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PROTEOLIPIDS V. THE ACTIVITY OF LIPID CYTOCHROME *C*

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SUMMARY

Lipid-cytochrome *c* prepared according to the procedure of DAS AND CRANE[1] was found to be highly active in the transfer of electrons from ascorbate *via* tetramethyl-*p*-phenylenediamine and the cytochrome oxidase system to O_2. The proteolipid can be assayed in aqueous medium after microdispersion by sonication. An assay method is described in detail. Lipid-cytochrome *c* micelles aggregate at or below pH 5.5, and the aggregate has no detectable activity. The pH optimum of lipid-cytochrome *c* oxidation was found at pH 6.0. High ionic strength which decomposes the lipid complex also impairs the activity. These observations are discussed in terms of the phospholipid requirements of the cytochrome-oxidase reaction.

INTRODUCTION

The requirement for phospholipids in cytochrome *c* oxidase is well known. The work of TZAGOLOFF AND MCLENNAN[2], GREENLEES AND WAINIO[3] and BRIERLEY AND MEROLA[4] have established that the binding of cytochrome *c* by the oxidase is phospholipid dependent. This result has been further confirmed in our laboratory with a lipid-free cytochrome-oxidase preparation[9,10]. Recent evidence has indicated that both cytochrome *c* and cytochrome oxidase are highly lipophilic[1,2] in nature and can exist as lipoprotein complexes. This leads to the suggestion that the entire reaction may proceed in a lipid milieu in which the electron carriers are held in close proximity.

Studies by REICH AND WAINIO[5] and DAS AND CRANE[1] have shown that cytochrome *c* can react with acidic phospholipids and produce isooctane-soluble proteolipid. The conditions for synthesis of this proteolipid have been studied quite extensively by DAS, HAAK AND CRANE[6]. The phospholipids appear to bind with cytochrome *c* stoichiometrically. The ratio of cytochrome *c* to the amount of acidic phospholipid bound approaches the average number of charged groups on the protein. When neutral phospholipid is present along with the acidic lipid, it is added to the acidic lipid-cytochrome *c* complex in a defined proportion. It has also been proposed that cytochrome *c* *in situ* acts as a proteolipid. GREEN AND FLEISCHER[7] suggested that lipid-cytochrome *c* is the true electron carrier which acts in the electron-transport chain to shuttle electrons back and forth between complex III (reduced coenzyme Q–cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase).

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

In order to study the role of phospholipids, we have tried to pin down the exact location of the lipid effects. Previous results have been entirely based on studies with soluble (lipid-free) cytochrome *c* and lipid-containing cytochrome oxidase. In this communication, we report the activities obtained with lipid-cytochrome *c* and various cytochrome *c* oxidase preparations containing different levels of phospholipids. We found that a low amount of lipids initially associated with cytochrome oxidase provides the best condition for achieving maximum activity with the lipid-cytochrome *c*.

MATERIAL AND METHODS

Horse-heart cytochrome *c* (Type III) and beef-heart cytochrome *c* (Type V) were obtained from Sigma Chemical. Tetramethyl-*p*-phenylenediamine hydrochloride (TMPD) was purchased from Eastman Kodak. Triton X-114 and Triton X-100 were kindly provided by Rohm and Haas.

Phospholipid preparation

Beef-heart mitochondrial phospholipids were extracted from fresh beef-heart mitochondria using chloroform–methanol (2:1, by vol.) and chloroform–methanol–ammonia (7:1:0.5, by vol.) essentially according to the method of ROUSER AND FLEISCHER[8]. The pooled extracts were evaporated to dryness, taken up in a small amount of chloroform–methanol (2:1, by vol.) and filtered. The filtrate was evaporated to dryness again and redissolved in a minimum amount of the same solvent. The entire procedure was carried out under pure N_2 atmosphere.

Neutral lipids were removed by cold acetone precipitation. The lipid extract was added drop by drop to approx. 20 vol. of dry acetone at —20°. The yellow supernatant was decanted, and the precipitates were washed four times with 10 vol. of cold dry acetone. The phospholipids were collected by low-speed centrifugation, and homogenized with 0.01 M Tris–HCl buffer (pH 7.5) in a glass homogenizer fitted with a teflon pestle. The homogenate was placed in a stainless steel cup and cooled to 0°. Two drops of γ-tocopherol were added to slow down the peroxidation of the lipids. The entire apparatus was placed in a large plastic bag filled with pure N_2 and sonicated for 20 min (Bronson Sonifer, maximum output). During the sonication period, the content of the cup was cooled in a rotary ice bath, and pure N_2 was continuously supplied. The lipid micelles were then centrifuged at 108000 $\times$ *g* for 1 h and the precipitate discarded. The final preparation was kept under N_2 and used within 10 days.

Preparation of lipid-cytochrome c

Lipid-cytochrome *c* was prepared essentially according to the procedure of DAS AND CRANE[1] with slight modifications. A routine procedure is briefly summarized as follows: 1 μmole, cytochrome *c* (type III); phospholipid micelles suspension (32.0 μatoms phosphorus); 300 μmoles, Tris–HCl (pH 7.8); 50 μg of γ-tocopherol and 9.0 ml of absolute ethanol are mixed with enough distilled water to make a final volume of 30 ml. Ethanol is added with vigorous stirring to avoid local concentration. The mixture is placed in a 100-ml glass stoppered bottle and mixed with 30 ml of isooctane. The contents of the bottle are gassed with pure N_2 for 3 min before being shaken for

30 min in a reciprocal shaker. After the shaking period, the mixture is centrifuged at the maximum speed of a clinical centrifuge to separate the layers. Isooctane layer which contains the lipid-cytochrome *c* is removed for further processing.

Lipid-cytochrome *c* was solubilized by sonic microdispersion. The isooctane solution was first concentrated to a small volume (less than 1 ml) by vacuum evaporation. This solution was transferred to a glass homogenizer and the proteolipid was evaporated to dryness. 5 ml of 0.01 M Tris–HCl buffer (pH 7.5) were added, and the entire contents were thoroughly homogenized with a teflon pestle. The cytochrome *c* was then reduced by adding 50 μmoles of potassium ascorbate (pH 7.0) and sonicated under N_2 atmosphere for 3 min as described in the section on phospholipids. The final pink solution is clear with slight opalescence. Any insoluble material present at this point was removed by centrifugation (maximum speed in a clinical centrifuge). Lipid-cytochrome *c* can be stored under N_2 for 2 days without any effect on the activity. However, experiments reported in this paper were carried out with freshly prepared lipid-cytochrome *c*.

Preparation of cytochrome oxidase

Lipid-free soluble cytochrome oxidase was prepared by the method of SUN AND JACOBS[9] without further modification. The final preparation contained 8 nmoles of heme *a* per mg of protein and less than 2 % (by wt.) of phospholipids. The enzyme was dissolved in 1 % Triton X-100 solution buffered with 0.2 M potassium phosphate (pH 7.4) in a concentration approx. 2–4 mg/ml. It was further diluted to 10 μg/ml with sucrose phosphate buffer (0.25 M sucrose, 0.01 M potassium phosphate (pH 7.4)) before assay.

Lipid-containing membranous cytochrome oxidase and soluble cytochrome oxidase were made according to a previously published method[10]. Cytochrome oxidase purified by bile salts was prepared by the method of FOWLER, RICHARDSON AND HATEFI[11]. All enzyme preparations were diluted to 10 ug/ml with 0.25 M sucrose; 0.01 M potassium phosphate buffer (pH 7.4) before assay.

Analytical methods

Cytochrome-oxidase activities were measured polarographically with a GME oxygraph fitted with a Clarke electrode covered with a teflon membrane. The reactions were carried out at 37° at pH 6.0. The assay mixture contained per ml: potassium phosphate, 10.8 μmoles; potassium citrate, 6.5 μmoles; EDTA, 0.108 μmole; cytochrome *c*, 15 μmoles; TMPD, 1.11 μmoles; potassium ascorbate, 8.33 μmoles; and cytochrome-oxidase protein in the range of 1–10 μg. The final volume was kept at 1.8 ml. The mixture of all components except the enzyme was added to the oxygraph cell and allowed 3 min for temperature equilibration. The enzyme was added to start the reaction. In order to obtain consistent results, the oxygraph cell was cleaned with chromic acid and the teflon membrane was changed every week.

The concentrations of cytochrome *c* and cytochrome *a* were determined by their differential spectra. The millimolar differential extinction coefficients were taken to be $\Delta\varepsilon$ 550—525 as 25.1 and $\Delta\varepsilon$ 605—630 as 13.1 for heme *c* and heme *a*, respectively[12]. Phospholipids were analyzed by thin-layer chromatography quantitatively after phosphorus analyses according to the method of BARTLETT[13]. Protein was analyzed by the modified biuret method of YONETONI[14].

In view of the small variations among different batches of cytochrome-oxidase preparations, experiments in which comparisons were made were carried out with one batch of cytochrome-oxidase preparation. Each set of experiments was repeated at least three times with different batches of cytochrome oxidase and cytochrome *c*. Control values of cytochrome-oxidase activity were always obtained for each batch of the enzyme.

RESULTS

The activities of lipid cytochrome c

The activities of six different forms of purified cytochrome oxidase with lipid-cytochrome *c* micelles as substrate are shown in Table I. The proteolipid demonstrated very high electron transfer capabilities in all cases. The effect is especially pronounced in the two lipid-deficient preparations where soluble cytochrome *c* would not function in the absence of phospholipids. In other cases, the physical state of the enzyme has an influence on the rate. It would appear that large-sized, proteolipid micelles may impede the interaction of the reactants and render the lipid-cytochrome *c* less active than its soluble form.

TABLE I

ACTIVITIES OF LIPID CYTOCHROME *c*

Rates are expressed in μmoles of O_2 uptake per min per mg of enzyme protein.

Form of cytochrome c	*Type of preparation of cytochrome oxidase*	*Activity*		K_m (μM)
		15 μM	*Infinite*	
Proteolipid	Lipid-free cytochrome oxidase (Triton)[9,10]	32.00	47.70	5.00
Free form	Lipid-free cytochrome oxidase (Triton)	5.00	5.12	2.50
Free form	Lipid-free cytochrome oxidase (Triton) *plus* phospholipids	26.38	31.51	1.87
Proteolipid	Lipid-deficient cytochrome oxidase preparation C (cholate phospholipase)[2]	7.49	—	—
Free cytochrome *c*	Lipid-deficient cytochrome oxidase preparation C (cholate phospholipase)	1.45	—	—
Free cytochrome *c*	Lipid-deficient cytochrome oxidase preparation C *plus* phospholipids	2.89	—	—
Proteolipid	Membranous cytochrome oxidase (Triton)[10]	6.04	8.40	7.50
Free cytochrome *c*	Membranous cytochrome oxidase	4.85	5.16	1.13
Proteolipid	Soluble lipid containing cytochrome oxidase (Triton)[20]	22.23	31.20	4.70
Free cytochrome *c*	Soluble lipid containing cytochrome oxidase (Triton)	39.72	42.30	1.32
Proteolipid	Soluble lipid containing[21] cytochrome oxidase (deoxycholate)	26.60	—	—
Free cytochrome *c*	Soluble lipid containing cytochrome oxidase (deoxycholate)	30.30	—	—

The kinetics of the lipid-cytochrome *c*-catalyzed oxidation is not different from that of the soluble cytochrome *c*. The polarographic trace recording the time course showed a straight line until the O_2 tension in the cell is nearly exhausted. However, due to the slow response of the membrane-covered Clarke electrode, there was approximately 5–10 sec lag time before the rate reached steady state after the enzyme was introduced.

Maximal rate obtained with lipid cytochrome *c* was highly dependent on the quality of the proteolipid. Properly prepared lipid cytochrome *c* will not catalyze the non-enzymatic oxidation of ascorbate appreciably. If the aqueous suspension of the proteolipid has been aged or contaminated, rapid O_2 uptake occurred in the absence of the enzyme and the rate thus obtained is no longer indicative of the enzyme-catalyzed reaction.

TABLE II

EFFECT OF THE PHYSICAL STATE OF LIPID-CYTOCHROME *c* ON ITS ACTIVITY

Activities are expressed in μmoles of O_2 uptake per min per mg of enzyme protein. The concentrations of cytochrome *c* were set at 15 μM in all four cases.

State of cytochrome c	*Enzyme*	*Activity*
Micellar state lipid-cytochrome *c*	Lipid free	56.31
Aggregated state lipid-cytochrome *c*	Lipid free	5.70
Free cytochrome *c*	Lipid free	3.80
Free cytochrome *c*	Lipid free + phospholipids	32.43

The physical state of the lipid-cytochrome *c* in aqueous suspension has great effects on its reactivity. Microdispersed lipid-cytochrome *c* aggregates out of solution when the pH is brought below 5.5. The aggregated form is essentially inactive even after the pH is brought back to neutral. It is also suspected that the variation observed in some aged lipid-cytochrome *c* preparations was also due to the partial aggregation into large micelles which functioned inefficiently (*cf.* Table II).

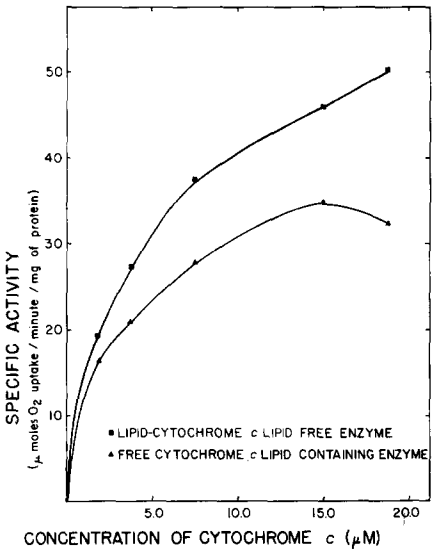
It is noteworthy that the lipid-cytochrome *c* micelles made by sonication exist in a quite stable state. Only a slight amount of precipitate was observed after 1 week of storage. Reduction of the cytochrome *c* with a small amount of potassium ascorbate (1 μM) helps to preserve the proteolipid in its micellar state. In this investigation, all experiments were carried out with fresh material. However, if the lipid-cytochrome-*c* suspension is to be preserved, it should be degassed and kept in Thunberg tubes under N_2 atmosphere.

The activity of lipid-cytochrome *c* decreased rapidly when kept in air. We have detected thiobarbituric-acid-positive material (malonaldehyde) in a 3-day-old suspension, which indicated peroxidation had taken plate. The addition of γ-tocopherol retards the peroxidation[15], but does not completely prevent it.

Effect of cytochrome-c concentration

In order to study the effect of each component in the reaction of lipid-cytochrome *c* and cytochrome oxidase, the lipid-free enzyme preparation made by the Triton X-114 procedure was selected because of its high activity. Control experiments using

soluble cytochrome *c* were also carried out. The lipid-free enzyme was allowed to incubate with enough micellar mitochondrial phospholipid to induce maximal activity when soluble cytochrome *c* was used. The effect of cytochrome-*c* concentration either in complexed form or in soluble form is shown in Fig. 1.



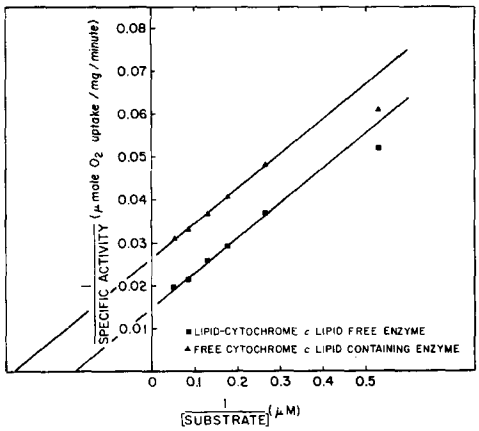


Fig. 1. Comparison of cytochrome *c*-oxidase activity with complexed lipid-cytochrome *c* and with cytochrome *c* and lipid added separately.

Fig. 2. Relation between substrate concentration and specific activity of cytochrome oxidase with complexed cytochrome *c* and free cytochrome *c* with lipid.

Lineweaver–Burk plots were used to study the effect of cytochrome *c* in this reaction. The result is indicated in Fig. 2. At cytochrome-*c* concentration below 15 μM, the rate increased as the concentration of cytochrome *c* increased. At high concentration of cytochrome *c*, the rate was slightly depressed. The apparent Michaelis constant for the lipid-cytochrome *c*–lipid-free cytochrome oxidase system was found to be higher than the free cytochrome *c*–lipid-enriched oxidase system. The extrapolated maximal rate, on the other hand, showed the opposite relationship. This indicated that the large size of the proteolipid micelles could cause a problem in entering the active sites, yet once they were attached, the lipid-cytochrome *c*–lipid-free oxidase system was much more effective in carrying out the electron-transfer process. This observation is also in good agreement with the concept postulated by OKUNUKI[16] that the active species in this reaction is the enzyme–substrate complex consisting of 1:1 molar ratio of cytochrome *c* and cytochrome oxidase.

The effects of other reactants

The concentration of ascorbate in the reaction mixture has been shown to influence the rate of oxidation of cytochrome *c* in the absence of TMPD. However, with the catalytic amount of TMPD added, we have found that the level of ascorbate does not affect the rate. Both lipid-cytochrome *c* and soluble cytochrome *c* are reduced instantaneously by the ascorbate–TMPD system. In the absence of TMPD, the re-

duction of lipid-cytochrome *c* is so slow that the rate of the enzyme reaction is greatly reduced. In this study, we have routinely used potassium ascorbate at concentrations between 0.5 to 1.0 μM. If the ascorbate concentration is too high, the rate is slightly depressed. This may be caused by the high ionic strength which leads to the dissociation of the enzyme–substrate complex.

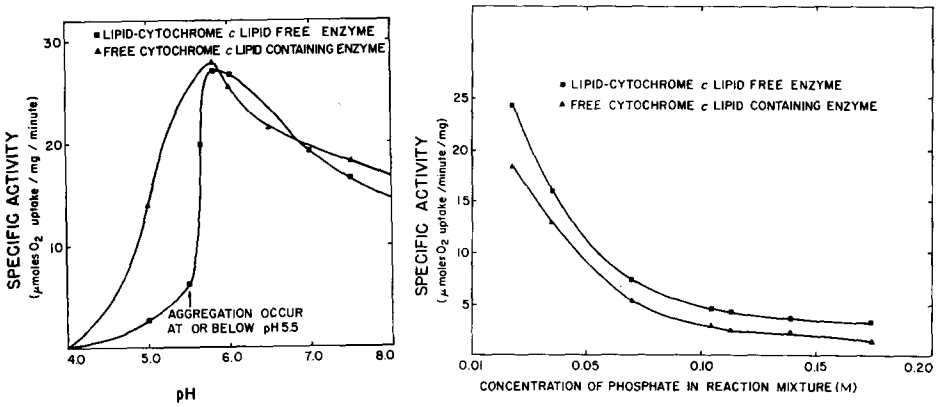


Fig. 3. Effect of pH on cytochrome oxidase with complex and free cytochrome *c* as substrate.

Fig. 4. Effect of ionic strength on cytochrome-oxidase activity using complex and free cytochrome *c*.

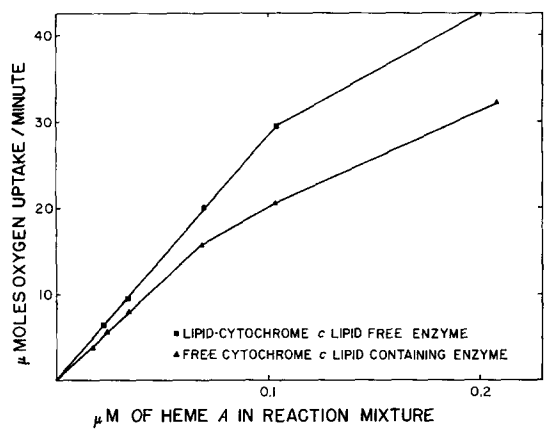


Fig. 5. Effect of cytochrome-oxidase concentration on rate of cytochrome *c* oxidation with lipid-bound and free cytochrome *c*.

Fig. 3 shows the effect of pH on the oxidase reaction using both lipid and free cytochrome *c* as substrate. The maximal rate was obtained between pH 5.7–6.0 in both cases. At pH below 5.5, lipid-cytochrome-*c* micelles aggregate, and the rate rapidly decreases to a very low level.

The effect of ionic strength of this reaction is plotted in Fig. 4. At high concentration of potassium phosphate, the reaction declined in both cases. This is inconsistent with the work of WAINIO AND MCGUINNESS[17] and DAVIS, SMITH AND WASSERMAN[18] using free cytochrome *c* and a lipid-containing enzyme.

Fig. 5 shows the effect of cytochrome-oxidase concentration on the reaction

rate. With lipid-cytochrome *c* as substrate, the rate is proportional to the enzyme concentration up to 100 μM.

The effect of ethanol in the preparation of lipid-cytochrome c

MARGOLIASH AND LUSTGARTEN[19] have reported that ethanol at 60% was able to convert monomer cytochrome *c* to its polymeric derivatives. The cytochrome *c* polymers reacts with both O_2 and CO and has considerably less activity than the monomeric form in the cytochrome oxidase system. Since 30% of ethanol was routinely used to prepare lipid-cytochrome *c*, a control experiment was carried out to see if ethanol has any effect in the preformed proteolipid. As shown in Table III lipid-cytochrome *c* made in the absence of ethanol has approximately the same reactivity as the same material made in the presence of ethanol. Furthermore, the lipid-cytochrome *c* made with ethanol added did not show any higher rate of autooxidation than native soluble cytochrome *c*. Therefore, it is concluded that cytochrome *c* in the lipid complex is not in the polymeric form. It is possible that phospholipids in the reaction mixture may have protective effect against polymerization under the condition cited.

TABLE III

EFFECT OF ETHANOL ON THE PREPARATION OF LIPID-CYTOCHROME *c*

Activities are expressed in μmoles of O_2 uptake per min per mg of protein.

Preparation	*Activity*		K_m (μM)
	15 μM	*Infinite*	
Lipid-cytochrome *c* prepared with 30% of ethanol added to the reaction mixture. Lipid-free enzyme	38.20	48.7	4.88
Lipid-cytochrome *c* prepared in the absence of ethanol. Lipid-free enzyme	30.38	39.0	4.88
Free cytochrome *c*. Lipid added to the enzyme preparation	26.55	33.7	1.87

TABLE IV

ACTIVITY DIFFERENCES BETWEEN HORSE-HEART AND BEEF-HEART CYTOCHROME-*c* PREPARATIONS

Rates are expressed in μmoles of O_2 uptake per min per mg of enzyme protein.

Species	*Form of cytochrome c and cytochrome oxidase*	*Activity*		K_m (μM)
		15 μM	*Infinite*	
Beef	Lipid-cytochrome *c*. Lipid-free cytochrome oxidase	24.83	35.00	5.7
Beef	Free cytochrome *c*. Lipid-free enzyme *plus* phospholipids	17.94	20.20	1.77
Horse	Lipid-cytochrome *c*. Lipid-free cytochrome oxidase	17.86	23.80	4.88
Horse	Free cytochrome *c*. Lipid-free enzyme *plus* phospholipids	17.13	19.50	1.87

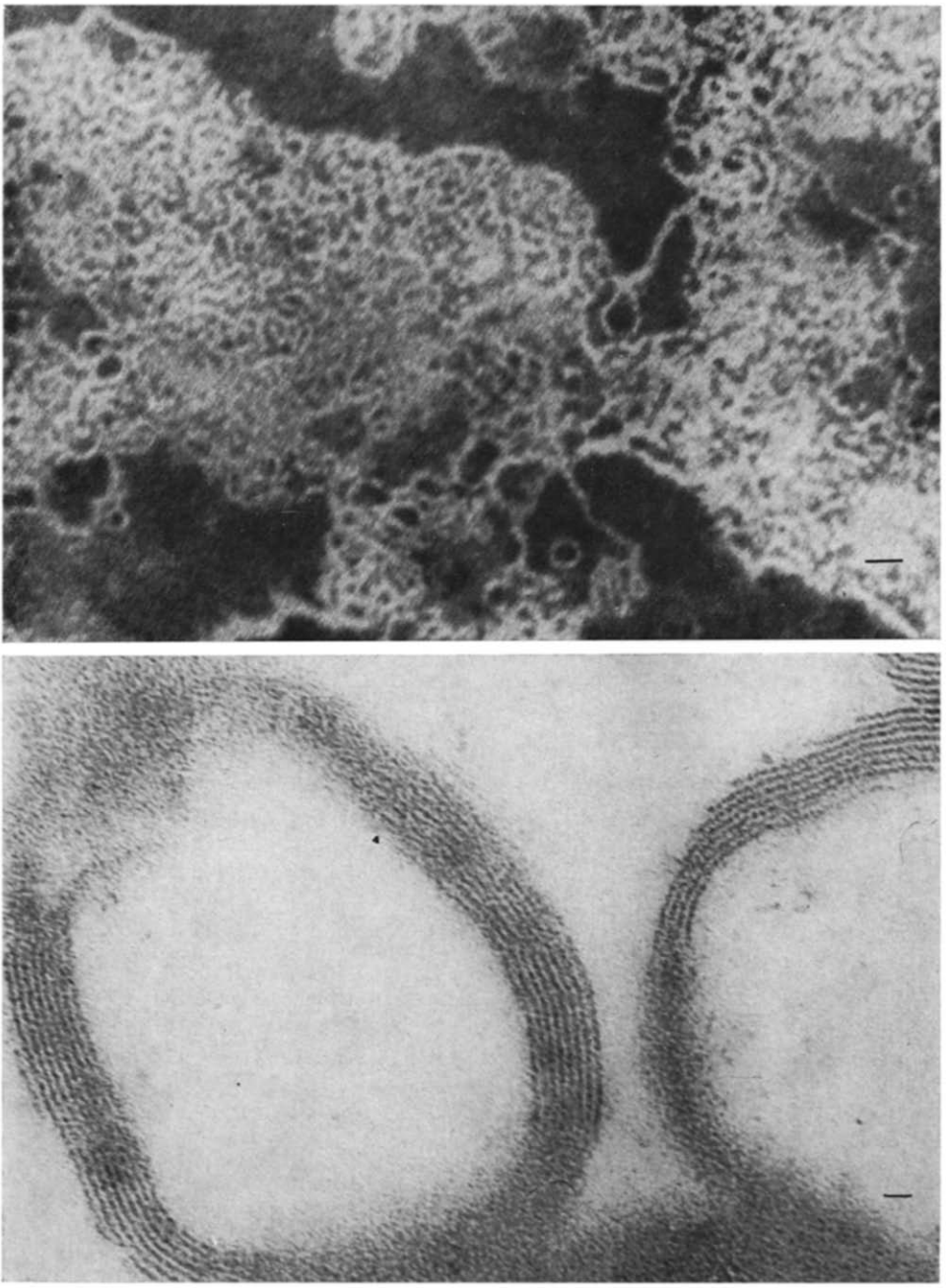


Fig. 6 (top). Lipid-cytochrome *c* complex negatively stained with phosphotungstate, pH 6.8. Marker indicates 200 Å.

Fig. 7 (bottom). Lipid-cytochrome *c* complex fixed for 2 h with OsO_4 and sectioned. Marker indicates 200 Å.

Differences in activity of cytochrome c preparations

It has been observed that there is no gross difference in the electron-transport capacities of various mammalian cytochrome. The same result was demonstrated with both beef-heart cytochrome *c* and horse-heart cytochrome *c* in the soluble form. However, when beef-heart cytochrome *c* was used to make the lipid complex, the resulting proteolipid demonstrated considerably higher activities than the horse-heart cytochrome *c* (*cf.* Table IV). On the other hand, the apparent Michaelis constants for both species are approximately identical. It appears that beef-heart cytochrome *c*–lipid complex is more efficient when the cytochrome oxidase from the same species is used, while the affinities of both lipid-cytochrome *c* are the same. One would be tempted to suggest that there is a species discrimination. However, factors in the preparation of the cytochrome *c* could also affect the activity, we feel that more experiments would be needed to firmly establish this point.

The effect of inhibitors

The activity obtained with lipid-cytochrome *c* as substrate is fully sensitive to conventional inhibitors such as CN^-, azide and NH_2OH. Complete inhibition was obtained with 1 mM KCN, 1 mM NaN_3, or 10 mM $NH_2OH \cdot HCl$. The same inhibitors also block the activity of free cytochrome *c* and lipid-containing cytochrome oxidase at equivalent concentration. This experiment ruled out the possibility that the observed O_2 uptake was caused by some unexpected nonenzymatic oxidation reactions.

Structure of lipid-cytochrome c

The micellar structure of lipid-cytochrome *c* is apparent in electron-microscope studies. Fig. 6 shows lipid-cytochrome *c* by negative staining with phosphotungstate. Fig. 7 shows a section of lipid-cytochrome *c* which has been fixed with OsO_4 and sectioned. Note that the interperiod spacing is 65 Å, which is thicker than the 40-Å spacing characteristic of pure phospholipid micelles.

DISCUSSION

The high efficiency of lipid-cytochrome *c* in transfer of electrons from artificial donors to cytochrome oxidase and O_2 strongly supports the thesis that this proteolipid may be the functioning form of cytochrome *c* in electron-transport chain. Since both cytochrome c_1 and cytochrome oxidase have been shown to exist in the form of lipoprotein, it is obvious that the intermediate electron carrier should take the same lipophilic form in order to attain maximal interaction. It is possible that during different phases of electron transport, there may be a shift in the functional form of cytochrome *c* from lipid bound to water soluble.

It should be pointed out that even the lipid-free cytochrome oxidase used in this study is not free of detergent molecules. The enzyme we used was dissolved in 1 % of Triton X-100 in a concentration of 2–4 mg per ml which was approx. 3 mg of Triton per mg of protein. We have found[20] that if the concentration of Triton is decreased, the activity obtained with lipid-cytochrome *c* declines accordingly. Activity can be raised in this lipid-free, detergent-deficient enzyme by the addition of phospholipid micelles, Triton X-100, potassium deoxycholate, or Emasol 4130. This indicated

that lipid material is also required on the cytochrome-oxidase side even when lipid-cytochrome *c* is used as substrate. In other words, addition of lipid-cytochrome *c* to the phospholipid-free cytochrome oxidase does not constitute an active system unless there is enough lipid material to hold the enzyme in the correct conformation. This observation also rules out the possibility that the activity obtained with lipid-cytochrome *c* was actually due to the transfer of phospholipids from lipid-cytochrome *c* to the oxidase. We believe that in native system, phospholipids have a dual action on this part of the chain, namely (1) to facilitate the interaction of cytochrome *c* and the enzyme, and (2) to maintain proper conformation of the cytochrome oxidase so it interacts with cytochrome *c* maximally. The first requirement was specific and cannot be replaced by detergents. The secondary requirement is nonspecific, and can be fulfilled by Triton or other detergents. A detail treatment of this additional lipid requirement will be presented in a later communication.

The inefficiency of lipid-cytochrome *c* in lipid-rich cytochrome oxidase was probably due to the unfavorable physical state of the enzyme. It has been shown in this laboratory that lipid-rich cytochrome oxidase tends to exist in membranous form which is not as active as the dispersed 'soluble' form of the enzyme. The membranous cytochrome oxidase shows only one tenth of the activity as the soluble dispersed oxidase as indicated in Table I. Thus the reaction of lipid-cytochrome *c* with lipid-rich cytochrome oxidase can be visualized as lipoprotein–lipoprotein interaction between two micellar phases. This interaction would obviously be affected by the size and geometry of the individual micelles. The use of lipid-free cytochrome oxidase reduces the number of variables involved in the assay by eliminating the problem of aggregation of the oxidase.

It is noteworthy that the lipid-cytochrome *c* prepared according to DAS AND CRANE[1] had a definite molar ratio of lipid phosphorus and heme which approached 32:1. Among the thirty batches of lipid-cytochrome *c* preparations used for this study, the ratio has been in the range from 32:1 to 40:1. The fresh sonic dispersed micellar proteolipid shows only one major band when eluted from an Agarose Bio-Gel A-50m column. This indicated that the micelles had uniform size. Under the electron microscope, the lipid-cytochrone *c* micelles appear as lamellar micelles both under negative staining and in sections similar to phospholipid micelles[21].

The center to center spacing of the dark bands in sections is 65 Å which is greater than the characteristic spacing of pure phospholipid micelles[21]. The addition of cytochrome *c* to the micelle thus appears to increase the spacing between phospholipid molecules.

From this study, it is clearly demonstrated that lipid-cytochrome *c* is active in the transfer of electron from ascorbate and TMPD to O_2 through the cytochrome-oxidase system. It remains to be shown if this active lipid-cytochrome *c* has unique properties as an electron carrier in the electron-transfer chain in mitochondria.

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