The Positional Specificity of Lysosomal Phospholipase A Activities

By H. WINKLER,* A. D. SMITH and FRANÇOISE DUBOIS

Department of Pharmacology, University of Oxford

and H. VAN DEN BOSCH

Department of Biochemistry, University of Utrecht, The Netherlands

(Received 22 August 1967)

The presence of phospholipase A in lysosomes of bovine adrenal medulla has recently been reported (Blaschko, Smith, Winkler, van den Bosch & van Deenen, 1967). Like many other lysosomal enzymes this phospholipase exhibited highest activity at low pH, the actual optimum being at pH 4.2. It was also stated by Blaschko *et al.* (1967) that the large-granule fraction from the adrenal medulla contained a second phospholipase A that had optimum pH 6.5. Further studies on the latter enzyme have shown that it has a distribution in a sucrose density gradient similar to that of typical lysosomal enzymes (H. Winkler & A. D. Smith, unpublished work). In view of these findings it was decided to see whether the phospholipases present in the lysosomal fraction differ not only in their pH optima but also in other aspects. This communication describes the different positional specificity of the two lysosomal phospholipases.

Methods. A fraction rich in lysosomes was isolated from homogenates of bovine adrenal medullae by centrifugation of the large-granule fraction ($2.42 \times 10^5 g \cdot min$. sediment) on a sucrose density gradient, as described by Smith & Winkler (1966). The fraction, corresponding to the density of 1.6M-sucrose, was dialysed against 25mM-glycylglycine-NaOH buffer, pH 6.5.

For use as substrates, lecithins containing radioactive fatty acids in known positions were prepared by chemical synthesis or by biochemical synthesis. A doubly-labelled distearoyl-lecithin, 1-[9,10- 3H]stearoyl-2-[1- ^{14}C]stearoylglycero-3-phosphorylcholine, was synthesized by the method of van den Bosch & van Deenen (1966). Rat liver homogenates were incubated with [9,10- 3H]stearic acid or with [1- ^{14}C]linoleic acid to prepare lecithins labelled in the 1- and 2-position respectively with trace amounts of [3H]stearic acid and [^{14}C]linoleic acid (van den Bosch, 1966).

Lysosomal phospholipase activity was determined by incubating up to 10 μg . of the labelled lecithin under the conditions described by Blaschko *et al.* (1967). For the assay of phospholipase activity at pH 6.5 a 50mM-glycylglycine-NaOH buffer was

used. After thin-layer chromatography (Skipski, Peterson & Barclay, 1964) the silica of the spots corresponding to lecithin, to lysolecithin and to the fatty acids was scraped off and the radioactivity was determined.

Results and discussion. At pH 4.2 1-[9,10- 3H]stearoyl-2-[1- ^{14}C]stearoylglycero-3-phosphorylcholine was hydrolysed by the lysosomal fraction at a rate only 7% of that of rat liver lecithin, whereas at pH 6.5 no hydrolysis could be detected (limit of analysis: 1% of the rate of hydrolysis of rat liver lecithin). However, even with this substrate, there was an indication that the enzyme acting at pH 4.2 preferentially hydrolysed the 1-acyl ester bond since 80% of the 3H in the hydrolysis products was recovered in the fatty acids.

The 3H - and ^{14}C -labelled lecithins prepared from rat liver proved to be better substrates. The distribution of the labelled fatty acids between the 1- and the 2-position of these substrates was first determined by using *Crotalus adamanteus* venom; the phospholipase A in this venom is known to be specific for the 2-position of lecithin (de Haas, Daemen & van Deenen, 1962). The results (Table 1) show that 85% of the [3H]stearic acid was in the 1-position and that 95% of the [^{14}C]linoleic acid was in the 2-position of the respective substrates. When the two lysosomal enzymes were tested with these substrates it was found that the enzyme active at pH 6.5 had a specificity similar to that of snake-venom phospholipase, whereas the enzyme active at pH 4.2 had a different site of action (Table 1).

The lysosomal enzyme that acts at pH 4.2 is almost entirely specific for the 1-position of lecithin: it released 3H -labelled fatty acids from the [3H]lecithin and [^{14}C]lysolecithin from the [^{14}C]lecithin. The presence in tissues of a phospholipase A acting at pH 4 and specific for the 1-position of lecithin has been described by Lloveras, Douste-Blazy & Valdiviú (1963) for calf spleen and by Gatt, Barenholz & Roitman (1966) for rat brain. The latter enzyme was present in a particulate fraction that was not further fractionated; it would, however, have contained the lysosomes. Phospholipases specific for the 1-position of lecithin, but whose

* Permanent address: Pharmakologisches Institut der Universität, Innsbruck, Austria.

Table 1. *Action of lysosomal phospholipases on rat liver lecithins compared with that of Crotalus adamanteus venom*

After incubation of 10 μ g. of substrate with the enzyme, the lipids were extracted and separated by thin-layer chromatography. The results are expressed as percentages of the recovered radioactivity found in the regions of the silica gel corresponding to lecithin, lysolecithin and fatty acids. The protein nitrogen content of the lysosomal fraction is given for each experiment in parenthesis after the pH. For the experiments with *Crotalus adamanteus* venom, an amount of venom was used that gave the same degree of breakdown of each substrate.

(a) Rat liver lecithin containing [3 H]stearic acid in the 1-position

	Lysosomal fraction		<i>Crotalus adamanteus</i>
	pH 4.2 (1.5 μ g. of N)	pH 6.5 (4.5 μ g. of N)	
Lecithin	84.0	79.2	61.0
Lysolecithin	2.2	12.4	33.1
Fatty acid	13.8	8.4	5.9

(b) Rat liver lecithin containing [14 C]linoleic acid in the 2-position

	Lysosomal fraction		<i>Crotalus adamanteus</i>
	pH 4.2 (1.5 μ g. of N)	pH 6.5 (13.5 μ g. of N)	
Lecithin	78.6	77.2	61.9
Lysolecithin	15.6	6.8	1.9
Fatty acid	5.8	16.0	36.2

activity was measured at or above pH 7, have been found in rat liver, lung and spleen (van den Bosch & van Deenen, 1964) and in rat and horse pancreas (van den Bosch, Postema, de Haas & van Deenen, 1965). Recently the microsomal fraction from rat liver has been shown to contain a phospholipase specific for the 1-position (Scherphof, Waite & van Deenen, 1966; Waite & van Deenen, 1967).

The lysosomal phospholipase acting at pH 6.5 preferentially removed the 2-acyl residue from lecithin, as shown by the release of 14 C-labelled fatty acid from the [14 C]lecithin and the formation of [3 H]lysolecithin from the [3 H]lecithin (Table 1). In addition, at this pH the lysosomal fraction catalysed the hydrolysis of the 1-acyl ester bond of lecithin, but at about half the rate at which it hydrolysed the 2-acyl ester bond. Three possible explanations of this finding are: first, that one enzyme acts at pH 6.5 and that this enzyme can hydrolyse both the ester bonds; secondly, that two enzymes with this pH optimum are present, one specific for the 1-position and the other specific for the 2-position; or thirdly, that the hydrolysis of the 1-acyl ester bond at pH 6.5 is due to the action of the other lysosomal enzyme with optimum pH 4.2. The third explana-

tion seems the most likely since at pH 4.2 lecithin is broken down by the lysosomal fraction two to three times more rapidly than it is at pH 6.5; and it can be calculated that if the activity of the 1-position-specific enzyme at pH 6.5 is 3% of its activity at pH 4.2 then this would account for the formation of [14 C]lysolecithin. It is therefore probable that lysosomes contain a phospholipase A specific for the 2-position of lecithin. Other tissue phospholipases with this positional specificity, but with different pH optima, have been found in pancreas (see van Deenen, de Haas & Heemskerk, 1963) and in rat liver (van den Bosch & van Deenen, 1965), and the latter enzyme is present in a mitochondrial fraction (Scherphof *et al.* 1966; Waite & van Deenen, 1967).

It is noteworthy that at pH 6.5 three times as much of the lysosomal fraction was required to give the same rate of release of the [14 C]linoleic acid from the 2-position of the [14 C]lecithin as was needed for the release of unlabelled fatty acids from the 2-position of the [3 H]lecithin. Although several explanations of this observation are possible, it is noteworthy that previous workers have found that some snake-venom phospholipases also liberate different fatty acids at different rates from the 2-position of lecithin (Moore & Williams, 1964; Nutter & Privett, 1966).

The results described above show that two phospholipases, each with a different positional specificity, are present in one type of subcellular particle: the lysosomes of the bovine adrenal medulla. We propose, in accordance with current usage (see van Deenen & de Haas, 1966), to call these enzymes lysosomal phospholipases A₁ and A₂. The products of the action of these enzymes on lecithin are two isomeric lysolecithins. This raises the question whether lysosomes can also metabolize lysophospholipids.

This work has been supported by a grant from the Medical Research Council. H.W. holds a J. H. Burn Research Fellowship and A.D.S. is a Royal Society Stothert Research Fellow.

- Blaschko, H., Smith, A. D., Winkler, H., van den Bosch, H. & van Deenen, L. L. M. (1967). *Biochem. J.* **103**, 30c.
 de Haas, G. H., Daemen, F. J. M. & van Deenen, L. L. M. (1962). *Biochim. biophys. Acta*, **65**, 260.
 Gatt, S., Barenholz, Y. & Roitman, A. (1966). *Life Sci.*, **24**, 169.
 Lloveras, J., Douste-Blazy, L. & Valdiguié, P. (1963). *C.R. Acad. Sci., Paris*, **256**, 1861.
 Moore, J. H. & Williams, D. L. (1964). *Biochim. biophys. Acta*, **84**, 41.
 Nutter, L. J. & Privett, O. S. (1966). *Lipids*, **1**, 258.
 Scherphof, G. L., Waite, M. & van Deenen, L. L. M. (1966). *Biochim. biophys. Acta*, **125**, 406.
 Skipski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.

- Smith, A. D. & Winkler, H. (1966). *J. Physiol.* **183**, 179.
- van Deenen, L. L. M. & de Haas, G. H. (1966). *Annu. Rev. Biochem.* **35**, 157.
- van Deenen, L. L. M., de Haas, G. H. & Heemskerk, C. H. Th. (1963). *Biochim. biophys. Acta*, **67**, 295.
- van den Bosch, H. (1966). Doctoral Thesis: University of Utrecht.
- van den Bosch, H., Postema, N. M., de Haas, G. H. & van Deenen, L. L. M. (1965). *Biochim. biophys. Acta*, **98**, 657.
- van den Bosch, H. & van Deenen, L. L. M. (1964). *Biochim. biophys. Acta*, **84**, 234.
- van den Bosch, H. & van Deenen, L. L. M. (1965). *Biochim. biophys. Acta*, **106**, 326.
- van den Bosch, H. & van Deenen, L. L. M. (1966). In *Advances in Tracer Methodology*, vol. 3, p. 61. Ed. by Rothchild, S. New York: Plenum Press.
- Waite, M. & van Deenen, L. L. M. (1967). *Biochim. biophys. Acta*, **137**, 498.