volume of 3.0 ml. Incubated at 30°C. The figures refer to μl. O_2/60 min./mitochondria or fraction from 1 gm. wet weight rat liver. The mitochondria were suspended in 0.44 M sucrose solution (Mt.) or in this sucrose solution containing 0.3 per cent DOC (Mt. DOC) and kept in an icebath until the 105,000 *g* fraction was obtained (approximately 75 min.).

	Substrate added						
	citrate (24 μM)	α-keto-glutarate (30μM)	fumarate (24 μM)	fumarate, pyruvate (18, 24 μM)	malate (24 μM)	β-hydroxy-butyrate (30 μM)	succinate (60 μM)
Mt.....	260	373	285	680	128	210	885
Mt. DOC..	35	28	35	8	20	10	180
105,000 *g*...	23	16	7	0	0	13	160

minutes at 4°C. in either 0.25 M or 0.44 M sucrose solutions resulted in no release of the enzyme. It has been previously postulated from metabolic data (26) that this enzyme is located at the mitochondrial surface. If adenylate kinase is indeed part of the membrane, it is very weakly held there.

Earlier findings (17, 27) that DPNH-cytochrome *c* reductase is concentrated both in rat liver microsomes and mitochondria, with the former having the larger amount and higher concentration of the enzyme, have been confirmed. Sonic disintegration of liver mitochondria results in approximately 40 per cent solubilization of the enzyme (non-sedimentable at 150,000 *g* for 60 minutes, (9, 28)), the rest being brought down as particles with a rate of sedimentation slower than that of the particles carrying the cytochrome oxidase of the mitochondria (28). From Table II it can be seen that while there was a 50 per cent inhibition of enzymatic activity by the 0.3 per cent DOC, all of the recoverable activity became non-sedimentable at 105,000 *g* for 60 minutes.[3] The results of Table II thus agree with the earlier results of Hogeboom and Schneider (28) in that the enzyme is not tightly associated either with cytochrome oxidase (27) or with succinoxidase.[4]

[3] With DOC treatment, 80 per cent of the mitochondrial protein becomes non-sedimentable at 105,000 *g* for 60 minutes, while with sonic treatment 60 per cent (9, 28) becomes non-sedimentable at 150,000 *g* for 60 minutes.

[4] The effects of antimycin A and amytal on DPNH-cytochrome *c* reductase are of interest. While there is general agreement (29, 30) that antimycin A does not inhibit the DPNH-chtochrome *c* reductase of the microsomes, the situation with respect to the mitochondrial enzyme is confused. It has been confirmed here that antimycin A (3 μg./ml.), or amytal (2×10^{-3} M), has no effect on the microsomal enzyme, nor on the enzyme which can be solubilized (22) from the microsomes by 0.3 per cent DOC. We have also found that these same concentrations of antimycin A or amytal have no effect on the enzyme activity of intact mitochondria nor on that activity which can be solubilized from mitochondria by 0.3 per cent DOC. Ernster *et al.* (31) found only a small inhibition by amytal of the oxidation of reduced DPN by mitochondria while de Duve *et al.* (30) found that antimycin A caused a 60 per cent inhibition of the DPNH-cytochrome *c* reductase activity of mitochondria which had been preincubated for 1 hour in water. Previously it had been postulated (32) that whereas antimycin A acts on the mitochondrion bound enzyme, a considerable quantity of DPNH seems to be oxidized *via* an antimycin A insensitive pathway. Also, the enzyme which has been solubilized from heart heart muscle preparations (33, 34) is not affected by antimycin A, though another heart preparation which has been treated with DOC (35) and a sedimentable breast muscle preparation (34) are both inhibited. However, there are indications that there are two forms of the mitochondrial DPNH-cytochrome *c* reductase (*cf.* review 36), an "external" one acting on exogenous DPNH and an "internal" one acting on the DPNH generated by the oxidations of substrates whose enzymes require DPN as a coenzyme. Experimental support for this hypothesis has been presented (31, 32, 37). Thus it might be that the enzyme which can be solubilized from mitochondria by 0.3 per cent DOC (Table II) is the "external" one attacking exogenous DPNH and is similar to the microsomal enzyme complex (containing a hemoprotein (38)) in not being inhibited by antimycin A. On the other hand, the "internal" enzyme which has been found responsive to antimycin A (32) and to amytal (31) might be that form of the mitochondrial enzyme complex (containing a flavoprotein (39)) which is inhibited by DOC

The distinctive feature of Table II is the marked concentration of succinoxidase and cytochrome oxidase in the 105,000 *g* fraction, the fraction which has been found (2) to comprise mitochondrial membranes. In various experiments, 25 to 38 per cent of the succinoxidase and 32 to 57 per cent of the cytochrome oxidase of the intact mitochondria were found in this fraction. In some cases there was as much as a sevenfold concentration of succinoxidase and an elevenfold concentration of cytochrome oxidase in this fraction. It is also apparent that more cytochrome oxidase activity can be solubilized by DOC than succinoxidase activity. These findings are in agreement with those of Hogeboom and Schneider (9) who found that succinoxidase was more inhibited by sonic treatment than was cytochrome oxidase, and that the latter enzyme was more easily solubilized. When the 105,000 *g* pellet was rehomogenized in 0.44 M sucrose solution without DOC and recentrifuged for 30 minutes at 105,000 *g*, 91 per cent of the enzyme activity was recovered in the combined resultant pellet and supernatant fluid, and out of this total 89 per cent was still found in the resultant pellet, indicating that the enzyme is firmly attached to the membranes comprising the pellet.

It was of interest to determine whether the supernatant from the 105,000 *g* centrifugation still contained some particulate (sedimentable) succinoxidase or cytochrome oxidase activity. Accordingly, this supernatant was centrifuged for one hour at 150,000 *g* and examined chemically and biochemically (Table III). In this fraction, the succinoxidase activity was equal to that in the 105,000 *g* fraction while the cytochrome oxidase activity was 30 per cent lower than in the heavier fraction. Since the amounts of protein (and phospholipide) were equal in these two fractions, the succinoxidase specific activities were the same while the specific activity of cytochrome oxidase was lower in the 150,000 *g* pellet. A comparison of Tables II and III reveals that approximately 70 per cent of the succinoxidase and cytochrome oxidase activities of the original mitochondria can be brought down in particulate form (105,000 *g* and 150,000 *g* pellets). When the activities of the other fractions are taken into account (*cf.* Table II), an almost full recovery of these two enzymes is obtained from the disrupted mitochondria. It is apparent that the inhibition of these enzymes by DOC is reversible in that nearly complete recovery of the enzyme activity can be obtained on washing out the DOC. It is possible that the DOC inhibition is due to reversible adsorption on the enzyme surface. Much the same conclusions have been voiced earlier (40, 41) with regard to inhibition of succinoxidase by surface-active reagents. Electron micrographs of these two high-speed pellets (2) revealed that they have a similar appearance, each containing vesiculated structures, and here and there, clusters of granules believed to be nucleoprotein

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treatment (Table II). That this hypothesis might be correct is indicated by the results in Table III which show the marked inhibition by DOC of the oxidations of those substrates requiring DPN.

granules from contaminating microsomes (2). The biochemical similarity of these two pellets is thus paralleled by their morphological similarity.

It is difficult to tell whether other mitochondrial enzymes are also part of the 105,000 *g* pellet. Table IV shows the marked inhibition by DOC of the oxidations of various substrates when tested under identical conditions. The oxidation of succinate was much less inhibited by DOC than were the oxidations of other substrates, and the recovery of succinic oxidation in the 105,000 *g* pellet was much greater. Thus, even with 80 per cent inhibition, the oxidation of succinate was concentrated threefold[5] in the pellet over the intact mitochondria, while there was little or no concentration in the cases of the oxidations of other

TABLE V

Effects of Cytochrome c and of Al^{+++} and Ca^{++} on Succinoxidase Activity of Intact Mitochondria and of 105,000 g Fraction

The conditions of assay are given in the Experimental section. The figures refer to μl. O_2/60 min./mitochondria or 105,000 *g* fraction from 1 gm. wet weight rat liver.

Experiment		Plus cyt. *c* (1)*	Minus cyt. *c* (2)	(1)/(2)	Plus Al^{+++}, Ca^{++} (1)‡	Minus Al^{+++}, Ca^{++} (2)	(1)/(2)
1	Mt.....	1710	1090	1.57	1920	1128	1.70
	105,000 *g*...	432	104	4.14	480	304	1.58
2	105,000 *g*...	603	394	1.53			
3	105,000 *g*...	208§	88§	2.36			

* Final conc. = 2×10^{-5} M.

‡ Final conc. of each ion = 4×10^{-4} M.

§ Determined in the absence of Al^{+++} or Ca^{++}.

substrates which were inhibited at least by 90 per cent. In no case when the mitochondria were incubated in the presence of 0.3 per cent DOC was there any evidence for coupled phosphorylation.

Since the complete succinoxidase complex seems to have been recovered in concentrated form in the membrane pellet, experiments were done to compare the effects of various experimental conditions on the activity in the membrane fraction to the corresponding effects on intact mitochondria. The results are given in Tables V to VII. It is known that the succinoxidase activity of rat liver is stimulated by the addition of cytochrome *c* (16, 42) or by the addition of Al^{+++} and Ca^{++} (16). Table V shows that the addition of Al^{+++} and Ca^{++} gave the same percentage increase in succinoxidase activity in the 105,000 *g* pellet as it did in the intact mitochondria. There has been speculation on the mode of action of Al^{+++} and Ca^{++} (16, 43, 44) but no definite conclusions can be drawn. Recent work (45) however, indicates that these ions act between cytochrome *c* and succinic dehydrogenase. Whatever the

[5] This figure differs from the sevenfold concentration of succinoxidase mentioned above because it was determined under suboptimal conditions (oxidative phosphorylation medium).

mechanism of activation may be, it is clear from Table V that the effect of these ions is quantitatively unchanged in the 105,000 *g* pellet; thus their action probably has the same significance for the succinoxidase activity of the pellet as it does for the same activity in the intact mitochondria.

TABLE VI

Effect of Treatment of Mitochondria with Water on Succinoxidase Activity Measured in Presence or Absence of Exogenous Cytochrome c

Mitochondria were prepared as described from 0.44 M sucrose homogenates. One aliquot was suspended in 0.44 M sucrose solution while the other aliquot was suspended in water. A calculated amount of solid sucrose was added to the media in the flasks containing the sucrose-suspended mitochondria, so that these media were rendered isotonic with 0.44 M sucrose solution; while the media in the other flasks containing the water-suspended mitochondria were isotonic with a 0.03 M sucrose solution. The figures are μl. O_2/60 min./eq. gm. Mt.

	Mt. in sucrose (1)	Mt. in water (2)	(1)/(2)
Plus cytochrome *c*	1630	1970	0.83
Minus cytochrome *c*	1030	675	1.53

TABLE VII

Effect of Antimycin A on Succinoxidase Activity of Intact Mitochondria and of 105,000 g Pellet

The antimycin A was dissolved for use in alcohol as described (30), and as controls all flasks contained the same concentration of alcohol (less than 0.5 per cent). The mitochondrial figures are expressed as μl. O_2/60 min./mitochondria from 100 mg. wet weight rat liver, while the figures for the 105,000 *g* fraction are expressed as μl. O_2/60 min./fraction from 400 mg. wet weight rat liver.

	Antimycin A added per flask, μg.					
	0.00	0.01	0.015	0.02	0.03	0.04
Mt	180	167	195	109*	0	0
105,000 *g*	113	124	121	99‡	0	0

* 61 per cent of value at zero antimycin A conc.

‡ 87 per cent of value at zero antimycin A conc.

The addition of cytochrome *c* increases the activity in both the 105,000 *g* pellet and in the intact mitochondria, though its effect is generally greater on the membrane pellet (Table V). Hogeboom and Schneider (28) also found that a fraction separated from sonically treated mitochondria had succinoxidase activity which was still apparent in the absence of added cytochrome *c*. The observation that the 105,000 *g* pellet has activity without exogeneous cytochrome *c* indicates that while some of the cytochrome *c* is lost from the pellet, a portion is still bound to the membrane structure after treatment with DOC and is still biologically active in the succinoxidase system.

As shown in the following experiments, structural variation in the membrane

does affect succinoxidase activity. It is known that mitochondria placed in water swell up, resulting in a considerable stretch of the limiting membranes (2). If it is assumed that the efficiency of the over-all succinoxidase system depends in part on the physical proximity of enzymes on the mitochondrial membrane (*cf.* reference 46), and further that this proximity depends on the state of the membrane, then in the absence of added cytochrome *c*, mitochondria having stretched membranes (*i.e.*, mitochondria placed in water) should show less succinoxidase activity than mitochondria having the original condensed membrane structure (*i.e.*, mitochondria placed in 0.44 M sucrose solution). That this is so is shown in Table VI. The swollen mitochondria show much less succinoxidase activity than do the unswollen ones in the presence of only endogeneous cytochrome *c*. They show more succinoxidase activity in the presence of added cytochrome *c* presumably because the exogeneous cytochrome *c* can more easily reach the site of enzyme activity. The results are similar to earlier ones of Schneider *et al.* (42, *cf.* reference 47) who made a comparison of succinoxidase activity of mitochondria from water homogenates and from NaCl homogenates. In the present case, the mitochondria to be tested were isolated from the same 0.44 M sucrose homogenate and presumably originally had the same content of cytochrome *c*. Thus it is clear that the endogenous cytochrome *c* was biologically less active in mitochondria suspended in water than in those suspended in 0.44 M sucrose solution.[6]

Antimycin A is an antimetabolite having a marked inhibitory effect on the succinoxidase system (32, 48) and which is thought (32) to act in the electron transport chain at a link (Slater factor) between the system involving DPN and diaphorase and the system involving succinic dehydrogenase and cytochrome *c* (49). Table VII gives the results of the effects of graded amounts of antimycin A on the succinoxidase activity of the intact mitochondria and of the 105,000 *g* pellet. When the amount of pellet material was increased to give succinoxidase activity in the same range as that obtained from the intact mitochondria (*cf.* reference 32), the antimycin titer (32) for both preparations was very similar.

The results of the experiments with Al^{+++}, Ca^{++}, cytochrome *c*, and antimycin A indicate that the succinoxidase of the isolated membrane pellet has in general the same relative concentrations of the various components making up the complex, as does the succinoxidase in the intact mitochondria. In other words, the system is apparently recovered intact in the membrane fraction.

In an endeavor to obtain a membrane fraction free of contaminating nucleoprotein particles (believed to be of microsomal origin), mitochondria from rat kidney cortex (having comparatively little endoplasmic reticulum which might act in the centrifuge as a carrier for the RNA granules) were isolated in the same way as liver mitochondria. In the kidney cortex, the elongated mitochondria contain more cristae than do liver mitochondria and are arranged in closely spaced arrays separated by deep invaginations of the cell membrane. Unfortu-

[6] It was found (42) that when mitochondria, isolated from water homogenates, were washed with saline much more cytochrome *c* was removed from them than when they were washed with water.

nately, while electron micrographs of intact kidney cortex mitochondria showed very little microsomal contamination, they showed a distinct contamination of membranous material believed to be derived from the cell membrane. When the kidney cortex mitochondria were treated with 0.3 per cent DOC and the fractions of the disrupted mitochondria separated as before, the isolated 105,000 *g* pellet contained about one-third of the total succinoxidase and cytochrome oxidase activity. Electron micrographs of this pellet showed only small and large membrane vesicles, with no nucleoprotein granules. Despite the fact that membranous contamination was present, the specific activity of both succinoxidase and cytochrome oxidase of this pellet was twice that of the intact mitochondria indicating the most of the vesicular material was of mitochondrial origin. It is of interest that succinoxidase and cytochrome oxidase of a kidney cortex mitochondria suspension were not inhibited at all by 0.3 per cent DOC, unlike the activities of liver mitochondria.

In order to determine whether similar membrane material could be obtained from mitochondria by means other than deoxycholate treatment, sonic treatment of liver mitochondria was tried. Mitochondria suspended either in 0.44 M sucrose or in water were treated in a Raytheon oscillator, Model S-101 at 10 kc./second for either 15 or 20 minutes. The disintegrated mitochondria were separated into fractions as before. The results obtained were very similar to those reported by Hogeboom and Schneider (9, 28): 20 per cent of the intact mitochondria succinoxidase and cytochrome oxidase activities came down at 105,000 *g*, but there was no concentration of enzyme activity in the pellet. Electron micrographs (2) showed a mixture of material in this pellet, as was to be expected, since 30 per cent of the mitochondrial protein came down in this fraction. However it was noted that these 105,000 *g* pellets from sonically disintegrated liver mitochondria contained vesicular material and in this respect resembled fractions from DOC-treated liver and kidney mitochondria. Thus, in these three cases, it would appear that succinoxidase and cytochrome oxidase were associated with vesicular membrane material, though only in the case of the DOC-treated liver mitochondria was the fraction pure enough to permit confident interpretation of the results.

DISCUSSION

Various means have been used to disrupt preparations containing mitochondria in an effort to solubilize the enzymes. Deoxycholate, cholate, or mixed bile salts have been employed in attempts to extract cytochrome oxidase (50–54), the cytochromes (52, 55), and succinic dehydrogenase (56–58), while other techniques have been used to bring a part of these enzymes into non-sedimentable form (9, 58–63). The difficulty in extracting these enzymes has prompted many workers to study them in particulate form (8, 9, 29, 64), following the classical pioneering work of Keilin and Hartree (7, 65, 43). These latter workers

found that by treating heart muscle with hypotonic conditions and with severe mechanical treatment, they could obtain a sedimentable fraction which exhibited all the properties of the intact succinoxidase complex. Thus, their preparation contained all the enzymes and cofactors which could oxidize succinate and transfer electrons to oxygen, the efficiency of the system depending on the "intactness of the colloidal structure which assures the mutual accessibility of the various enzymes involved" (43). After studying various morphological and enzymatic properties of the Keilin-Hartree preparation, Cleland and Slater (8) concluded that it consisted of small particles derived from fragments of the sarcosmal (mitochondrial) membranes.

A comparison of the properties of the Keilin-Hartree preparation with those of the sediment from deoxycholate-treated rat liver mitochondria indicates their great similarity. Both preparations contain the complete system which transports electrons from succinate to oxygen, but lack the phosphorylating activity associated with this transfer in intact mitochondria and are deficient in oxidative ability towards many of the other substrates of the Krebs's cycle. They differ in that the Keilin-Hartree preparation also contains fumarase activity and the enzymes which oxidize DPNH, while such activities were not found in the deoxycholate preparation. The properties of these preparations are also similar in general to preparations of this type obtained by other workers (9, 28, 62). Recently, using 1 per cent digitonin, Lehninger and coworkers (37, 66) have isolated a mitochondrial fragment containing oxidative phosphorylation properties and capable of oxidizing β-hydroxybutyrate, ferrocytochrome *c*, succinate, and DPNH, but incapable of oxidizing other Krebs's cycle substrates. Since this preparation gave a fivefold concentration of enzymatic properties and was high in phospholipide, it is probably very similar to the 0.3 per cent DOC preparation. Whether the additional enzymatic properties contained in the digitonin preparation are part of the mitochondrial membrane is yet to be demonstrated (*cf.* below). The major contribution of the combined morphological and biochemical effort described in this and the preceding paper (2) is the demonstration of the intimate association of the intact succinoxidase complex with mitochondrial membranes. Therefore, most probably the other succinoxidase preparations derive their activity from the presence of fragments of the mitochondrial membranes.

Of the enzyme systems tested, it can be stated unequivocally that the membrane pellet contains the complete succinoxidase system but lacks adenylate kinase and the enzyme which oxidizes exogenous DPNH. It must be emphasized that the absence in the membrane pellet of enzymes oxidizing other substrates of the Krebs's cycle does not mean that the enzymes are not a part of the intact mitochondrial membrane. The marked inhibition by DOC of the oxidation of these substrates by mitochondria prevents us from determining whether or not any of the enzyme complex from dehydrogenase through

flavoprotein has been dislodged from the membranes by the action of DOC (*cf.* reference 66). By the same reasoning, the absence of phosphorylating enzymes from the membrane pellet should not be construed to mean that these enzymes are not associated with the membrane in intact mitochondria (*cf.* reference 66 and below). Adenylate kinase, for example, might be held to the membrane by weak bonds since earlier metabolic data (26), indicated it to reside at the mitochondrial surface. On the other hand, DPNH-cytochrome *c* reductase might be held by stronger bonds which are disruptable by DOC, since in the digitonin preparations of Lehninger *et al.* (37, 66), this enzyme could be washed out by dilute digitonin solutions.

The role played by the phospholipides, (highly concentrated in the membrane fraction) on the structure and function of the succinoxidase complex is poorly understood. Edwards and Ball (67) found that a phospholipase inhibits succinoxidase much more than succinic dehydrogenase or cytochrome oxidase and came to the conclusion that the lipase interferes with the interaction of the various enzymes in the chain. Finding that lecithinase A markedly inhibits succinoxidase, with a much slower inhibition of succinic dehydrogenase and cytochrome oxidase, Nygaard and Sumner (68) came to the conclusion that lecithin may be a part of the component between succinic dehydrogenase and cytochrome *c*. There are indications (69) that lecithinase A also causes some disruption of the mitochondrial membranes. Ball and Cooper (44) believe phospholipide to be a cementing substance holding together the various components making up the complex. They found that 2 per cent DOC caused a dispersal of the various components of the complex. In the present work it is seen that a lower concentration of DOC leaves the complex relatively intact, while solubilizing about 80 per cent of the remainder of the mitochondrial protein. Furthermore, the inhibition produced by the lower concentration of DOC can be removed by washing, showing that no permanent dispersal or damage was done to the complex. Even with 0.5 per cent DOC, the mitochondrial phospholipide is still sedimentable at 105,000 *g* for one hour, while nearly all of the phospholipide of the microsomes is not sedimentable under these conditions (22), indicating a marked difference between these two alcohol-ether extractable phospholipides. All these results and interpretations are in agreement with the conclusions of the present paper, that the succinoxidase complex is part of the lipoprotein material comprising the mitochondrial membrane.

The localization of cytochrome oxidase in the membrane pellet gives rise to the question of how much of the total terminal electron transport in the intact mitochondria goes through the cytochrome system of the membrane fraction. It was thought (70) that succinate is oxidized *via* the chain: succinic dehydrogenase, cytochrome *b*, Slater's factor, cytochromes *c*, *a*, a_3, and oxygen. The DPN-linked dehydrogenases were thought (70) to be linked in a similar chain (dehydrogenase, DPN, diaphorase (or DPNH oxidase or flavoprotein), Slater's

factor, cytochromes *c*, *a*, a_3, and oxygen). Recent work by Chance and Williams (71) indicates, however, that the respiratory chain coupled to phosphorylation from substrate oxidation onwards consists of DPN, flavoprotein, cytochromes *b*, *c*, *a*, a_3, and oxygen. These authors are of the opinion that non-phosphorylating systems like the Keilin-Hartree preparation (and, presumably, the present mitochondrial membrane preparation) are artifacts in which cytochrome *b* is missing. Thus the electron transport chain from cytochrome *b* to oxygen might be common to both the intact succinic dehydrogenase system and the DPN-linked dehydrogenase systems. The evidence so far is therefore indicative (*cf.* reference 36) that the non-phosphorylating preparations are fragments of the entire phosphorylating electron transport mechanism in the intact mitochondria (*cf.* reference 66). It seems likely that the mitochondrial membrane preparation, described here, contains an integral part of the electron transport chain of the mitochondria. In view of the large amount of cytochrome oxidase recoverable in this fraction, it is suggested that all the electrons involved in mitochondrial oxidation go to oxygen *via* the terminal chain of components found in the membrane. Much of the phosphorylation coupled to substrate oxidation occurs during electron transport after dehydrogenase action (36, *cf.* reference 72), with one phosphorylation coupled to oxidation of reduced cytochrome *c* (73). Since the phosphorylating enzymes are intimately associated with electron transport, it is probable that these enzymes lie adjacent to the mitochondrial membrane, like adenylate kinase and DPNH-cytochrome *c* reductase (*cf.* above). The link of these phosphorylating enzymes to the membrane can be broken by DOC, but apparently not by digitonin (37, 66).

Since the ATP synthesized in the mitochondria is not stored by them (26, 74), the occurrence of the terminal electron transport system bound to the mitochondrial membrane, together with the phosphorylating system which may be attached to it in the intact mitochondria, would facilitate the rapid utilization of motochondrially synthesized ATP by other parts of the cell. The fact that the terminal electron transport chain may be part of an adhesive membrane structure and that the enzymatic activity of mitochondria can be experimentally modified by treatments affecting the membrane (Table VI, and reference 36), suggests that natural biological agents which affect the rate of metabolism may do so by modifying the structure of the mitochondria, and specifically, the structure of the mitochondrial membrane (*cf.* reference 75).

SUMMARY

1. Mitochondria isolated from rat liver were disrupted with 0.3 per cent deoxycholate and a number of subfractions were isolated from this preparation by differential centrifugation.

2. The protein N, RNA and phospholipide content, as well as the succinoxidase, cytochrome *c* oxidase, adenylate kinase, and DPNH-cytochrome *c* reductase of these fractions were determined.

3. Two of these subfractions, found to consist of mitochondrial membranes (2), contained ~ 12 per cent of the protein N and ~ 35 per cent of the phospholipide of the whole mitochondria and accounted for ~ 70 per cent of the succinoxidase and cytochrome *c* oxidase activity of the original mitochondrial preparation. There was no discernible adenylate kinase, DPNH-cytochrome *c* reductase, or phosphorylating activities in these fractions, nor could they oxidize other substrates of the Krebs's cycle.

4. The most active fraction (60 minutes at 105,000 *g* pellet) had a higher phospholipide/protein value than the whole mitochondria and showed a seven- to elevenfold concentration of succinoxidase and cytochrome *c* oxidase activities.

5. Evidence has been given to indicate that the various components of the succinoxidase complex are present in this membrane fraction in the same relative proportions as in the whole mitochondria.

6. The implications of these findings are discussed.

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