THE Ca^{2+} STIMULATED INCORPORATION OF CHOLINE INTO MICROSOMAL LECITHIN SUBSPECIES *IN VITRO*

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1. Introduction

Recent investigations have indicated that free choline can be incorporated into lecithin bypassing phosphorylcholine and/or CDP-choline [1–4], and that this pathway is quantitatively the most important [4]. Several authors have suggested that the Ca^{2+} stimulated incorporation of free choline into lecithin found by Dils and Hübscher [5] in liver homogenates may represent this direct pathway of lecithin biosynthesis *in vivo*.

In vivo experiments have shown that oligounsaturated lecithins are more rapidly labelled from labelled choline, than are the polyunsaturated lecithins. One would therefore expect that the Ca^{2+} stimulated choline incorporation would preferentially label the oligounsaturated species.

In the present paper, the specificity of the Ca^{2+} stimulated choline incorporation into lecithin subspecies has been investigated. In the presence of Ca^{2+} choline is incorporated up to 12 times faster into microsomal lecithins containing polyunsaturated fatty acids, than into monounsaturated lecithins *in vitro*. It seems unlikely therefore that the Ca^{2+} stimulated incorporation of choline into lecithin is quantitatively important *in vivo*.

2. Materials and methods

Choline chloride-CH_3-^{14}C, 32 mCi/mM was purchased from the Radiochemical Centre, Amersham, England. All organic solvents were of analytical grade. Butylated hydroxytoluene was added as an antioxidant (50 mg/l). Microsomes were prepared from Wistar male male rat livers by homogenization in 0.25 M sucrose in a Potter-Elvehjem homogenizator. Microsomes were obtained from the 243,000 g_{min} supernatant by centrifugation at 198,000 g_{max} for 30 min. The microsomes were finally suspended in 0.25 M sucrose. The incubations were stopped by adding 15 ml of methanol–chloroform (2:1). Then 3 ml of 1% choline chloride were added, and lipids extracted according to Bligh and Dyer [6]. Lecithins were isolated by TLC on Silica gel H with chloroform–methanol–water (65:25:4) as solvent. Spots were located after spraying with 0.2% dichlorofluorescein in ethanol. Lipids were eluted from the gel according to Arvidson [7] with a small modification to remove traces of dichlorofluorescein. After adding 4 M NH_4OH, the organic phase was washed once with pure upper phase prepared by mixing the eluting solvent and 4 M NH_4OH (3:1). Thereafter, the organic phase was washed once with 50% methanol in water.

Lecithins were subfractionated according to their degree of unsaturation on silver nitrate plates essentially as described by Arvidson [8]. Plates were activated at 195° for 3 hr, and developed with chloroform–methanol–water (65:25:4). Spots were located in longwave UV-light after spraying with 0.02% dichlorofluorescein in ethanol. The use of dilute dichlorofluorescein was essential for the detection of minor components. The triene fraction consisted of three to four narrow bands which were eluted from the plates together. The hexaene fraction consisted of two or three distinct bands which were eluted together. Phospholipids were eluted from the gel as described above.

Phospholipids were transmethylated in 14% (w/v) BF_3 in methanol [9]. After addition of water, fatty

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acid methyl esters were extracted with hexane, and purified by TLC as described by Mangold [10]. Fatty acid composition was determined by GLC on a Varian model 1400 gas chromatograph, equipped with a glass column, 180 cm long, 4 mm i.d., filled with 10% EGSS-X on Gas Chrom P. Fatty acid methyl esters were identified by comparison with known standards or by combined GLC-mass spectrometry. Quantitation was obtained by triangulation. Table 1 shows the fatty acid composition and relative abundance of the five different lecithin subfractions examined. The values are very similar to those reported by Arvidson [7].

Phosphate was determined according to Bartlett [11] after digestion of the lipids in 72% perchloric acid. Protein was determined by the method of Lowry et al. [12]. Radioactivity was determined in a Tri Carb 3003 liquid scintillation spectrometer as previously described [1].

3. Results and discussion

Fig. 1 shows the incorporation of free choline into microsomal lecithin subspecies. After 5 min of incubation, the specific activity of the hexaene fraction was 12 times higher than in the monoene fraction. The specific activities of the other species were intermediary, and increased with increasing unsaturation.

In 1968 Arvidson showed that radiolabelled choline predominantly was incorporated into the monoene and diene lecithin species in rat liver [13]. Similar results were obtained by Treble et al [4]. In *in vivo* experiments with ^{32}P and choline-^{3}H they showed that free

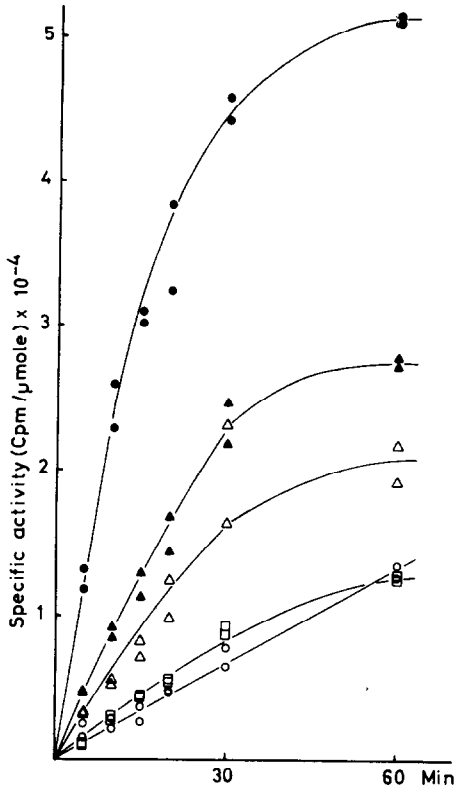

Fig. 1. Ca^{2+} stimulated incorporation of choline into microsomal lecithin subspecies. The incubation medium contained 4 mM $CaCl_2$; 60 mM imidazolebuffer, pH 7.6; 0.5 μCi of choline chloride-CH_3-^{14}C (32 mCi/mM); microsomes (4.1 mg of protein) and 150 mM sucrose. Total volume was 1 ml, and the incubation temperature was 37°. ○——○ monoene, □——□ diene, △——△ triene, ▲——▲ tetraene and ●——● hexaene licithin subspecies.

Table 1

Fatty acid composition of microsomal lecithin subfractions (weight %). Lecithin subfractions were eluted from the TLC plates, transmethylated and analyzed on GLC. The recovery of P from the plates was approximately 90%. Especially the triene species contained numerous minor fatty acid components with low carbon atom number, which are not included in the table.

	% of total P	16:0	18:0	16 :1	18:1	18:2	20:3	20:4	20:5	22:5	22:6
Monoene	12.1	36.6	15.4	5.4	35.9	5.4	–	–	–	–	–
Diene	28.3	20.3	21.4	0.8	8.6	44.9	–	–	–	–	–
Triene	5.9	15.8	22.7	2.5	8.5	4.8	18.8	–	2.2	–	–
Tetraene	35.4	14.0	30.1	0.8	5.4	0.9	–	48.3	–	–	–
Hexaene	18.3	17.6	22.8	1.5	5.8	1.4	–	7.4	7.2	4.8	31.3

choline is incorporated relatively much faster into a combined fraction of oleyl and linoleyl lecithins and arachidonoyl lecithins, than into docosahexaenoyl lecithins. Their data also indicated that labelled choline *in vivo* may be incorporated into lecithin by a mechanism bypassing phosphorycholine and CDP-choline. They estimated that the choline was incorporated approximately 4 times faster into lecithin by this mechanism, than by way of phosphorylcholine and CDP-choline. They suggested that this might be due to an exchange between free choline and the choline of preformed lecithin, as previously found *in vitro* by Dils and Hübscher [5]. Since this direct incorporation of choline into lecithin apparently is the quantitatively most important, one should expect that the Ca^{2+} stimulated incorporation of choline preferentially would label the oligoene lecithin species.

In the present *in vitro* work, docosahexaenoyl lecithin is labelled 10–12 times faster than oleyl and linoleyl lecithins. This shows that at least *in vitro*, the specificity of the Ca^{2+} stimulated choline exchange does not agree with the specificity expected from *in vivo* experiments [4]. It is therefore questionable whether the Ca^{2+} stimulated exchange mechanism can explain the rapid choline incorporation, and the very low specific activities of CDP-choline in relation to lecithin, found after choline labelling *in vivo* [1, 3, 4].

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