PROTEOLIPIDS III. PROTECTION OF LIPID-CYTOCHROME C BY AN ANTIOXIDANT FRACTION FROM BEEF HEART

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Received September 15, 1964

When complexes are prepared between cytochrome c and crude phospholipids as previously described (Das et al. 1962) they retain their characteristic properties and enzymatic activity for weeks. When purified phospholipids are used for formation of the complexes, there is a rapid change from soluble to insoluble form with decline of enzymatic activity in a few days and along with this change malonaldehyde appears as an indication of lipid peroxidation. We would now like to report that we have isolated a lipid material from the crude phospholipid fraction which protects the purified phospholipids from peroxidation. This compound is even more effective than α-tocopherol in protecting the cytochrome c - phospholipid complex from peroxidation and loss of enzyme activity. The active compound is purified from an ethanol - ether (2:1) extract of heart muscle lipids. The ethanol ether extract is reduced to small volume under vacuum and the lipids extracted into petroleum ether. Phospholipids are removed by precipitation in acetone at - 15° C overnight. The supernatant remaining after removal of phospholipids is taken up in chloroform after removal of acetone under vacuum and placed on a silicic acid - supercell column. Elution with chloroform is continued until the first yellow band is completely removed. This eluate is evaporated and taken up in acetone. The acetone solution is cooled in an acetone-dry ice bath and then rapidly filtered to remove white material. This process is repeated to remove as much of the sterols and fats as possible.

Supported in part under grant AM 04663 from the National Institutes of Health.

The amber colored supernatant is then chromatographed on thin-layer silica gel G in long strips using N-heptane-ether (170:30, V/V). When the plate is protected except for a small spot on one side and is sprayed with Emmerie-Engel reagent (1938) as many as six compounds which reduce ferric chloride are visualized. After the removal of acetone from the amber colored supernatant, the residues were taken up in benzene. The benzene solution was then placed on a florisil column. Elution of this column with benzene removes the active fraction. This eluate was now chromatographed again on thin-layer plates in the same system as before. Only one Emmerie-Engel spot appears corresponding to α-tocopherol, but several spots appear when the plate is sprayed with phosphomolybdic acid. The phosphomolybdate-positive fraction with an R_f of 0.63 contains most of the active material although some activity has been found in a compound at R_f 0.90. The section of silica gel containing the active material is eluted into ethanol.

The active material showed a broad absorbance band in the UV with maximum between 271-274 mμ ($E^{1\%}_{1cm}$ = 4.9). The purified fraction does not give Emmerie-Engel reaction.

When a purified sample of beef heart phosphatidyl-ethanolamine prepared by the method of Hanahan et al. (1957) is sonicated in water it forms a finely dispersed suspension. When this sonicated suspension is left at 0° C for 6 days there is steady increase in peroxidation of the lipid as measured by the thiobarbituric acid test (Tappel and Salkin 1959). If α-tocopherol or the active lipid fraction described above is added to the phospholipid suspension the rate of peroxidation is reduced. An even better protective effect is given by γ-tocopherol which is one of the Emmerie-Engel-positive components found on the first thin-layer chromatogram (cf. Table I). On the other hand we do not find any γ-tocopherol in the lipids from the heart mitochondria, but only in the lipids from whole heart.

When a sonicated suspension of phosphotidylethanolamine-cytochrome c complex is prepared (Brierley and Merola 1962) and stored there is an even

Table I

Effect of the Antioxidant Lipid Fraction and α-tocopherol in Protection of Phospholipid and the Cytochrome c Complex from Peroxidation

Form of Phospholipid	Addition*	TBA values after storage ** 1 day	3 days	6 days
Phosphotidylethanolamine (PE)	none	0.140	0.230	0.300
"	α-tocopherol	0.110	0.150	0.190
"	antioxidant	0.110	0.170	0.190
"	γ-tocopherol	0.070	0.085	0.100
Lipid cytochrome c (PE)	none	0.190	0.270	0.330
"	α-tocopherol	0.150	0.200	0.270
"	antioxidant	0.130	0.150	0.150
"	γ-tocopherol	0.070	0.080	0.100

*Addition to phospholipid at the start of the storage period. Tocopherols at 4 μgrams per ml per 60 μgrams phospholipid phosphorus. Antioxidant fraction added equivalent to the amount obtained from a portion of the original extract containing 6 μgrams of α-tocopherol.

**Thiobarbituric acid test: Values given as absorbancy at 525 mμ for 10 μgrams phospholipid phosphorus.

faster rate of peroxidation than with purified phospholipid alone. This peroxidation rate is decreased by addition of α-tocopherol, and is almost eliminated by the addition of the active lipid.

The freshly prepared phosphotidylethanolamine-cytochrome c complex is soluble in isooctane. Whe peroxidation occurs the complex becomes insoluble in isooctane. Both α-tocopherol and the active lipid prevent conversion to the insoluble form in proportion to their antioxidant activities.

When a purified cytochrome c oxidase preparation is extracted with acetone according to the method of Brierley and Merola (1962) addition of phospholipid is necessary for the restoration of activity. As phosphotidylethanolamine becomes peroxidized it looses its ability to restore activity. Addition of α-tocopherol to the phospholipid prevents this loss of activity. When the

active lipid antioxidant is added to the phospholipid there is a partial inhibition of cytochrome c oxidase, but there is no further decrease in activity during aging (cf. Table II). We feel that the initial inhibitory effect of the antioxidant may indicate that the interaction of phospholipid with cytochrome c or the enzyme is modified in the presence of the antioxidant. After the new rate is established there is no further loss of activity on storage because peroxidation has been prevented.

Table II

Effect of Aging on the Ability of Phospholipid to Restore Cytochrome Oxidase Activity after Acetone Extraction

Addition	Cytochrome c oxidase activity*		
	0 days	5 days	18 days**
None	0.07	0.00	0.00
Phosphotidylethanolamine (PE)	1.73	1.02	0.77
PE-α-tocopherol	1.73	1.75	1.84
PE-antioxidant	0.55	0.62	0.64

*Cytochrome oxidase assay and acetone extraction as described by Brierley and Merola (5). Activity expressed as $\mu A\ O_2$/min/mg protein.

**The time indicates the length of storage of the phospholipid alone or with the protective agents.

We wish to acknowledge the excellent assistance of Mr. D. Myers.

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