Lecithinase and Lysolecithinase of Intestinal Mucosa

By B. EPSTEIN* and B. SHAPIRO
Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 25 *June* 1958)

Enzymes, capable of hydrolysing lecithin to liberate inorganic phosphate, are present in the kidney and intestinal mucosa (King, 1931). Since several pathways may lead from lecithin to inorganic phosphate and more than a single enzyme must have been involved, it is uncertain which enzyme was the rate-limiting one in this investigation. In a recent paper Schmidt, Bessman & Thannhauser (1957) described a mitochondrial preparation of rat intestinal mucosa, which catalysed the splitting of fatty acids from kephalin. Some activity, though a much smaller one, was found with lecithin as substrate. The low activity of similar intestinal-mucosa preparations towards lecithin was attributed by us, in a preliminary communication (Epstein & Shapiro, 1957), to the fact that lecithin degradation by the intestinal enzyme depends upon the presence of fatty acids. This activation by one of its reaction products puts the enzyme involved into a category apart from other lecithinases. As will be seen in the present paper, intestinal lecithinase differs from known lecithinases in several other respects.

EXPERIMENTAL

Materials

Ovolecithin. This was prepared according to Hanahan, Turner & Jayko (1951). A 0·5% (w/v) suspension was prepared by mixing the lecithin with hot water in a Waring Blendor. The suspension was kept frozen.

Lysolecithin. After preparation according to Belfanti (1941), 10 mg. was suspended in 1 ml. of water. The suspension was kept frozen.

Acyl ester. Content of acyl ester was determined according to Shapiro (1953).

L-α-*Glycerylphosphorylcholine.* The cadmium salt of this substance was kindly supplied by Dr G. Schmidt and was freed of cadmium according to Tattrie & McArthur (1955). It was determined in reaction mixtures according to Shapiro (1953).

Fatty acids. The acids were dispersed in boiling water to give a 25 mM-solution of pH 8–9 after addition of 0·1 N-KOH. The solution was kept in a refrigerator and was warmed before use to dissolve the fatty acids. Their concentration in the reaction mixtures was determined by titration according to Dole (1956).

Albumin. Bovine serum albumin (Pentex Co. Incorp., Kankakee, Ill., U.S.A.) was used.

Protein. This was determined by a modification of the method of Herriot (1941) according to Kaufman & Wertheimer (1957).

Enzyme preparations

Preparation of S_1 and P_1. Rats were killed by decapitation, the small intestines were removed and rinsed through with 0·9% NaCl soln. After the intestines were cut open, the mucosa was scraped off with a blunt spatula. In all the following steps the preparation was kept below 5°. The mucosa was ground with an equal weight of sand, and with water (2 ml./g. of mucosa); the mixture was then centrifuged at 800 *g* for 5 min. The supernatant (S_1) was brought to pH 5·6 with 0·1 N-acetic acid or 0·1 N-HCl, centrifuged at 12 000 *g* and the precipitate (P_1) was resuspended in 0·02 M-sodium phosphate buffer (2 ml./g. of original mucosa) so that the final pH was 6·5.

'Mitochondrial' and 'microsomal' fractions. The mucosa was homogenized in a tight-fitting glass homogenizer with 10 vol. of 1·15% (w/v) KCl. In all the following steps the preparation was kept below 5°. The suspension was centrifuged at 12 000 *g* for 20 min. The precipitate was homogenized again with 5 vol. of 1·15% KCl and centrifuged at 700 *g* for 5 min. The supernatant was centrifuged at 12 000 *g* for 20 min. and the precipitate, called the 'mitochondrial fraction', was resuspended in 0·02 M-sodium-phosphate buffer (1 ml./g. of original mucosa), so that the final pH was 6·5. The first and last supernatant fractions were combined and re-centrifuged at 40 000 *g* in a Spinco centrifuge; the resulting precipitate, called the 'microsomal fraction', was resuspended as above.

Reaction mixtures. Unless otherwise stated these were composed of 5 μmoles of lecithin, 5 μmoles of sodium oleate, 40 μmoles of sodium phosphate buffer, pH 6·5, and 0·3 ml. of P_1 and were made up to 1 ml.

With lysolecithin, 7 μmoles of substrate and 0·1 ml. of P_1 were used.

Incubation. This was at 37° for the indicated time intervals and the reaction was stopped by adding 4 ml. of trichloroacetic acid soln. (5%, w/v) for glycerylphosphorylcholine estimation or with 4 ml. of ethanol–ether mixture (3:1, v/v) for determination of the acyl ester content.

RESULTS

As already reported in our preliminary note (Epstein & Shapiro, 1957) breakdown of lecithin with the enzyme preparation described was preceded by a lag phase. This lag phase could be abolished by increasing the enzyme concentration or by adding fatty acids (Fig. 1).

* Part of Ph.D. thesis submitted to the Senate of the Hebrew University, Jerusalem, Israel.

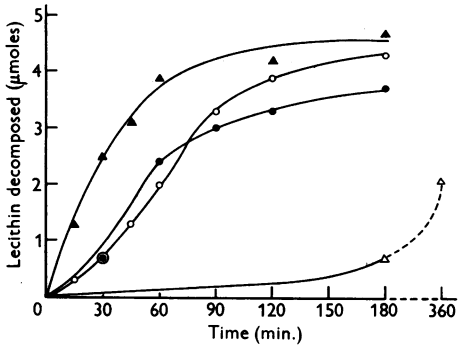

Fig. 1. Effect of palmitic acid and enzyme concentration on intestinal lecithinase activity. △, With 0·3 ml. of enzyme; ○, with 0·3 ml. of enzyme + 4 μmoles of palmitic acid; ●, with 0·6 ml. of enzyme; ▲, with 0·6 ml. of enzyme + 4 μmoles of palmitic acid. The rate of reaction was determined by following the decrease in acyl ester content.

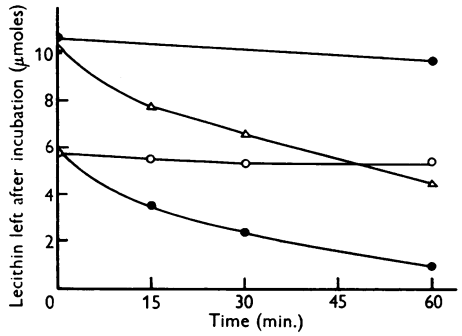

Fig. 2. Effect of lecithin concentration on the concentration of oleic acid required for activation. ○, Without added oleic acid; ●, with 5 μmoles of oleic acid; △, with 10 μmoles of oleic acid.

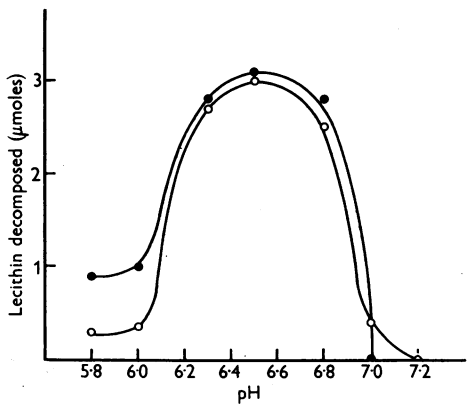

Fig. 3. pH–Enzyme activity curve of intestinal lecithinase in the presence of oleic acid. ○, Glycerylphosphorylcholine formation; ●, decrease in ester content. Time of reaction: 45 min.

Table 1. *Effectiveness of various fatty acids in shortening the lag period of lecithin degradation*

Fatty acid (5 μmoles) added	Time required for the splitting of 1·4 μmole of lecithin (min.)
Linoleic acid	10
Oleic acid	10
Lauric acid	26
Myristic acid	58
Decanoic acid	96
Palmitic acid	144
Stearic acid	177
Octanoic acid	196
Hexanoic acid	210
Butyric acid	246
Acetic acid	300
Without acid	360

Table 2. *Breakdown of lecithin and lysolecithin by various enzyme preparations from intestinal mucosa*

Enzyme system	Hydrolysis of substrate (μmoles/hr./mg. of protein) With lecithin	With lysolecithin
S_1	0·84	7·0
P_1	1·84	10·0
'Microsomes'	1·60	20·0
'Mitochondria'	1·10	12·4

The amount of fatty acid required for optimum activity was tested and it was found that this point was reached when amounts nearly equimolar to those of the lecithin were added. When the lecithin concentration was increased to 10 μmoles/ml. the usual amount of oleate (5 μmoles/ml.) was not enough to start the reaction and larger amounts of fatty acid were required (Fig. 2).

The effectiveness of the various homologous fatty acids in shortening the lag period was tested (Table 1). Oleic and linoleic acid were found to be the most active and the only acids which abolished the lag completely (the shortest time tested was 10 min.). The order of effectiveness in shortening the lag period in the saturated fatty acids series tested was:

$$C_{12} > C_{14} > C_{10} > C_{16} > C_{18} > C_8 > C_6 > C_4 > C_2.$$

In all subsequent experiments reported in this paper oleic acid was used.

The pH–enzyme activity curve in Fig. 3 shows a very sharp pH optimum at 6·5 and little activity at pH 6 or pH 7.

Generally close agreement was found between the decrease in ester content and the formation of glycerylphosphorylcholine. This is due to the large excess of lysolecithin-splitting enzyme in all the preparations used (Table 2). Lysolecithin breakdown proceeded without addition of fatty acid and

the rate of the reaction was unaffected by increasing the substrate concentration from 5 to 10 mM.

Endogenous fatty acids as activators. The activity found with the intestinal lecithinase in the absence of external fatty acids and the shortening of the lag period by increasing the enzyme concentration (Fig. 1) were thought to be due to endogenous fatty acids in the enzyme preparation. Estimation of free fatty acids in the enzyme preparation revealed that it contained 7 μmoles/ml. (the high content of endogenous fatty acids is due to hydrolysis of endogenous lipids during the preparation and storage of the enzyme). Various methods were tried to remove the endogenous fatty acids from the enzyme. Extraction of fresh mucosa, S_1 or lyophilized S_1, with acetone was found to inactivate the enzyme irreversibly; lyophilization, itself, had no detrimental effect on the activity. On the other hand, the endogenous fatty acids could be bound and inactivated by the addition of albumin or rat serum to the reaction mixture (Table 3) and finally it was shown that by washing P_1 with albumin solution the endogenous fatty acid content could be decreased to 0·5 μmole/ml. (Table 4) and only negligible lecithin breakdown could be demonstrated even after incubation for 24 hr. Addition of oleic acid to the albumin-washed enzyme restored about 50 % of the original activity, but butyric acid had no such activating effect.

Effect of metals on intestinal lecithinase. This is shown in Table 5. Hg^{2+}, Cd^{2+} and Ca^{2+} ions had a considerable inhibitory effect at the concentrations used. Ca^{2+} ion was not required for activity and the hydrolysis was not impaired by the addition of sodium ethylenediaminetetra-acetate (EDTA), pH 6·5, or by dialysis for 24 hr. against 0·02 M-sodium phosphate buffer, pH 6·5, at 5°. The inhibition by 0·4 mM-Cd^{2+} ion was not reduced by doubling the amount of oleic acid or lecithin. On the other hand, it could be reversed with EDTA (Table 6). Even after removal of possible inhibitory cations in the reaction mixture, the enzyme activity was still dependent on fatty acids.

Stability of lecithinase and lysolecithinase. This was examined when the compounds were incubated at 30° at various pH values. It was found that the enzymes were the most stable at pH 6·5 and that at the other pH values the activity dropped (Table 7).

Heat stability of intestinal lecithinase and lysolecithinase at pH 6·5. This is shown in Table 8. Heating the enzyme to 50° for 10 min. caused 100 % inactivation, at 40° for 10 min. 50% inactivation. Lecithin protected the enzyme from heat-inactivation and pre-incubation at 40° for 10 min. with lecithin even caused some activation. Since pre-incubation with lecithin was carried out without added fatty acids, no detectable hydrolysis occurred during this period. Lysolecithinase activity followed the same stability pattern as intestinal lecithinase activity.

Table 3. *Influence of albumin and rat serum on lecithin breakdown by intestinal lecithinase*

Addition	Hydrolysis (μmoles of lecithin/45 min.)
None	3·4
Albumin soln. 7·5%	
0·1 ml.	3·1
0·2 ml.	0·6
0·3 ml.	0
Rat serum	
0·1 ml.	3·3
0·2 ml.	2·4
0·3 ml.	0

Table 4. *Removal of endogenous fatty acids by washing with albumin solution*

Expt. no. 1. Albumin solution (30 %, w/v) was brought to pH 5·6 with 0·1 N-acetic acid; 4 ml. of this solution was mixed with 4 ml. of P_1 in a glass homogenizer. The mixture was centrifuged at 13 000 *g*. Residual albumin was removed by treating the resulting precipitate as above with 4 ml. of 0·02 M-sodium phosphate buffer, pH 5·6. The final precipitate was resuspended in 4 ml. of 0·02 M-sodium phosphate buffer, pH 6·5. Expt. no. 2. As in Expt. no. 1 except that P_1 was washed with 16 ml. of albumin solution (8 %, w/v).

Expt. no.	Enzyme system	Fatty acid (μmoles/ml. of enzyme)	Hydrolysis: Without added oleate (μmoles of lecithin/ 24 hr.)	Hydrolysis: With 5 μmoles of oleate (μmoles of lecithin/ 60 min.)	Hydrolysis: With 5 μmoles of oleate (μmoles of lecithin/60 min./ mg. of protein)
1	P_1	7	—	3·6	—
	Washed P_1	0·5	0·4	1·5	—
2	P_1	7	2·1	2·7	0·93
	Washed P_1	0·7	0·5	1·3	0·68
			0·4*	—	—

* With 5 μmoles of butyric acid.

Effect of detergents. Oleic acid could not be replaced by detergents such as Tween 80, cholic acid or saponin. The first two substances were even found to be inhibitory in the presence of added oleic acid (Table 9).

DISCUSSION

The obligatory requirement of fatty acids for the activity of intestinal lecithinase presents an unusual case of activation of an enzyme by its reaction product. The mechanism of this activation is not yet clear. It is not due merely to an emulsifying action of the fatty acids, since much more efficient emulsifiers, such as Tween 80 or cholic acid, are devoid of activity. The fact that the amount of fatty acid required for optimum activity depends upon the amount of substrate, and is roughly equimolar to it, points to a substrate–activator complex as a possible basis of activation. An alternative explanation that fatty acids may bind inhibitory cations, such as Cd^{2+} or Ca^{2+} ions, is unlikely, since these ions retained their inhibitory effect in the presence of excess of fatty acids and, moreover, after removal of Ca^{2+} ion inhibition by treatment with EDTA, fatty acids were still required for activity.

The enzyme obtained from intestinal mucosa differed from other lecithinases in several other respects. It was bound to particles, was highly

Table 5. *Influence of various metals on intestinal lecithinase activity*

Incubation was carried out for 45 min. Breakdown of lecithin without addition of metal was 3·4 μmoles.

Cation added	Concn. (mM)	Inhibition (%)
Hg^{2+}	0·1	14
Hg^{2+}	0·4	100
Cd^{2+}	0·4	70
Cu^{2+}	0·4	30
Zn^{2+}	0·4	20
Ca^{2+}	5·0	50
Fe^{3+}	3·0	26
Ni^{2+}	4·0	9
Co^{2+}	4·0	9

Table 6. *Influence of sodium ethylenediaminetetraacetate on inhibition by cadmium ions*

Breakdown of lecithin without addition of metal: 2·7 μmoles/45 min.

Addition to reaction mixture (without oleate)	Relative activity
5 μmoles of oleic acid	100
5 μmoles of oleic acid + 0·4 μmole of Cd^{2+}	55
5 μmoles of oleic acid + 1 μmole of EDTA	100
5 μmoles of oleic acid + 0·4 μmole of Cd^{2+} + 1 μmole of EDTA	100
1 μmole of EDTA	0

Table 7. *Stability of the enzyme at 30° at various* pH *values*

Enzyme (0·3 ml.) was pre-incubated at the indicated pH values for 30 min. at 30° and the reaction mixture was then readjusted to pH 6·5. Hydrolysis of the substrates without pre-incubation was 3·5 μmoles/45 min. for lecithin and 4·0 μmoles/20 min. for lysolecithin. The relative activities are percentages of these values.

pH of pre-incubation	Relative activity: With lecithin	Relative activity: With lysolecithin
5·5	20	—
5·7	74	72
6·0	83	89
6·5	100	100
6·7	94	—
7·0	83	—
7·5	77	55
8·0	23	—

Table 8. *Stability of the enzyme to pre-incubation at various temperatures*

Enzyme (0·3 ml.), with or without lecithin, was pre-incubated at pH 6·5 as indicated; the reaction mixture was then completed. Incubation time and hydrolysis without pre-incubation was 2·6 μmoles/45 min. for lecithin and 5·0 μmoles/20 min. for lysolecithin. Results are expressed relative to the hydrolysis after pre-incubation for 30 min. at 30°.

Temperature	Time (min.)	Relative activity: With lecithin: Pre-incubated without substrate	Relative activity: With lecithin: Pre-incubated with substrate	Relative activity: With lysolecithin. Pre-incubated without substrate
30°	30	100	—	—
37	30	27	142	—
40	10	50	150	88
45	10	23	84	62
50	10	0	19	11
55	10	0	11	6

Table 9. *Effect of detergents on intestinal lecithinase*

Addition to reaction mixture (without oleate)	Hydrolysis (μmoles of lecithin/45 min.)
4 μmoles of oleic acid	2·6
4 μmoles of Tween 80	0
4 μmoles of Tween 80 + 4 μmoles of oleic acid	0
1 μmole of Tween 80	0
1 μmole of Tween 80 + 4 μmoles of oleic acid	2·6
1 mg. of saponin	0·2
5 μmoles of cholic acid + 4 μmoles of oleic acid	1·0

thermolabile and did not require Ca^{2+} ions as treatment with EDTA or dialysis had no effect on the activity.

Lysolecithinase activity was not separated from the lecithinase by the procedures used. Both enzymes showed similar pH-dependence and heat-susceptibility. However, lysolecithinase did not require fatty acids for activity. Contrary to the findings with pancreas lysolecithinase (Shapiro, 1953) and liver lysolecithinase (Dawson, 1956), no self-inhibition by the substrate was found with intestinal lysolecithinase up to a concentration of 10 mM-lysolecithin. It is of significance that lysolecithinase activity was always much greater than that of the lecithinase. No considerable amounts of lysolecithin can therefore accumulate during splitting of lecithin in the intestinal mucosa.

It may be of significance for the biological function of intestinal lecithinase that the optimum conditions for its activity are those which can be expected to prevail in the mucosal cells during absorption of fat, i.e. an abundance of free fatty acids. On the other hand, the conditions in the intestinal lumen, which favour lipase action, namely the presence of bile acids and Ca^{2+} ions, are unsuitable for lecithinase activity.

SUMMARY

1. A particulate enzyme system which decomposes lecithin and lysolecithin into glycerylphosphorylcholine and fatty acids was isolated from the mucosa of rat intestines.

2. Lecithin breakdown was found to depend upon the presence of fatty acids. The optimum concentration of fatty acids depends upon the concentration of the substrate and is roughly equimolar to it. With suboptimum concentrations (e.g. with the endogenous fatty acids present in the enzyme preparation) the reaction is autocatalytic, starting with a lag period. This lag period can be prolonged to more than 24 hr. by removing most of the endogenous fatty acids from the enzyme by washing it with serum albumin solution.

3. Linoleic and oleic acid were the most efficient activators found, followed by lauric and decanoic acid of the saturated acid series. Tween 80, cholic acid and saponin could not replace fatty acids.

4. Ca^{2+} ion was not required for activity and was inhibitory at a concentration of 5 mM. Other bivalent cations, especially Hg^{2+} and Cd^{2+} ions, were also strongly inhibitory.

5. Lecithinase and lysolecithinase action had a pH optimum at 6·5 which was also the point of greatest stability. The enzyme was inactivated by short incubations at 30–50° but lecithin gave some protection.

6. Activity with lysolecithin was always greater than with lecithin and no fatty acids were required for breakdown of the former compound.

REFERENCES

Belfanti, S. (1941). In *Die Methoden der Fermentforschung*, vol. 1, p. 89. Ed. by Bamann, E. & Myrbäck, K. Leipzig: Georg Thieme.

Dawson, R. M. C. (1956). *Biochem. J.* **64**, 192.

Dole, V. (1956). *J. clin. Invest.* **35**, 150.

Epstein, B. & Shapiro, B. (1957). *Nature, Lond.*, **180**, 387.

Hanahan, D. J., Turner, M. B. & Jayko, M. E. (1951). *J. biol. Chem.* **192**, 632.

Herriot, R. M. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 642.

Kaufman, E. & Wertheimer, E. (1957). *Amer. J. Physiol.* **190**, 133.

King, E. J. (1931). *Biochem. J.* **25**, 799.

Schmidt, G., Bessman, M. J. & Thannhauser, S. J. (1957). *Biochim. biophys. Acta*, **23**, 138.

Shapiro, B. (1953). *Biochem. J.* **53**, 663.

Tattrie, N. H. & McArthur, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 761.