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# THE FUNCTION OF PHOSPHOLIPASE $A_2$ IN THE METABOLISM OF MEMBRANE LIPIDS

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## SUMMARY

The phospholipase-2 inhibitor, p-bromophenacyl bromide, has been shown to inhibit strongly the elongation of endogenous fatty acids in preparations of brain mitochondria and microsomes. On the other hand, it does not inhibit the elongation of added palmitic or linoleic acids. The implication is that the normal first step in alteration of membrane lipid fatty acids is their release to other membrane-bound enzyme systems by a membrane-bound phospholipase A.

## INTRODUCTION

In recent studies on the elongation systems of brain fatty acids it was found (1) that in the mitochondrial system with no added fatty acid substrate, the principal product from added [1-$^{14}$C]acetyl-CoA, under anaerobic conditions, was 22:4ω6. In the microsomal preparation, with added [1,3-$^{14}$C]malonyl CoA, the principal products were 22:4ω6, 24:4ω6 and the saturated fatty acids, 16:0 and 18:0, which appeared from tracer distribution to be synthesized de novo. The addition of a substrate fatty acid or acyl CoA derivative resulted, in both cases, in the formation of an additional product two carbons longer than the substrate.

The source of the endogenous 20:4, the precursor of 22:4, is most likely to be the fatty acid at the 2-position of a membrane phosphoglyceride. Thus, the first step in the elongation of 20:4 to 22:4 may be the hydrolysis of the 2-acyl group and its transfer to the first enzyme of the elongation system, probably acyl CoA synthetase.

If this is the case, then a phospholipase $A_2$ inhibitor should inhibit the elongation of endogenous 20:4 but should have little effect on the elongation of added substrate fatty acids. Such an inhibitor, p-bromophenacyl bromide, has

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been used effectively by Roberts et al. (2). It was therefore applied as a tool to investigate the present problem.

## MATERIALS AND METHODS

[1-$^{14}$C]palmitic acid, [1-$^{14}$C]linoleic acid, [1,3-$^{14}$C]malonyl CoA and [1-$^{14}$C]acetyl CoA were obtained from New England Nuclear Corp. ATP, NADPH and NADH were products of Sigma Chemical Co. The inhibitor, p-bromophenacyl bromide was obtained from Aldrich Chemical Co. All other reagents used were Baker analyzed reagents. Fatty acid standards were obtained either from Hormel Fatty Acid Laboratories or Applied Science Laboratories.

Preparation of Subcellular Fractions from Rat Brains - Five or six young rats (16 days old) of the Sprague Dawley strain were killed by decapitation and the brains quickly removed and washed with ice cold 0.32 M sucrose. Preparations of mitochondria and microsomes were as described by Yatsu and Moss (3). The whole brains were weighed and homogenized in 9 volumes of 0.32 M sucrose with a Potter-Elvehjem type homogenizer equipped with a teflon pestle. The homogenate was centrifuged at 1000 x g for 10 min to remove cellular debris. A crude mitochondrial fraction was obtained by centrifugation of the supernatant fluid at 12,000 x g for 30 min. The crude mitochondrial pellet was suspended in 0.32 M sucrose and applied to a discontinuous density gradient (consisting of 6 ml each of 0.8 M and 1.2 M sucrose per tube) in the SW-27 head of the Beckman L5-50 ultracentrifuge at 50,000 x g for 2 hr (4). The microsomal fraction was conveniently prepared at the same time by centrifuging the supernatant at this speed. The pellet was used for the microsomal incubations described below. All the above procedures were done at 4°C. Preparation of enzymes and incubations were performed on the same day. The subcellular fractions were suspended in 0.32 M sucrose and the protein contents of the suspension were determined by the Folin method as modified by Lowry et al. (5).

Incubations - Unless otherwise indicated, the incubation mixture contained 30 nmol of acetyl-CoA or 30 nmol malonyl-CoA, 8 μmol ATP, 1 μmol NADPH, 1 μmol NADH, 60 μmol of potassium phosphate buffer, pH 6.5 and 1-3 mg of mitochondrial or microsomal protein in a final volume of 0.5 ml. The fatty acid substrates were prepared in micellar solution by dispersal in twice their molar amount of 0.1 N $NH_4OH$ and diluting to the desired volume with 1% triton WR 1339 (Ruger Chemical Co., Irvington on Hudson, New York). A stock solution of inhibitor in ethanol at a concentration of 4 x $10^{-2}$M was used when needed. All the incubations were carried out in a nitrogen atmosphere. The incubation vials were capped with rubber stoppers and nitrogen was flushed through for at least 10 min. The vials were shaken in a Dubnoff Metabolic Shaker at 37°C.

Extraction and Separation of Lipids - The total lipids from the reaction mixtures were extracted with 20 vol of chloroform-methanol (2:1, v/v). The suspension was centrifuged and to the supernatant was added 0.2 vol of water. The upper phase was removed, and the chloroform-rich lower phase was washed three times with Folch's theoretical upper phase (6). The lower phase, containing the lipids, was reduced to dryness and an aliquot was taken for counting. The whole lipid extracts were transmethylated by treatment with 5% methanolic HCl solution for 2-1/2 hr at 80-90°C in screwcap vials under $N_2$. The fatty acid methyl esters were extracted with pentane and washed three times with water. The pentane extract of radioactive fatty acids was taken to dryness under a stream of nitrogen.

Gas Liquid Chromatography and Identification of Fatty Acids - Gas liquid chromatography was carried out in a Packard Gas Chromatograph Model 7400 equipped with dual flame ionization detectors and a 4 mm x 6 ft coiled glass column. Fifteen percent DEGS on 60/80 mesh Supelcoport and 10% Apolar 10C on 100/200 mesh Gaschrom Q were generally used for the analyses. The Packard Model 894 proportional counter was used for monitoring $^{14}$C activity. Identification of

the radioactive peaks was accomplished by comparison with known standard fatty acid methyl esters (7).

## RESULTS AND DISCUSSION

Inhibition of elongation of endogenous fatty acids by increasing concentrations of p-bromophenacyl bromide is shown in Table I. It can be seen that incorporation of label from acetyl CoA into fatty acids in the absence of added fatty acid precursor is reduced almost to zero in the mitochondria and that incorporation of malonyl CoA was reduced to 25% of the control in the microsomal preparation by $4 \times 10^{-4}$M inhibitor. It was found in previous experiments (1) that some total synthesis of palmitate and stearate occurs in the latter preparation and it would not be expected that this type of incorporation would be inhibited. However, if this were the explanation, the products of the most inhibited reaction should be 16:0 and 18:0, whereas, they were similar to those of the uninhibited reaction. Possibly other unaffected hydrolytic pathways are involved in the microsomes.

To test the possibility that p-bromophenacylbromide might inhibit some step in the elongation path, incorporation of acetyl CoA by mitochondria and malonyl CoA by microsomes was carried out in the presence of added fatty acid precursor and $1 \times 10^{-3}$M inhibitor. This concentration of inhibitor was over twice as great as the highest concentration showing complete inhibition of mitochondrial elongation of endogenous fatty acid. It can be seen in Table II that elongation of added 16:0 or 18:2 proceeds normally in the presence of this concentration of inhibitor.

The significance of these results is that a membrane-bound phospholipase, acting on its supporting membrane lipids, may carry out the first step in the transfer of a fatty acid from a phospholipid (in this case, the 2-position) to other membrane-bound enzyme systems for alteration (in this case, elongation). The fatty acid released from the 2-position of a membrane phosphoglyceride is the normal substrate for a number of additional important reactions such as desaturation (8), prostaglandin formation (9), esterification to other lipids or

TABLE I

Incorporation of 1-$^{14}$C Acetyl CoA by Brain Mitochondria and 1,3-$^{14}$C malonyl CoA by Brain Microsomes into Endogenous Fatty Acids in the Presence of p-Bromophenacyl Bromide

| Concentration of inhibitor (mM) | Incorporation of label into endogenous fatty acids* (cpm/total) | |
|---|---|---|
| | Mitochondria | Microsomes |
| 0 | 11,000 | 82,500 |
| 0 + 5µl ETOH | 10,500 | 79,000 |
| 0.1 + 5µl ETOH | 2,500 | 38,500 |
| 0.2 + 5µl ETOH | 2,000 | 33,000 |
| 0.4 + 5µl ETOH | 0 - 2,000 | 23,000 |

Conditions of incubation described in Materials and Methods.

*Composition of fatty acid mixtures, in terms of % of total radioactivity of major components in the uninhibited incubations is (1) mitochondria: 18:0-12; 20:0-10, 22:4-50; microsomes: 16:0-37; 18:0-20, 22:4-24; 24:4-19.
For the 75% inhibited microsomal incubations, the % composition is: 16:0-3, 18:0-34, 22:4-55, 24:4-8.

TABLE II

Elongation of Added 1-$^{14}$C Palmitic Acid and 1-$^{14}$C Linoleic Acid by Brain Mitochondria and Microsomes in the Presence of 1 x $10^{-3}$M p-Bromophenacyl Bromide

| Precursor | Inhibitor | Elongation Product (as % of labeled fatty acids after incubation)* | |
|---|---|---|---|
| | | Mitochondria | Microsomes |
| 16:0 | - | 9.9 | 13.8 |
| | + | 10.5 | 12.2 |
| 18:2 | - | 7.0 | 12.8 |
| | + | 6.6 | 11.3 |

Conditions of incubation described in Materials and Methods.

*Labeled elongation products were 18:0 and 20:2 (from 16:0 and 18:2, respectively). 22:4 and 24:4 were not labeled under these conditions.

release from the membrane. The present study does not consider the means by which the direction of transfer occurs. It is possible that activation, as the

CoA ester, would lead to elongation, desaturation, esterification or (in mitochondria) oxidation, whereas the unactivated fatty acid could be a substrate for radical attack and the cyclooxygenase involved in prostaglandin synthesis. This explanation, however, would not be particularly satisfying since it could account for only two of several directions.

Another possibility, which has not been tested, is that each enzyme system is coupled to a phospholipase which feeds directly into it and thus determines the pathway followed by the released and transferred fatty acid.

An additional point of interest lies in the finding (1) that the products, as well as the normal substrates of elongation, are incorporated into phospholipids. Accomplishment of this "exchange" requires that there be a reservoir of lysophospholipid present in the membrane to accept the altered fatty acid in the presence of an acyl transferase, which may also be coupled to the enzyme system.

This study serves to emphasize the existence of a considerable coupling of membrane-bound enzymes, permitting rapid and efficient pathways of transformation. How general it is and the mechanisms of regulation remain to be investigated.

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