Diethyl Phosphate Binding to Mitochondrial Cardiolipin after Intraportal Infusion in Rats

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20 min after intraportal infusion of diethyl phosphate (DEP) in rats, the mitochondrial cardiolipin (CL; 1,3-bisphosphatidyl glycerol) fraction is labelled with DEP. The obtained mixture of CL and DEP-labelled CL, which up to now has not been separated, behaves chromatographically (fractogel; HPLC; TLC), in respect to release of DEP and also in respect of the ^{31}P NMR spectrum as a mixture of CL and cardiolipin ketone diethylenol phosphate. The latter compound was also obtained by photochemical reaction of CL ketone with diethyl phosphoric acid. After cautious hydrogenation (palladium charcoal) of the total mitochondrial lipid fraction of rats treated with DEP and subsequent extraction with NaCl solution (0.005 mol/l), glycerol-2-DEP has been identified in the water phase. It is supposed that this phosphoric acid derivative is formed from CL-DEP by elimination of two molecules of phosphatidic acid.

Introduction

In previous experiments it has been demonstrated that methyl radicals – originating from the *tert*-butoxy radical – react with triethyl phosphate to form diethyl methyl phosphate (Levin *et al.*, 1970). The replacement of ethyl by methyl apparently results from the reaction of the methyl radical with the phosphoryl oxygen which is identical with the reversal of the β-scission process. Phenyl radicals react obviously in the same way (Levin *et al.*, 1974), which can be basically formulated by the formation of a tetraalkoxyphosphoranyl radical as the intermediate state:

$$R^{\cdot} + O{=}P(OR')_3 \longrightarrow RO\dot{P}(OR')_3 \longrightarrow RO\overset{\overset{O}{\|}}{P}(OR')_2 + R'^{\cdot}.$$

Abbreviations: CL, 1,3-bisphosphatidyl glycerol/cardiolipin; CL-DEP, cardiolipin diethyl phosphate; CLK, cardiolipin ketone; CLK-DEEP, cardiolipin ketone diethylenol phosphate; DEClP, diethyl chlorophosphoric acid; DEIPP, diethyl isopropyl phosphate; DEMVP, diethyl methylvinyl phosphate; DEP, diethyl phosphate; DEPA, diethyl phosphoric acid; DME, 1,2-dimethoxy ethane; G-DEP, glycerol-2-diethyl phosphate; MPG, 1-monophosphatidyl glycerol; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine.

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In this way phosphoric acid can be brought into an organic bond by a radical reaction. The reversal of β scission, which usually appears not to be possible for thermodynamic reasons (Bentrude, 1990), becomes explainable by the "Marcus-inverted region" (Marcus and Sutin, 1985; Grampp, 1993) (increase of the reaction rate with decreasing exoergicity (Levin, 1981)).

We have previously shown that diethyl phosphoric acid (DEPA) reacts in 1,2-dimethoxy ethane (DME) with photochemically excited acetone – presumably after hydrogen abstraction from the solvent – to give diethyl isopropyl phosphate (DEIPP). The same compound is likewise formed when acetone is reduced with magnesium amalgam in the presence of DEPA under the conditions of pinacol synthesis (Schole *et al.*, 1994). In both cases, a reaction of the ketyl radical – formed as intermediate during the acetone reduction – with DEPA has to be assumed, the product being further reduced to DEIPP. DEPA is used in these experiments because tertiary esters of phosphoric acid – especially its enol esters – are more stable than the corresponding primary esters (Lichtenthaler, 1959, 1961).

Since the structure of acetone is embedded in the oxidized CL molecule, the possibility should be considered that this phospholipid, which is essential for the oxidative phosphorylation, reaches

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a corresponding "radical state" in the respiratory chain of the mitochondria by a one-electron transfer process *via* redox clusters of the respiratory-chain complexes. This radical, then, could spontaneously react with inorganic phosphate to form a high-energy CL phosphate. The phosphate could then be accepted, in a proton-catalyzed reaction, by ADP under formation of ATP (according to the Theory of Chemical Coupling; Slater, 1953). Photochemically excited CL ketone (to a certain extent a biradical state) reacts in fact with DEPA to form CLK diethylenol phosphate (CLK-DEEP; see Syntheses).

Since up to now a "cardiolipin phosphate" has not been isolated, we have to assume that such a compound is extremely unstable, and that it, if formed, would be hydrolyzed in the course of the phospholipid preparation. As already mentioned, especially enol phosphates are – with exception of phosphoenol pyruvate – extremely labile (Lichtenthaler, 1959, 1961). However, the former reports indicate that esters of both dimethyl or diethyl phosphate are essentially more stable than esters of the unsubstituted phosphoric acid. Therefore, we administered diethyl phosphate (DEP) to the rat liver *via* the *Vena portae,* and after 20 min we isolated the phospholipids from mitochondria, and tried to detect CL-bound DEP in the CL fraction. There is a twofold advantage in using DEP: CL phosphate is stabilized and labelled. *In vitro* experiments with DEP are problematic because isolated mitochondria denature a short time after addition of this phosphate derivative.

Materials and Methods

Materials

All phospholipids were obtained from Sigma. Fractogel (TSK HW-40 (S), particle size 0.02–0.04 mm, for gel chromatography), fractogel ion exchanger (TSK DEAE-650 (S), Cl^- form (0.025–0.050 mm)), silica gel H 60, TLC aluminium sheets silica gel 60 (20×20 cm and 7.5×5 cm, respectively; 0.2 mm), and TLC cellulose plates (10×20 cm; 0.1 mm) were purchased from Merck. Solvents (Merck and Aldrich) were of chromatography or HPLC grade, respectively. Other reagents were of the purest grade commercially available.

Rats: Adult male Wistar rats (350 g body weight) were fed about 14 days before use with a special phosphate-poor diet (C 1048, Altromin, Lage/Lippe, *P* level 14.4 mg/kg) to increase the mitochondrial binding of DEP.

HPLC: pump (Waters 501); variable wavelength monitor (Knauer, Scientific Instruments, Basel, Switzerland); column (250×4 mm Hibar RT packed with LiChrospher Si 100 (10 μm), Merck); detection at 206 nm. – *Irradiation:* Hg high-pressure diving lamp (150 W; quartz vessel; Heraeus, Hanau). – *^{31}P NMR:* Bruker AM-250, 101.26 MHz (spectrometer A) and Bruker WH-270, 109.26 MHz (spectrometer B); $CDCl_3$; 85% H_3PO_4 extern.

Methods

Rats (10 animals per experiment) were anaesthetized with pentobarbitale (15 mg/animal), opened under irradiation with red light, and 50 mg of DEP (in 0.2 ml of H_2O, pH 7.0) per animal were infused into the *Vena portae.* After 10 min, another portion of 50 mg DEP was infused, and after 20 min, the liver was extirpated. If a rat came *ad exitum* before the 20 min were over, the liver was immediately extirpated. The mitochondria were prepared according to Myers and Slater (1957) in a medium containing sucrose (0.25 mol/l) and tris-HCl (2 mmol/l; pH 7.4). Washing of the particles was performed without tris. To prevent degradation of the phospholipids, subsequent manipulations were carried out as much as possible in a nitrogen atmosphere and at 4 °C. The mitochondria were extracted by the method of Folch *et al.* (1957), the extract was washed (NaCl solution, 0.005 mol/l) and evaporated to dryness. The separation of unpolar lipids by the method of Eberhagen and Betzing (1962) was carried out overnight. 500 mg of the phospholipid fraction were chromatographed on a fractogel column (80×4 cm, freshly prepared 24 h before use; $CHCl_3$/MeOH 1:1 (v/v)). Chromatography on silica gel was not possible due to the high instability of cardiolipin ketone diethylenol phosphate (CLK-DEEP; see Discussion). The separated phospholipid classes were identified by HPLC following the method of Patton *et al.* (1982) (eluent: hexane/2-PrOH/phosphate buffer (25 mmol/l, pH 7.0)/EtOH/acetic acid 367:490:62:100:0.6, by vol.). The entire solvent was filtered through a 0.22 μ type FH Millipore filter (Millipore Corp., Bedford,

MA). All peaks were additionally rechecked by TLC on silica gel plates ($CHCl_3$/MeOH/H_2O 65:25:4, by vol.; detection with H_2SO_4/acetanhydride/EtOH 5:5:90, by vol., and ninhydrin). All phospholipid fractions were hydrolyzed and examined for DEP as follows: the eluting solvent was evaporated to dryness and the residue was transferred into a screw-capped test tube. 1 ml of an acid preparation (5 ml $HClO_4$ (70%) + 20 ml HCl (1 mol/l) + 75 ml H_2O) was added, and the mixture was heated at 85 °C for 2 h. Under these conditions DEP is quite stable. After heating the mixture was neutralized with KOH and the formed $KClO_4$ was precipitated by centrifugation at 4 °C. The supernatant was decanted, evaporated to dryness, and the residue was stirred with 50 µl of MeOH. Different volumes of the MeOH phase were chromatographed on cellulose plates (2-PrOH/NH_3 (28–30%)/H_2O 8:1:1, by vol.). After development, the plates were sprayed with $(NH_4)_2MoO_4$, heated at 75 °C for 10 min, and irradiated at 254 nm (Lichtenthaler, 1959). Blue spots at R_f 0.56 indicated DEP. Only diacyl glycerol phosphate (phosphatidic acid; PA), detectable in case of a too mild hydrolysis, runs to a level directly below DEP (R_f 0.43). Moreover, the DEP zone was removed from the cellulose plates, rechromatographed on the same plates, and DEP was unequivocally identified.

A part of the CL fraction was additionally chromatographed preparatively on TLC aluminium sheets silica gel 60 ($CHCl_3$/MeOH/H_2O 65:25:4, by vol.). The CL zone at R_f 0.57 (salt; the free acid runs with a R_f of 0.73) was eluted with $CHCl_3$/MeOH 2:1 (v/v) and hydrolyzed with $HClO_4$/HCl. DEP was detected on cellulose plates (see above).

The phosphate determination was carried out according to a standard procedure (Anderson and Davis, 1982).

Syntheses

Chemical synthesis of CL-DEP

To 100 mg of CL dissolved in 1 ml of THF (abs.) + 15 µl of triethylamine (4 °C; magnetic stirrer; $CaCl_2$ tube), 15 µl of diethyl chlorophosphoric acid (DEClP, dist.) were added in 3 portions. After 27 min, the mixture was quickly evaporated to dryness, and the residue chromatographed on fractogel (column 30×3 cm; $CHCl_3$/MeOH 1:1 (v/v)). The CL fraction was identified by HPLC and TLC (silica gel). Pyrophosphates formed during the reaction with DEClP were unstable and well eliminated by chromatography. A part of the pure CL-DEP fraction was hydrolyzed, and DEP was detected on a cellulose plate (see above). The CL fraction was additionally investigated by ^{31}P NMR spectroscopy.

Photochemical synthesis of CLK-DEEP

a) CL ketone (CLK). In a solution of 100 mg of CL in 20 ml THF, 34 mg of AgO were suspended. Afterwards, 0.066 ml of HNO_3 (65%; d = 1.40) in 10 ml THF were added in drops within 15 min, and the mixture was stirred for another 15 min (black AgO became colourless) (Snyder and Rapoport, 1972; Kraus and Neuenschwander, 1980). The mixture was diluted with $CHCl_3$ and H_2O, and the organic layer was extracted twice with H_2O. The organic phase was dried ($MgSO_4$), evaporated, and the residue was chromatographed on a silica gel column (22×2.5 cm; $CHCl_3$/MeOH 65:25 (v/v); detection at 254 nm). The fractions were evaporated to dryness, and CLK was identified by the UV spectrum (maximum at 278 nm like acetone; Fig. 1). The R_f value on silica gel plates

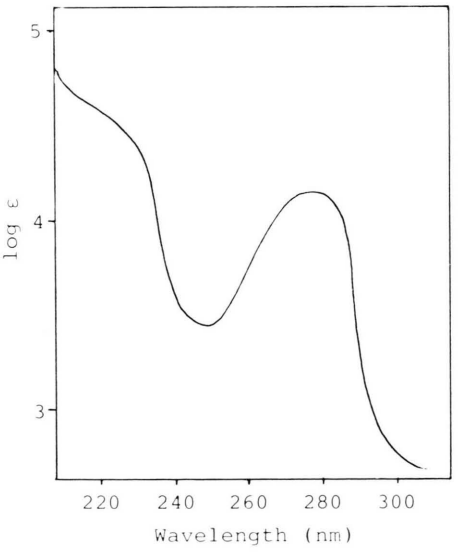

Fig. 1. UV spectrum of CL ketone in *n*-hexane. 100 mg of CL were oxidized with AgO (see Materials and Methods) and chromatographed on a silica gel column H 60 (22×2.5 cm; $CHCl_3$/MeOH 65:25 (v/v); detection at 254 nm).

corresponds with that of CL (R_f 0.73). Eventual oxidations in the region of unsaturated fatty acids were not analyzed.

b) CLK-DEEP. The residues of the CL ketone fractions (three preparations) were transferred into an irradiation apparatus (Schole *et al.*, 1994; Schole and Schole, 1994) with 1,2-dimethoxy ethane (DME; 200 ml). After addition of 1 ml DEPA, the mixture was irradiated for 24 h (room temperature; N_2; magnetic stirrer). Excess of DEPA was separated by neutralization with KOH (10 mol/l). The DME layer was washed (H_2O), dried ($MgSO_4$), and evaporated. The residue was redissolved in $CHCl_3$/MeOH 1:1 (v/v), and chromatographed on both fractogel column and silica gel plates as described before. A part of the pure "CL fraction" was hydrolyzed, and DEP was identified on a cellulose plate. The other analytic data of the latter synthesis product (retention time of HPLC; R_f on silica gel) agreed with the data obtained from CL-DEP synthesized with DEClP. By ^{31}P NMR spectroscopy, the compound has been identified as cardiolipin ketone diethylenol phosphate (CLK-DEEP; see Results).

Catalytic hydrogenation of the lipid extract

1.3–1.4 g of mitochondrial lipids of rats treated with DEP were dissolved in DME/MeOH 20:30 (v/v) and shaken under H_2 atmosphere with 400 mg of palladium charcoal (10% Pd). After consumption of 7.5 ml of H_2, the catalyst was separated, the organic layer evaporated, and the lipids were redissolved in $CHCl_3$/MeOH 2:1 (v/v). After both extraction with NaCl solution (0.005 mol/l, "water phase") and dialysis (Eberhagen and Betzing, 1962), the phospholipids were chromatographed on fractogel (see above).

Identification of glycerol-2-diethyl phosphate (G-DEP)

The "water phase" was evaporated to dryness (reduced pressure; 30 °C), and the residue was exhaustively extracted with THF. The combined THF extracts from 6 "water phases" (60 rats) were evaporated, redissolved in water (pH 8–9), and passed through a fractogel ion exchanger column (20×2.5 cm) in order to separate free DEP. G-DEP was monitored by TLC (cellulose plates, 2-PrOH/NH_3/H_2O 8:1:1, by vol., R_f 0.83; silica gel plates, cyclohexane/ethyl acetate/2-PrOH 4:1.5:2.25, by vol., R_f 0.42) and chromatographed on a silica gel column (cyclohexane/ethyl acetate/2-PrOH 2:1:1.5, by vol.).

Syntheses of G-DEP, diethyl isopropyl phosphate (DEIPP), and diethyl methylvinyl phosphate (DEMVP)

G-DEP was prepared by phosphorylation of glycerol-1,3-dibenzyl ether with diethyl chlorophosphate and elimination of the protective groups by hydrogenation (Schole and Schole, 1994). DEIPP was obtained by photochemical reaction of acetone with DEPA (Schole *et al.*, 1994). DEMVP was synthesized according to the Perkow reaction (Jacobson *et al.*, 1957).

Results

Fig. 2 shows the elution diagram of mitochondrial phospholipids of DEP-treated rats from the fractogel column. The individual peaks were identified by HPLC and TLC on silica gel. The proportional distribution of the individual compo-

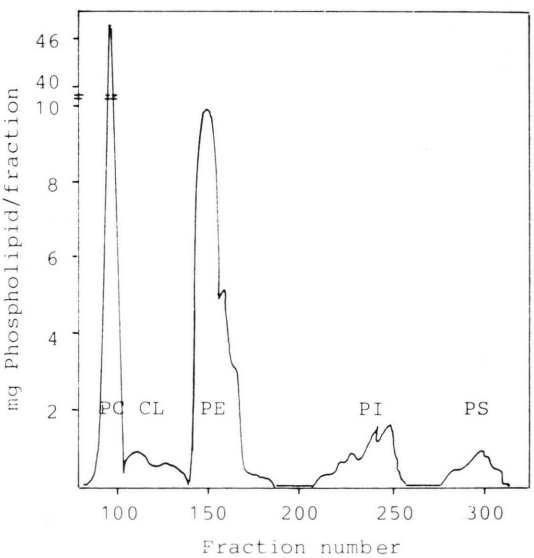

Fig. 2. Chromatography of mitochondrial phospholipids of rats treated with DEP (2×50 mg per animal, intraportal) on fractogel. 500 mg of phospholipids were charged on a fractogel column (TSK HW 40S, 80×4 cm), and the column was developed with $CHCl_3$/MeOH 1:1 (v/v) (fraction volume 3.5 ml). Identification of phospholipids was obtained by HPLC (Patton *et al.*, 1982) and TLC (silica gel 60; $CHCl_3$/MeOH/H_2O 65:25:4, by vol.).

nents agrees with the data from the literature (Bruckdorfer and Graham, 1976; Daum, 1985; Ishankhodzhaev *et al.*, 1987), with exception of the relative contributions of CL and phosphatidyl serine (PS) (see also Discussion).

All fractions were separately hydrolyzed and checked for DEP. Chemically bound DEP was found only in the CL fractions (major part in the second half of the peak), and in the PS fractions. The DEP released from the PS fractions by hydrolysis is partly bound to a substance (test tubes No. 295 through to about No. 325) containing PA, reacting positively for aldehydes, and moving on the silica gel plate like 1-monophosphatidyl glycerol (MPG) ($CHCl_3$/MeOH/H_2O 65:25:4, by vol.; R_f 0.86). Another part at the end of the peak is linked with a low-molecular compound containing no PA. The structure of these compounds has up to now not been clarified. Moreover, free DEP has been identified approximately up to test tube No. 450. The "water phase", obtained by extraction of the Folch medium with diluted NaCl solution, contains DEP too. Here the DEP is partly linked to a low-molecular compound which apparently corresponds to the compound being bound to the DEP that is released from the fractions at the end of the PS peak.

DEP derivatives of CL obtained photochemically or by conversion of CL with DEClP and comparably chromatographed both on a fractogel column as well as on silica gel plates behave in HPLC and TLC (silica gel) in the same way as the product isolated from mitochondria. All products were hydrolyzed and DEP was identified on a cellulose plate.

Table I shows the results of the ^{31}P NMR analyses of the synthesis products. In the chemically synthesized CL-DEP, the signal of the CL phosphorus is shifted by −0.74 ppm. This is in agreement with the shift after benzoylation of the free glycerol–OH group (−1.0 ppm (Schlame and Otten, 1991)). The shift is considerably larger after photochemical synthesis (−2.02 ppm). On further consideration this difference becomes understandable. On the basis of these analyses, differentiation between CL-DEP and CLK-DEEP is possible: measurements without ^1H broadband decoupling produce 6 splitting lines for DEIPP as well as for the "DEP component" of CL-DEP (chemical synthesis). For DEMVP as well as for the DEP component of the photochemical synthesis product, only 5 splitting lines are obtained because there is no proton at C-2 of the glycerol (Fig. 3). The CL phosphorus shows no characteristic splitting lines. Accordingly, in the chemical synthesis with DEClP, the reduced CL-DEP is formed – as was to be expected. In the photochemical synthesis, however, enol phosphate (CLK-DEEP) is the predominating product.

Table I. ^{31}P NMR analyses of CL and DEP derivatives. The compounds were synthesized as described in Methods.

Compound	Spectrometer	ppm (0.0 = H_3PO_4 (85%))	Line splitting without ^1H broadband decoupling (see Fig. 3)
CL (Sigma)	A	+2.21	–
	B	+1.74	–
DEP	A	+0.71	–
G-DEP	A	+0.19	–
DEIPP	B	−1.18	6
DEMVP	B	−6.20	5
CL-DEP (chemically synthesized)			
P of the CL component	A	+1.47	–
DEP component	A	+0.57	6
CL-DEP (photochemically synthesized)			
P of the CL component	B	−0.28	–
DEP component	B	−2.14	5

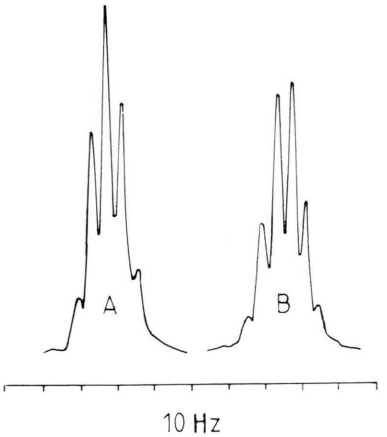

Fig. 3. Line splitting in the ^{31}P NMR spectra of diethyl phosphate derivatives. ^{31}P NMR spectroscopy (spectrometer B) of DEMVP (A) and DEIPP (B) without ^1H broadband decoupling. The corresponding components of CL-DEP and CLK-DEEP split up in the same way (see Table I).

Separation of these compounds – CL-DEP and CLK-DEEP – was not possible by the methods used here. Therefore, only *via* ^{31}P NMR spectroscopy it can be determined whether after infusion of DEP in rats the CL fraction of the liver mitochondria contains the reduced form – CL-DEP – or the enol form – CLK–DEEP. The ^{31}P NMR spectroscopic measurements (with ^{1}H broadband decoupling) of the mitochondrial CL fraction, with and without hydrogenation of the total lipid fraction, are shown in Fig. 4. The width of the spectral bands is dependent on formation of micelles. It can be noticed that the hydrogenated lipid fraction contains only one broad band (+0.4 ppm) shifted a bit to the negative area relative to the normal CL band, with a little asymmetry in direction to negative ppm values. A separation of the signals of CL and of the DEP component (Table I), lying relatively closely, is apparently not possible. After hydrolysis, DEP can also be identified in this fraction. The CL band obtained from non-hydrogenated lipids (at about −0.9 ppm), however, presents not only a further shift of the CL signal to negative ppm values, but shows, moreover, a shoulder at about −2.9 ppm lying in the region of the "DEP component" of CLK-DEEP as obtained photochemically (−2.14 ppm; Table I).

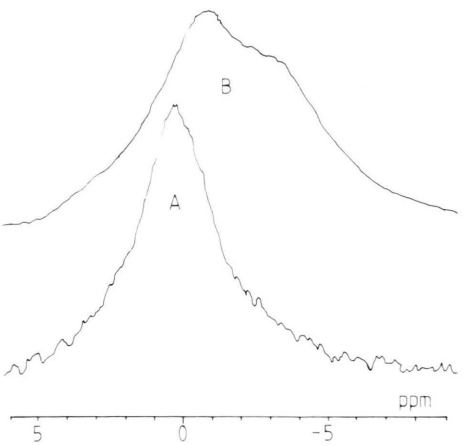

Fig. 4. ^{31}P NMR spectra (spectrometer B) of the CL fraction of rat liver mitochondria prepared 20 min after intraportal infusion of 2×50 mg DEP. Purification and separation of the lipid extract (Folch *et al.*, 1957) with or without hydrogenation was performed as described in Methods. A, with hydrogenation (Pd/C); B, without hydrogenation.

In the hydrogenated mitochondrial lipid fraction, G-DEP has been identified in the "water phase" (TLC: cellulose plate 2-PrOH/NH_3/H_2O 8:1:1, by vol., R_f 0.83; silica gel plate cyclohexane/ethyl acetate/2-PrOH 4:1.5:2.25, by vol., R_f 0.42). The identification has been established by corresponding synthesis (Schole and Schole, 1994). The R_f values, the phosphate content and the ^{31}P NMR spectrum (Table I) agreed with the data obtained from the synthesis product. This "cleavage product" has not been found without hydrogenation.

Discussion

The attempt to detect the fast incorporation of DEP into CL is extremely complicated by the extraordinary sensibility of this highly unsaturated phospholipid. Its high hydrolyzability represents an additional difficulty. Therefore, the mitochondrial phospholipid fraction was not separated on silica gel but with fractogel. The DEP ester appears to be relatively stable but by binding of this phosphate derivative as enol ester, bonds between the central glycerol part and the phosphate groups of the PA molecules are labilized. An enol diester is formed which according to Lichtenthaler (1959) is more sensitive than an enol triester. This causes formation of numerous degradation products. Thus it may be explainable that CL – in comparison with the normal spectrum of mitochondrial phospholipids – is found in a too low concentration. On the one hand, the decrease in the CL part appears to be caused by the phosphate-depleted diet lowering the CL concentration also without DEP treatment (data not shown). On the other hand, CLK-DEEP appears to be partly decomposed during the Folch extraction, the Eberhagen dialysis, and the fractogel chromatography. In the fractogel elution, the larger degradation products are found in the PS peak, causing an apparent extension of this fraction. A compound behaving similarly to MPG and apparently containing an aldehyde group might represent monophosphatidyl glycerol aldehyde DEP. The formation of this "degradation product" may be due to loss of one molecule of PA from CLK-DEEP. The structure of this compound, however, is not yet unequivocally established.

The ^{31}P NMR spectrum of the mitochondrial CL fraction, non-hydrogenated, brings about an ad-

ditional indication for the supposition that the DEP-labelled CL, formed *in vivo,* really is the enol phosphate (CLK-DEEP). CL ketone, photochemically excited, obviously reacts with DEPA to form preferentially CLK-DEEP, and the obtained ^{31}P NMR signal of the "DEP component" is situated in the same area as is the corresponding signal of the mitochondrial CL fraction after treatment of rats with DEP. The signal of the CL phosphorus, however – dependent on formation of micelles –, is very broad so that the signal of the "DEP component" can be seen only as a shoulder.

So at present, only three phenomena can be considered as certain:

1. In DME, photochemically excited CL ketone reacts with DEPA to give CLK-DEEP.

2. In rats, after intraportal treatment with DEP, the CL fraction of the mitochondrial phospholipids is labelled in a relatively short time. DEP can be released by hydrolysis and identified by TLC (cellulose plates). This DEP-labelled CL, non-separable from the free CL by the methods used here, behaves chromatographically (fractogel, HPLC, TLC (silica gel)) and in respect of the ^{31}P NMR spectrum like CLK-DEEP obtained by photochemical reaction of CL ketone with DEPA.

3. If the total lipid spectrum after the Folch extraction of the liver mitochondria is cautiously hydrogenated with palladium charcoal, followed by extraction of the organic layer with diluted NaCl solution, G-DEP can be detected in the "water phase". The structure of this "cleavage product" has been confirmed by TLC, phosphate analysis, and synthesis. It is quite likely that this phosphoric acid derivative is formed from CL-DEP by elimination of two molecules of PA.

Because G-DEP may be analyzed only after hydrogenation of the mitochondrial lipids and the ^{31}P NMR spectrum of the CL fraction of non-hydrogenated lipids differs significantly from that of the hydrogenated fraction, one may finally discuss that *in vivo,* under physiological conditions, the compound with the highest energy – cardiolipin ketone enol phosphate – is formed. Another argument for this conclusion would be obtained if monophosphatidyl glycerol aldehyde DEP could be positively detected as a cleavage product. However, this detection problem may be solved only by further experiments. A further stabilization of CLK-DEEP or of the CL ketone enol phosphate, as probably found *in vivo,* may possibly be obtained by methylation of the phosphate residues with diazomethane (Schlame and Otten, 1991).

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Anderson R. L. and Davis S. (1982), An organic phosphorus assay which avoids the use of hazardous perchloric acid. Clin. Chim. Acta **121,** 111–116.

Bentrude W. G. (1990), in: The Chemistry of Organophosphorus Compounds, **Vol. 1** (F. R. Hartley, ed.). John Wiley & Sons Ltd., Chichester, pp. 531–566.

Bruckdorfer K. R. and Graham J. M. (1976), in: Biological Membranes, **Vol. 3** (D. Chapman and D. F. H. Wallach, eds.). Academic Press, London, pp. 103–152.

Daum G. (1985), Lipids of mitochondria. Biochim. Biophys. Acta **822,** 1–42.

Eberhagen D. and Betzing H. (1962), An improved technique for dialysis of lipids. J. Lip. Res. **3,** 382–383.

Folch J., Lees M. and Sloane Stanley G. H. (1957), A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226,** 497–509.

Grampp G. (1993), Der inverse Marcus-Bereich – von der Theorie zum Experiment. Angew. Chem. **105,** 724–726; Angew. Chem., Int. Ed. Engl. **32,** 691–693.

Ishankhodzhaev T. M., Bornikov V. T., Zainutdinov B. R. and Saatov T. S. (1987), Influence of changes in the lipid composition of membranes on the activity of the enzymes of the respiratory chain of rat liver mitochondria. Biokhimiya **52,** 220–224.

Jacobson H. I., Griffin M. J., Preis S. and Jensen E. V. (1957), Phosphonic acids. IV. Preparation and reactions of β-ketophosphonate and enol phosphate esters. J. Am. Chem. Soc. **79,** 2608–2612.

Kraus G. A. and Neuenschwander K. (1980), A general synthesis of functionalized hydroxy quinones. Synth. Commun. **10,** 9–16.

Levin Ya. A., Trutneva E. K., Gozman I. P., Abul'khanov A. G. and Ivanov B. E. (1970), Free radical addition to the phosphoryl group. Bull. Acad. Sci. U.S.S.R. Div. Chem. Sci., 2687.

Levin Ya. A., Trutneva E. K. and Ivanov B. E. (1974), Homolyting reactions of organophosphorus compounds. I. Kinetics and mechanism of the reactions of phosphoryl compounds with phenyl radicals. J. Gen. Chem. U.S.S.R. **44,** 1418–1425.

Levin P. P. (1981), Effect of structure of donor and acceptor on kinetic spectral characteristics of triplet exciplexes. Bull. Acad. Sci. U.S.S.R. Div. Chem. Sci. **30,** 1970–1972.

Lichtenthaler F. W. (1959), Über die Reaktivität von Enolphosphaten. Thesis, University of Heidelberg, Germany.

Lichtenthaler F. W. (1961), The chemistry and properties of enol phosphates. Chem. Rev. **61,** 607–649.

Marcus R. A. and Sutin N. (1985), Electron transfers in chemistry and biology. Biochim. Biophys. Acta **811,** 265–322.

Myers D. K. and Slater E. C. (1957), The enzymic hydrolysis of adenosine triphosphate by liver mitochondria. Biochem. J. **67,** 558–572.

Patton G. M., Fasulo J. M. and Robins S. J. (1982), Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. J. Lip. Res. **23,** 190–196.

Schlame M. and Otten D. (1991), Analysis of cardiolipin molecular species by high-performance liquid chromatography of its derivative 1,3-bisphosphatidyl-2-benzoyl-*sn*-glycerol dimethyl ester. Anal. Biochem. **195,** 290–295.

Schole J., Schole Chr., Eikemeyer J. and Krebs H. Chr. (1994), Diethyl isopropyl and isopropenyl phosphate *via* differently generated ketyl radicals of acetone reacting with diethyl phosphoric acid. Tetrahedron **50,** 1125–1128.

Schole J. and Schole Chr. (1994), Glycerin-2-diethylphosphat durch photochemische Umsetzung von Dihydroxyaceton mit Diethylphosphorsäure. Tetrahedron **50,** 3743–3746.

Slater E. C. (1953), Mechanism of phosphorylation in the respiratory chain. Nature **172,** 957–978.

Snyder C. D. and Rapoport H. (1972), Oxidative cleavage of hydroquinone ethers with argentic oxide. J. Am. Chem. Soc. **94,** 227–231.