THE ROLE OF SOLUBLE LIPID IN MITOCHONDRIAL ENZYME SYSTEMS*

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Basford and Green (1959) prepared from mitochondria a soluble preparation composed of 96% lipid and 4% protein which was referred to as the Q lipoprotein (Q-LP), by virtue of its CoQ content (0.3-0.6%). Quite apart from its water solubility (a remarkable property for a predominantly lipid system) this preparation showed high activity in reactivating various submitochondrial fractions (Hatefi, 1958, 1959; Lester and Fleischer, 1961).

By modification of the Basford and Green procedure, the protein content of the final preparation could be lowered to less than 0.5% protein and it thus became clear that the soluble state of the lipid could not be referred to the protein. Indeed, we were able to solubilize protein-free lipid directly by applying the same procedures that were used in the preparation of Q-LP from mitochondria. The following is one such procedure. Mitochondrial lipid*** (25 mg) is dissolved in 1 ml of a mixture containing butanol and 20% potassium cholate (pH 7.5) or deoxycholate) in a ratio of 87:13 (v/v) and the solution is dialyzed on a rocker-dialyzer against at least 100 volumes of a solution (pH 8.0), 0.02 M with respect to Tris-acetate and 0.001 M with respect to EDTA for 7 days at 5°C (the dialysis fluid being changed each 24 hours). The resulting solution of mitochondrial lipid which is clarified by

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***Mitochondrial lipids were obtained by extracting beef heart mitochondria by the ethanol-ether extraction method of Hanahan (1957) or by the method of Folch (1957) as modified by Rouser, et al. (1961). The modifications introduced by Rouser, et al. insure more complete extraction and minimize lipid peroxidation.

centrifugation for 30 min. at 35,000 rpm in the Spinco ultracentrifuge shows the same two properties which seemed to be unique to the Q-LP: (1) solubility in water; and (2) the capacity to reactivate various submitochondrial enzyme systems.

The solutions of solubilized lipid are water clear or faintly opalescent. The ratio of the optical density values at 500 and 700 mμ respectively (Table I) were found to approximate the theoretical ratio calculated from the Raleigh Rule for light scattering.

TABLE I

Optical Density of Solubilized Lipids*

Solubilized Lipid**	OD_{700}	OD_{500}	$\frac{OD_{700}}{OD_{500}}$***	μg P/ml
Mitochondrial phospholipids	.075	.273	0.27	295
Asolectin	.058	.217	0.27	248
Lecithin	.026	.083	0.31	279
Cardiolipin	.007	----	----	341

* The amount of opalescence is somewhat variable. These optical densities represent typical values. The optical densities were measured in a Beckman DU spectrophotometer with a slit width of 0.035 mm.
** Asolectin (soybean phosphotides) was first obtained as a gift from Dr. George Rouser, City of Hope Medical Center, Duarte, California, and later from Associated Concentrates, Woodside, Long Island. The lecithin and cardiolipin was obtained from the Sylvana Chemical Company, East Orange, New Jersey.
***According to the Raleigh Rule, ($K = C/\lambda^4$, K = extinction due to light scattering; C = scattering constant; λ = wavelength in mμ), for scattered light the ratio of $\frac{OD_{700}}{OD_{500}}$ should equal $\frac{(500)^4}{(700)^4} = 0.26$.

The activity of soluble mitochondrial lipid as well as of soluble phospholipid from the soybean (asolectin) was tested in three enzyme systems: (1) reduced coenzyme Q oxidase (Table II); (2) cytochrome c oxidase (Table II); and (3) succinate-cytochrome c reductase (Figure 1). The two soluble lipid preparations were as active as Q-LP in restoring the full catalytic capacity of these three systems and showed their activity at about the same concentra-

TABLE II

Reactivation of Cytochrome c Oxidase and Reduced CoQ Oxidase with Solubilized Lipid

Additions	μMoles cytochrome c oxidized/min/mg	μMoles QH_2 oxidized/min/mg
None	21	.081
Q-L.P.	62	.260
"Solubilized" mitochondrial lipids	64	.280
"Solubilized" asolectin	58	.230

The assay conditions for and the preparations of cytochrome c oxidase and reduced coenzyme Q oxidase are described by Griffiths and Wharton (1961) and Hatefi (1959) respectively. One mg of lipid was used for the reactivation of 130 mμmoles of cytochrome oxidase (20 micrograms) in 1.5 ml. We are indebted to Drs. D. E. Griffiths and D. Wharton for performing the cytochrome c oxidase assays. In the reduced CoQ_{10} oxidase assay, 50 μg of lipid were used to fully reactivate 170 μg of the enzyme preparation.

tion levels. It is to be noted that the restored activities of the three test systems were inhibited by the specific inhibitors for the particular activity (antimycin for succinate c reductase activity, etc.).

These data show clearly that the active component in Q-LP is soluble phospholipid and that the mixture of soluble phospholipid and coenzyme Q can duplicate the effect of Q-LP in the succinic-cytochrome c reductase assay system (Figure 1). Thus, it is neither the residual protein in Q-LP nor the coenzyme Q which have unique properties, but rather the soluble phospholipid. The reactivation of the three submitochondrial enzyme systems is not specific for mitochondrial phospholipid since solubilized asolectin (soybean phosphatides) works equally well. Solubilized lipid is effective in relatively small amounts because the lipid is readily accessible to the particulate preparations which were used in the assays. Homogenized asolectin which forms a milky, turbid solution is not very effective in small quantities (Figure 1). Lester and Smith (1961) in our laboratory have found that lipid sonicated almost to clarity (Rouser, 1958) is as effective as soluble lipid or the Q-LP. It should be mentioned that as early as 1930 Thierfelder and Klenk (1930) were

aware that plant phospholipids could form soluble aqueous solutions under appropriate conditions.

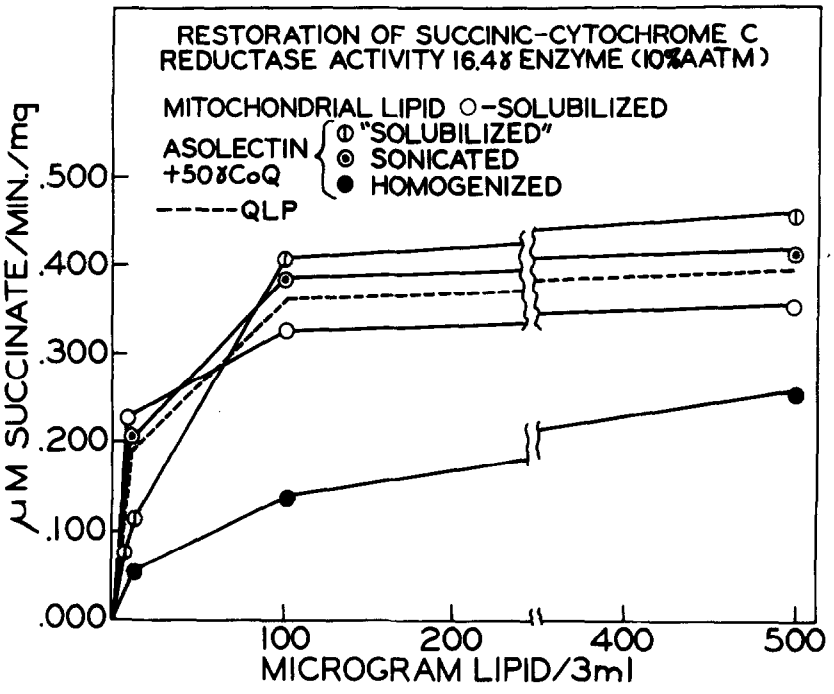

Fig. 1. Reactivation of succinate-cytochrome c reductase activity of aqueous acetone treated mitochondria (AATM) by solubilized lipids. The assay conditions, as well as the preparation of AATM are described by Lester and Fleischer (1961). Aqueous acetone treatment of mitochondria removes 75-80% of the mitochondrial phospholipid, as well as all of the CoQ_{10}. Both lipid and CoQ_{10} are therefore required for reactivation. CoQ is present in solubilized total mitochondrial lipid, since CoQ is solubilized in the presence of phospholipids (Fleischer and Brierley, 1961). Where there is no CoQ solubilized together with the lipid (asolectin or mitochondrial phospholipid devoid of neutral lipid) CoQ_{10} must be added separately to the assay mixture. Micromoles is abbreviated as μM.

Solubilized lipids can be stored at 0-4° C for considerable periods without significant deterioration. Prolonged storage causes some deposition of the lipid. Several samples recentrifuged after six months storage retained about 70% of the original lipid in solution and still activated the enzyme preparations.

After dialysis of solubilized mitochondrial lipid for seven days, the concentration of residual cholate is 5.3 mg per 100 mg of lipid. This amount is not reduced appreciably by further dialysis (respectively, 4.9 and 4.6 mg per 100 mg of lipid after 14 and 20 days of dialysis). The concentration of

cholate was determined by measurement of radioactivity. These studies were carried out with C^{14}-cholate, a gift from Dr. S. Bergstrom to Dr. Helmut Beinert. No detectable lipid (measured as phosphorus) is lost during the dialysis procedure.

The extent to which lipid can be solubilized in water is a function of the nature of the lipid preparation. It is not unusual to be able to solubilize 80-100% of mitochondrial and soybean phospholipids. The rocker motion of the dialysis tubing is most critical at the start when the butanol layer decreases in volume and is being replaced by water. Individual phospholipids from mitochondria [lecithin, phosphatidyl ethanolamine, cardiolipin, and phosphatidyl inositol (still contaminated with cardiolipin)] have been effectively solubilized by the butanol-cholate procedure described above, but neutral lipids (such as coenzyme Q and cholesterol) are refractory in this regard unless solubilized in the presence of phospholipids (Fleischer and Brierley, 1961). Solutions of solubilized phospholipids can readily be concentrated by dialysis against polyvinylpyrolidone.

When phospholipids of natural origin are hydrogenated, the capacity to form micelles (or at least micelles sedimentable only in a strong gravitational field) is greatly reduced if not abolished. A sample which contained 2.6 μmoles double bond per mg could be solubilized to the extent of 90% whereas the same sample after hydrogenation (0.33 μmoles double bond/mg, i.e., 13% residual unsaturation) could be solubilized only to the extent of 9%. Rouser (1958) has made similar observations on phosphatidyl ethanolamine. The biological significance of the occurrence of unsaturation in mitochondrial phospholipids will probably find its explanation in terms of the physical orientation of the lipid within the mitochondrion.

The sedimentation characteristics of solubilized lipid strongly suggest that the dispersion of lipid in such preparations comes close to (if it does not reach) the stage of individual molecules and that lipid no longer exists as a bulk phase. While it is true that the individual molecules of phospho-

lipid are held together in micelle formation in preparations of soluble lipid, this type of molecular association is utterly different from that which obtains in bulk phase. The lipid in effect is water soluble (i.e., manageable biochemically) and permits exceedingly rapid equilibration with the external phase -- a property of the greatest biochemical importance. The possibility that mitochondrial lipid (> 90% phospholipid) exists in a similar oriented, micellular state requires serious consideration.

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