# CARDIOLIPIN DEGRADATION BY RAT LIVER LYSOSOMES *

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## SUMMARY

The lysosomal subcellular fraction of rat liver contains acid hydrolases which can carry out the degradation of cardiolipin to yield water-soluble products and free fatty acids. The time course of appearance of the products indicates that the major catabolic route involves the sequential removal of three of the fatty acids, followed by hydrolysis to acylglycerophosphoryl glycerol (from which the fatty acid is subsequently removed) and d-glycerophosphate (which is hydrolysed to give free phosphate and glycerol). The phospholipase A activity responsible for removal of the first fatty acid is located in the lysosomal fraction.

## INTRODUCTION

Phospholipases D which are specific for cardiolipin (diphosphatidylglycerol) have recently been discovered in bacteria (1,2), and peanut seed phospholipase D is effective against cardiolipin (3). However little is known about the degradation of cardiolipin in mammalian systems except that cardiolipin has a much lower turnover rate than other phospholipids (4). In this study cardiolipin-degrading activity was demonstrated in rat liver and shown to be localised in the lysosomal fraction. The time course of appearance of hydrolysis products during degradation indicates that the initial attack on cardiolipin at acid pH is by a lysosomal phospholipase A.

## METHODS

[$^{32}$P] Cardiolipin was prepared by injecting 250g Wistar rats intraperitoneally with 3.0 ml 0.9% saline containing 20 mCi [$^{32}$P] phosphate pH 7.0 (1.25 mCi/μmole). After three days the rats were killed and cardiolipin was extracted from the livers by the method of Courtade and McKibbin (5). Cardio-

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lipin was obtained free of other lipids as shown by thin-layer chromatography in several systems. Cardiolipin concentration was determined as phosphorus by the method of Bartlett (6). An enzyme substrate suspension was prepared by sonication of 12.5 μmoles of a mixture of [$^{32}$P] cardiolipin and commercial beef heart cardiolipin (Sigma) in 10 ml 0.4% Triton X-100.

Aliquots of the [$^{32}$P] cardiolipin suspension were incubated with subcellular fractions of rat liver in the presence of 0.05 M acetate buffer. Specific reaction conditions are shown in the results section. The reaction was stopped by lipid extraction as described by Bligh and Dyer (11). Chloroform-soluble products were separated by thin-layer chromatography on silica gel G developed with $CHCl_3$: $CH_3OH$:$H_2O$:14.0 M $NH_4OH$ (140:50:7:1). Identification of products was by comparison of Rf values, and cochromatography with lyso (deacylated) derivatives prepared from commercial cardiolipin by the method of Robertson and Lands (12) except that Naja naja venom was used. Water-soluble products were separated by thin-layer chromatography (1) and identified by comparison with standards prepared by the complete or partial mild alkaline methanolysis of phosphatidic acid, phosphatidylglycerol and cardiolipin. Identification was verified by 2-dimensional chromatography on thin layers of silica gel, the solvent in the first dimension being chloroform:methanol:90% formic acid: pyridine:water (45:45:5:0.35:5), and in the second dimension chloroform:methanol:water:14M ammonium hydroxide:glacial acetic acid (90:90:10.3:6.0:3.6) containing 0.17g tetrasodium EDTA per 100 ml.

The deacylation of cardiolipin (phospholipase A activity) was measured by a procedure similar to that used for enzymatic hydrolysis of cardiolipin, the chloroform-soluble products being separated by thin layer chromatography as described before.

Radioactive products were located on chromatograms by autoradiography and were estimated by liquid scintillation counting.

Subcellular fractions were prepared from the livers of 150-250 Wistar rats by the method of Trouet (7) as modified by Leighton et al (8). Protein was assayed by the method of Lowry et al (9) and β-N-acetylglucosaminidase by the method of Beck and Tappel (10).

| | N-acetyl-β-D glucosaminidase | | Cardiolipin Degrading Activities | | |
|---|---|---|---|---|---|
| | | | Release of Aqueous-methanol-soluble phosphorus | Phospholipase A | |
| | | | | 40 minute Incubation | 5 minute Incubation |
| | Units per mg protein | % Recovery | % | % | % |
| Homogenate | 5.2±1.2 | 100 | 3.7±0.2 | 14.6±3.6 | 3.0±1.9 |
| Nuclei | 5.6±2.1 | 45±24 | 4.0±0.7 | 15.6±1.6 | 3.9±2.0 |
| Mitochondria | 7.2±2.1 | 11.6±4.5 | 3.2±1.0 | 11.8±1.3 | 1.6±1.2 |
| Lysosomes | 164±42 | 9.3±3.9 | 23.4±8.4 | 44.9±5.3 | 19.9±2.8 |
| Microsomes | 12.8±2.3 | 28.2±3.8 | 3.4±0.8 | 13.5±1.5 | 6.7±1.4 |
| Supernatant | 2.5±1.0 | 13.2±5.0 | 1.5±0.8 | 10.1±2.1 | 3.1±3.1 |

Table 1. Enzyme activities in rat liver subcellular fractions. The cardiolipin degrading activities are expressed as percent total $^{32}P$ recovered in the aqueous-methanol-soluble fraction, after a 7 hour incubation at pH 4.0, or (for phospholipase A) percent total $^{32}P$ recovered in deacylated derivatives of cardiolipin after incubation at pH 5.0. Incubation conditions were as described in the legend to figure 2 except that 0.24 mg protein was used. Controls contained no enzyme. Units of N-acetyl-β-D glucosaminidase are nmoles per minute. Data shows mean± s.d. for 6 individual subcellular fractionations.

## RESULTS

The lysosomal fraction of rat liver was found to be many times more effective at releasing water-soluble phosphorus from cardiolipin than any other subcellular fraction, none of which were any more effective than the homogenate. (Table 1). The release of water-soluble phosphorus is not first-order with respect to time (fig. 1) or protein concentration because of the multiple enzymes involved, so no accurate estimate can be made of the purification of the activity in the lysosomal fraction. However the results do indicate that the activity is mainly lysosomal.

Thin layer chromatograms of the chloroform-soluble degradation products of cardiolipin showed a total of eight radioactive spots. Three of these, corresponding to cardiolipin, lysocardiolipin and dilysocardiolipin, contained the bulk of the radioactivity. None of the other spots contained more than 2.5% of the radioactivity at any time. The time course of appearance of radioactivity in the

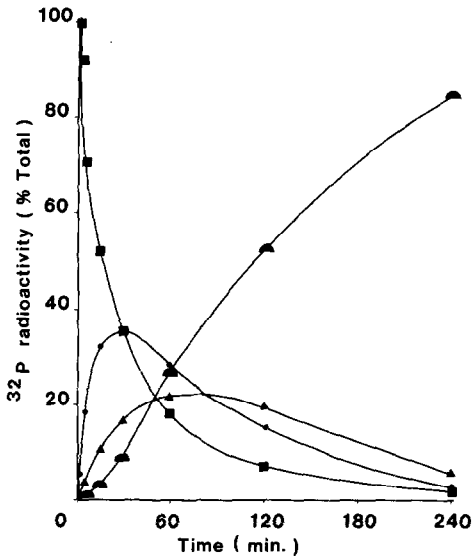



Figure 1. Time course of degradation of cardiolipin by lysosomal enzymes. The ordinate expresses the percent total $^{32}P$ recovered in a particular compound. ■——■, cardiolipin; ●——●, lysocardiolipin; ▲——▲, dilysocardiolipin; ▲——▲, aqueous-methanol-soluble phosphorus. Samples of 0.2 ml were removed at the indicated times from a mixture of 370 nmoles cardiolipin, 2 mg Triton X-100, 0.1 mmoles sodium acetate buffer pH 3.7, 10 μmoles $CaCl_2$, and 2.5 mg lysosomal protein in a total volume of 2 ml, incubated at 37°.

products clearly indicates that the major pathway for the initial breakdown of cardiolipin is by successive removal of three of the fatty acid residues. The product acyl-di-(glycerophosphoryl)-glycerol was the major aqueous-methanol-soluble product after 60 minutes. Other radioactive products identified in the aqueous-methanol phase were acylglycerophosphorylglycerol, di-(glycerophosphoryl)-glycerol, glycerophosphorylglycerol, α-glycerophosphate and inorganic phosphate. Of these, glycerophosphorylglycerol, which apparently accumulates and is not significantly degraded further, and inorganic phosphate were major products at 240 minutes. It is not obvious whether di-(glycerophosphoryl)-glycerol, which contained 8% of the radioactivity at 240 minutes, is degraded further. From consideration of these products it is apparent that acyl-di-(glycerophosphoryl)-glycerol can be degraded in two different ways: by a phosphodiesterase, to give α-glycerophosphate (further degraded to glycerol and phosphate) and acylglycerophosphoryl-glycerol, the fatty acid residue being subsequently removed to leave glycero-

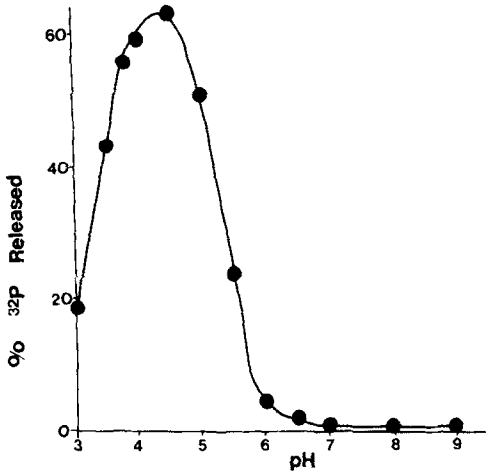



Figure 2. pH curve for the release of aqueous-methanol-soluble phosphorus from cardiolipin by lysosomal enzymes. 37 nmoles [$^{32}$P] cardiolipin, 1 mg Triton X-100, 50 µmoles sodium acetate buffer and 575 µg lysosomal protein, in a total volume of 1 ml, were incubated at 37° for 30 minutes.

phosphorylglycerol; or by a phospholipase to leave the complete di-(glycerophosphoryl)-glycerol backbone.

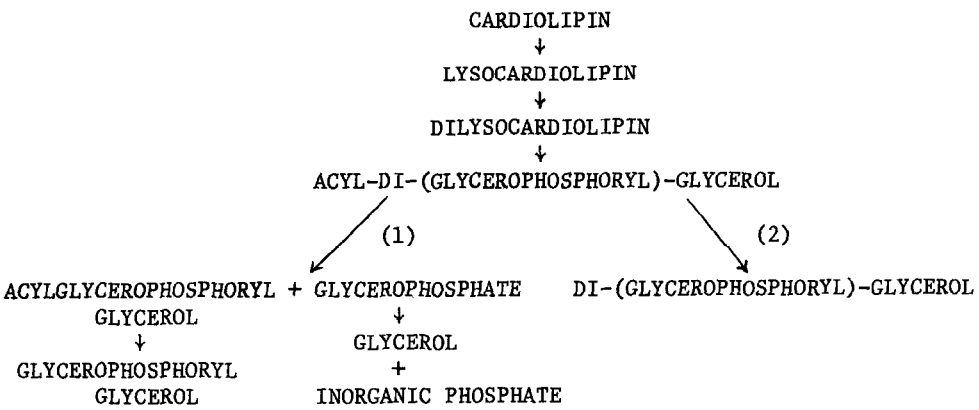
The phospholipase A responsible for the initial hydrolysis was found to have a pH optimum of 4.5-5.0 whereas the pH optimum for production of aqueous methanol-soluble phosphorus from cardiolipin was 4.0-4.5 (fig.2). The location of the cardiolipin-degrading phospholipase A was found to be lysosomal (table 1) as for the aqueous-methanol-soluble-phosphorus releasing activity. The assay was not carried out under optimal conditions and is not first order with respect to the protein concentration. This explains why the apparent purification of the enzyme in the lysosomal fraction was greater for the shorter period of incubation. The mean ratio of percent hydrolysis by the lysosomal fraction to percent hydrolysis by the homogenate during the shorter (5 minute) incubation was 9.8, this figure being a minimum value for the mean purification of the phospholipase A in the lysosomal fraction.

## DISCUSSION

The demonstration that lysosomes contain enzymes which degrade cardiolipin at acid pH is in accordance with the previous localisation in rat liver lysosomes

of several phospholipid-degrading enzymes with acid pH optima, including phosphatidate phosphatase (13), phospholipase (14) and sphingomyelinase (15). The specificities of mammalian phospholipases towards cardiolipin are largely unknown, but it is interesting that in the absence of ether Naja naja venom phospholipase A2 has little activity towards cardiolipin, and Crotalus adamanteus venom phospholipase A2 shows none (16). However it is possible that rat liver lysosomal phospholipases hydrolyse both lecithin and cardiolipin.

No evidence was found of a phospholipase C or D active against cardiolipin in rat liver lysosomes. If these had been present then phosphatidyl glycerophosphate, or phosphatidic acid and phosphatidyl glycerol, would have been among the degradation products. On the basis of the evidence presented above we suggest that the lysosomal degradation of cardiolipin proceeds as shown in the following scheme (fatty acids are not shown):-




Of the two routes shown, (1) appears to be the most important, accounting for about 90 percent of the end products.

While much work remains to be done on the properties, including substrate and positional specificities, of the several enzymes involved in the degradation of cardiolipin it is clear from the present study that lysosomes play an important role in the catabolism of this compound.

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