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PN 1250

A time study of the hydrolysis of lecithin by snake-venom phospholipase A

It has been established[1–3] that snake-venom phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) specifically hydrolyses the ester bond at the β-position of the lecithin molecule irrespective of the type of fatty acid occupying the β-position[3]. Although lecithin obtained from most natural sources is generally believed to consist of molecules with saturated acids occupying the α-position and unsaturated acids occupying the β-position, there have been suggestions[2,4] that a small proportion of native lecithin molecules does not possess this configuration. The results of the study now reported lend support to these suggestions and in addition indicate that phospholipase A hydrolyses certain types of lecithin molecules more readily than others.

The lecithin used in these experiments was prepared from the yolks of fresh eggs by the method of SAUNDERS[5] and was found to give rise to one spot only when chromatographed either on paper impregnated with silicic acid[6] or on thin-layer chromatoplates of Silica Gel G containing 10% (w/w) ammonium sulphate[7] with a solvent system of chloroform–methanol–water (65:25:5, v/v). Enzyme hydrolysis of the lecithin was carried out essentially as described by LONG AND PENNY[8]. To each reaction vessel containing 17.5 μmoles of lecithin and 0.25 μmole of ammonia dissolved in 5 ml of peroxide-free diethyl ether was added 0.05 ml of a solution (0.1%, w/v) of dried snake venom (*Crotalus adamenteus*, L. Light Ltd., Colnbrook, Great Britain) in aqueous 0.005 M $CaCl_2$. After shaking, the reaction mixtures were incubated for periods varying from 2 to 80 min at 18°. The reactions were stopped by the addition of 10 ml of methanol and the mixtures taken to dryness by means of a rotary film evaporator attached to a supply of nitrogen. As rapidly as possible the dry residues were dissolved in chloroform and the resulting solutions filtered. To determine the extent of hydrolysis during the reaction, portions of the chloroform solutions were analysed by thin-layer chromatography with the system described above. The bands of Silica Gel G containing the lysolecithin and unchanged lecithin were then eluted separately with methanol. After removal of the methanol, the phospholipids were digested with $HClO_4$ and the phosphorus content of the digests

determined by the method of CHEN[9]. The amounts of lysolecithin formed after the various time intervals are given in Table I from which it may be seen that the reaction was almost complete after 80 min but about 8 % of lecithin still remained unchanged. For fatty acid analysis, the three lipid components of the reaction mixtures were separated on 3-g columns of silicic acid[10]. After application of the chloroform solutions of the lipids to the columns, free fatty acids were eluted with chloroform, unchanged lecithin with chloroform–methanol (3:2, v/v) and lysolecithin with methanol[11,12].

TABLE I

THE AMOUNTS OF LYSOLECITHIN FORMED DURING THE HYDROLYSIS OF EGG LECITHIN BY SNAKE VENOM (*Crotalus adamenteus*) PHOSPHOLIPASE A

Time (min)	*Lysolecithin (μmoles)*
2	1.9
4	5.9
6	8.1
8	9.1
10	10.0
20	13.2
30	14.2
40	15.1
50	15.4
60	15.8
70	15.9
80	16.1

The efficiency of the separations was checked by thin-layer chromatography. The free fatty acids and the fatty acids of the lecithin and lysolecithin were converted to the corresponding methyl esters by the method of STOFFEL *et al.*[13]. The methyl esters were analysed by gas–liquid chromatography in an Argon Chromatograph (W.G. Pye Ltd., Cambridge, Great Britain) with a column (122 cm × 4 mm) consisting of 10 % (w/v) Apiezon L. grease on 100–120 mesh Celite. The column was operated at 200° with a gas flow of 60 ml/min. Major peaks on the recorder chart were identified by comparison of their retention times with those of the methyl esters of pure acids (obtained from Calbiochem Inc. New York, U.S.A.) and the composition of each sample was calculated by the method of CARROLL[14].

The concentrations of the major fatty acids in the free fatty acids and lysolecithin produced by the enzymic hydrolysis of the lecithin after various reaction times are shown in Figs. 1 and 2, respectively. Marked changes in the composition of the free fatty acids and lysolecithin fatty acids occurred during the first 10 min of the reaction. The concentrations of palmitic (16:0) and stearic (18:0) acids in the free fatty acids decreased but increased in the lysolecithin whereas the concentrations of oleic (18:1) and linoleic (18:2) plus linolenic (18:3) acids increased in the free fatty acids but decreased in the lysolecithin. The results for the unchanged lecithin are not given in detail but it may be of interest to note that the concentration of the saturated fatty acids in the unchanged lecithin increased as the reaction progressed. For instance, the fatty acid composition (molar percentage of the total fatty acids) of the lecithin remaining after 80 min was; palmitic, 40.1; stearic, 29.4; oleic, 19.4; and linoleic plus linolenic, 8.2. Although these results were obtained with a single

preparation of egg lecithin similar findings have been obtained in identical experiments with other preparations.

SAITO AND HANAHAN[15] could find little difference in the composition of the fatty acids released from egg lecithin during hydrolysis by phospholipase A but they did not examine the fatty acids released during the first 10 min.

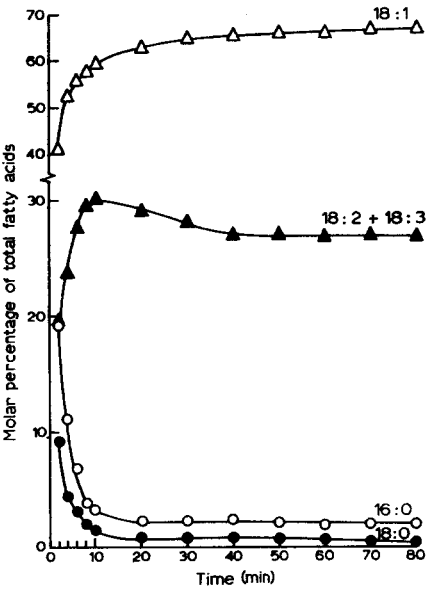

Fig. 1. Variations in the composition of the free fatty acids liberated during the hydrolysis of lecithin by phospholipase A. O—O, palmitic acid; ●—●, stearic acid; △—△, oleic acid; ▲—▲, linoleic + linolenic acids.

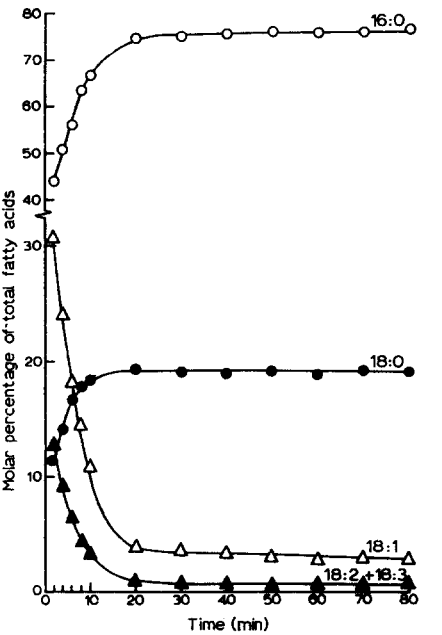

Fig. 2. Variations in the composition of the fatty acids of lysolecithin liberated during the hydrolysis of lecithin by phospholipase A. O—O, palmitic acid; ●—●, stearic acid; △—△, oleic acid; ▲—▲, linoleic + linolenic acids.

It appears therefore that the lecithin used in our experiments contained a small proportion of molecules with an unsaturated fatty acid occupying the α-position and a saturated fatty acid occupying the β-position and that these molecules were hydrolysed more readily than the bulk of the lecithin molecules, *i.e.*, those possessing the reverse ("conventional") configuration. The fatty acid composition of the unchanged lecithin points to the presence of a small amount of lecithin with saturated acids occupying both the α- and β-positions. This type of molecule was apparently hydrolysed less readily than those molecules containing unsaturated acids. These results also imply that the nature of the fatty acid occupying the α-position of the lecithin molecule exerts a considerable influence on the ease with which the ester bond in the β-position is cleaved by the enzyme.

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Metabolism of lysolecithin and lecithin in a yeast supernatant

Lysolecithin was found to cause a very limited ionic leakage from yeast cells, this in contrast to the strong action of synthetic detergents of the quaternary amine type. With the aid of chromatography on silica-impregnated paper it was demonstrated that after a short incubation time the amount of egg lysolecithin added was greatly diminished, while lecithin was found to be present in the supernatant fluid or adsorbed at the cell surface. This conversion of lysolecithin into lecithin was also effected by a supernatant freed from yeast cells by centrifugation, and this ability appeared to be lost after heating the supernatant for 10 min at 100°. Theoretically the formation of lecithin might proceed by an acylation of lysolecithin as observed by LANDS[1] and WEBSTER[2] to occur in the microsomal and mitochondrial fractions of animal tissues. Since phospholipase C (EC 3.1.4.3.)[3] and choline phosphate cytidyl transferase (EC 2.7.7.15.)[4] have been reported to be present in yeast another pathway may play a part as well. Presuming that phospholipase C-formed monoglyceride is converted into diglyceride, lecithin might be formed according to a synthesis *de novo* as established by KENNEDY[5]. However, our experiments showed that the noticed formation of lecithin from lysolecithin in the yeast supernatant involves a quite different mechanism.

An amount of 100 g of bakers yeast (Koningsgist, Delft) was suspended in 300 ml of water and stored at 0° for 48 h. A tracer amount of [^{32}P]lysolecithin was emulsified with 0.5 ml of the clear yeast supernatant fluid together with 0.2 ml of a 0.1 M phosphate buffer (pH 7.4) and the mixture was incubated under shaking at 37°. At various time intervals adequate samples were subjected to chromatography on silica-impregnated paper[6]. Lysolecithin was found to give rise to the formation of labeled lecithin and a radioactive water-soluble compound, which was demonstrated to be identical to glycerylphosphorylcholine (R_F 0.44, propanol - ammonia - water;