Estimation and subcellular distribution of lecithinase activity in rat intestinal mucosa

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SUMMARY A method for the quantitative estimation of lecithinase has been developed using rat intestinal mucosa as the lecithinase source. Optimal conditions for lecithinase preparation and assay involve the use of buffered glycerol media with cysteamine and EDTA to stabilize the activity. Lecithin hydrolysis proceeds with apparent first order reaction kinetics when sonicated lecithin-oleate suspensions are used: in these conditions the rate of hydrolysis is proportional to the lecithinase concentration. The method gives satisfactory results in subcellular distribution studies. Fractionation by differential centrifugation and by treatment with polymixin or pH change strongly suggest that the enzyme activity is bound to ribosomal structures.

THE PRESENCE of phospholipase activity in rat intestinal mucosa was first demonstrated by Schmidt et al. (1) using cephalin as a substrate. Epstein and Shapiro (2) later showed that lecithin and lysolecithin are hydrolyzed by rat intestinal mucosa with liberation of fatty acids and glycerylphosphoryl choline (GPC). Lecithinase, defined (2) as the enzyme (or enzyme system) that attacks lecithin with liberation of 2 moles of fatty acid and 1 mole of GPC, has been found in other tissues as well (3, 4). Evidence bearing on the existence of active and inactive lecithinase in rat tissues other than mucosa or in the mucosa under special conditions (X-irradiation) has been presented (5–8). A quantitative method for the estimation of lecithinase and data concerning the subcellular distribution and partial purification of the enzyme are reported in this paper.

MATERIALS AND METHODS

Preparation of Lecithin

Lecithin was prepared from egg yolks and purified on silicic acid columns according to the method of Rhodes and Lea (9). After removal of traces of organic solvent under vacuum and nitrogen, the lecithin was dispersed at 30–35° in an aqueous solution (pH 8 to 9 with 0.2 N KOH) containing an equimolar amount of oleic acid. This procedure is essentially identical to the one used by Epstein and Shapiro (2), who showed that intestinal lecithinase requires added fatty acids to initiate the reaction. The final volume was adjusted to a concentration of 12 μmoles of phospholipid P per ml and the suspension was divided into aliquots for storage at −15°. After thawing the frozen samples in a water bath at 37°, adequate dispersion of the lecithin–oleate mixture for the lecithinase assay was obtained by sonication. Sonication conditions were as follows: Raytheon 200 Watt, Magnetostrictive Oscillator, Model DF-101, (Raytheon Manufacturing Co., Waltham 54, Massachusetts); average output current 1.1 amp; lecithin suspension volume (minimum) 20 ml; sonication time 3 min, circulating water temperature 5°.

Preparation of Lecithinase and Assay Procedure

The directions given here represent the method finally adopted for testing lecithinase activity as a result of the studies presented in detail in the following section. Not all conditions can be considered as absolute [i.e., choice of homogenization medium, addition of ethylenediamine tetraacetic acid (EDTA)]; the procedure given here is the one which in our experience can be used in a variety of experimental conditions to give comparable and reproducible results.

Rat intestinal mucosa homogenates were obtained by the following procedure. Male Osborne-Mendel rats of 175–200 g were starved overnight and killed by cervical dislocation followed by decapitation. The small intestine was removed, slit longitudinally and rinsed in water to free it from the gross contents and then washed more thoroughly in ice-cold isotonic KCl. The intestine was dried on folded cheesecloth and scraped with a blunt

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glass slide; the mucosal sample thus collected was weighed and homogenized in 10 volumes of cold 25% (v/v) glycerol, 2×10^{-3} M in both cysteamine and EDTA and buffered at pH 6.4 with potassium phosphate (the phosphate buffer was made up in two-thirds the final volume of solvent required for an ionic strength value of 0.1, in order to compensate for the dilution taking place in the test procedure).

For assay the homogenate or fractions of it were diluted with buffer containing cysteamine and EDTA to adjust the glycerol concentration to 12.5%. Routinely the test for lecithinase was conducted in 15-ml glass-stoppered centrifuge tubes by mixing 1 ml of the enzymatic preparation and 0.5 ml of the lecithin–oleate mixture equilibrated at 37° for a few minutes prior to addition. Incubation at 37° was continued and the reaction was stopped by addition of 0.1 ml of 2 N H_2SO_4. The fatty acids liberated by lecithinase action were estimated after extraction in hexane according to the method of Dole (10). Occasionally the hydrolysis of lecithin was measured by the hydroxamate method described by Magee and Thompson (11): smaller aliquots of materials (final reaction mixture 0.3–0.4 ml) are required in this method which gives, as a rule, reliable results but is somewhat less adaptable for experiments involving high protein and salt concentrations.

In a few critical experiments the GPC formed during hydrolysis of the lecithin was determined (6). In all tests the molar ratio of liberated fatty acids and GPC was 2:1, thus verifying the assumption that "lecithinase activity," as designated here, involves splitting of both fatty acid ester bonds and that lysolecithin does not accumulate as the reaction proceeds. Similar results and conclusions have been presented by Epstein and Shapiro (2) and by Robertson and Lands (3).

Polymixin Precipitation

Mitochondrial or microsomal supernatant fractions were added to 0.3–0.4 volumes of 1% polymixin B sulfate, kept for 10 min at 0°, and then brought to 3 times the original volume by addition of water containing cysteamine and EDTA. After a further 20 min at 0°, the precipitated enzyme was separated by a short centrifugation (10 min) at 30,000 × *g*. The pellet was finally resuspended in concentrated phosphate buffer (0.3–0.4 M), and adjusted for test conditions.

Protein content was determined by the method of Lowry (12) or by ultraviolet absorption (13).

RESULTS

Choice of Homogenization Medium

The stabilizing effect of glycerol on phospholipases has already been reported (14, 15) and has been confirmed for the intestinal lecithinase. A definite optimum of glycerol concentration could not be established: in media containing cysteamine and EDTA (2×10^{-3} M) no loss of activity could be detected over a 2 hr period at temperatures of 0° and 25°, when 25% glycerol (v/v) was present. Lowering the glycerol concentration to 12.5% had little effect on recovery of activity after storage at 0°, but appreciably decreased the activity after storage at 25°. Higher concentrations of glycerol up to 37.5% showed no definite advantage over the lower range of concentrations (12–25%), which was finally adopted for the homogenization medium.

The requirement for added SH groups (glutathione, cysteamine) was first observed in a preliminary series of fractionation experiments involving prolonged centrifugation in different media. In all instances addition of SH compounds to a final concentration of 2×10^{-3} M increased the amount of activity found in each fraction and the over-all recovery. It was also found that EDTA (1 to 2×10^{-3} M) had some additional stabilizing effect on the lecithinase. These findings are in agreement with the experiments of Epstein and Shapiro (2) on the inhibitory effect of cations and with our own evidence concerning the reversible inactivation of the enzyme with SH-group blocking agents (*p*-chloromercuribenzoate, dithiodiformamidine).

Conditions for Lecithinase Assay

Preparation of Lecithinase

The preparations used in these studies were obtained by centrifugation of the mucosal homogenate for 10 min at 12,000 × *g* at 0–5° to remove clumps of homogenized mucosa. Approximately 60% of the total activity was in the supernatant fraction and practically all the lecithinase of the homogenate was recovered by two washings of the pellet. Routinely the pellet washing was omitted and the first supernatant solution was used without further treatment.

Preparation of Substrate, Reaction Rates, K_m

Epstein and Shapiro have shown that addition of fatty acids is necessary to obtain high initial rates of hydrolysis of lecithin by intestinal lecithinase. Formation of "lecithin–oleate complexes" and consequent facilitation of enzyme–substrate interaction was suggested as a probable mechanism for this effect. We have confirmed these findings with the additional observation that whereas sonication of an aqueous suspension of lecithin alone does not abolish the requirement for oleate, sonication of lecithin–oleate mixtures is necessary in order to obtain maximal initial rates of hydrolysis. With unsonicated lecithin–oleate mixtures (Fig. 1) the reaction exhibited a definite lag period which was

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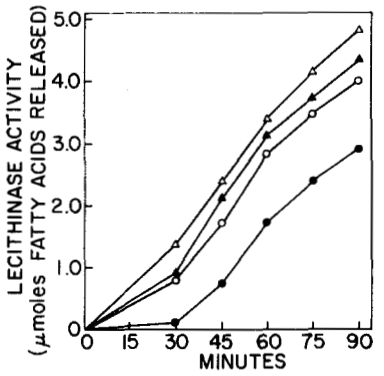

FIG. 1. The effect of ionic strength on lecithinase activity in unsonicated lecithin–oleate mixtures. ●——● μ = 0.10; ○——○ μ = 0.20; ▲—▲ μ = 0.36; △—△ μ = 0.48. μ = ionic strength. All other experimental conditions as given under Methods.

more pronounced at relatively low ionic strength (μ = 0.06–0.1) and was progressively reduced by increasing the ionic concentration of the medium. No ion specificity appears to be involved in this effect, which was reproduced by the addition of equivalent amounts, in terms of ionic strength, of a variety of salts (Na and K chlorides, sulfates, and K phosphates). In contrast, varying the ionic concentration of the medium had no effect on the reaction rate when sonicated lecithin–oleate mixtures were used (Fig. 2). With these preparations the reaction was found to proceed according to first order kinetics up to 25% completion, with a subsequent decline in reaction rate probably due to enzyme inhibition by the accumulated fatty acids (2) (Fig. 3).

Although in view of the complexity of the system no definite conclusions can be advanced, these findings suggest that the best reaction conditions are realized when the size of the lecithin–oleate micelles is below a certain value, as in the sonicated mixtures; in these conditions other factors, presumably involved in the ionic strength effect (dissociation of the oleic acid could possibly be one), become less critical. In support of this contention are the results of a comparison of reaction rates (Fig. 4) in samples of lecithin–oleate sonicated for different intervals of time, showing that the maximum rate of hydrolysis is already reached in samples sonicated for only 30 sec: prolonging the sonication up to 6 min further decreased the optical density of the samples (used here as a gross indication of dispersion) but was without effect on the rate of the reaction. Similar conclusions regarding the importance of the physicochemical state of the substrate in the hydrolysis of the lecithin by snake venom have been presented by Dawson (16).

Using sonicated lecithin–oleate mixtures, lecithin hydrolysis is proportional to the amount of added enzyme over a rather extended range of concentrations. Figure 5 shows the data from an experiment in which lecithinase activity was estimated in duplicate samples by the Dole and by the Stern and Shapiro methods. (A detail of some practical value is that a definite increase in cloudiness can be detected when the reaction is approximately 10% complete. We have found this phenomenon useful in judging the appropriate time of terminating the incubation when samples of different enzymatic activity were being tested.) An apparent K_m of 1.8×10^{-3} M for the lecithinase was calculated from the Lineweaver-Burk plot of initial rates of hydrolysis and substrate concentration shown in Fig. 6.

Effect of pH and Other Variables

The pH optimum for the lecithinase found in our studies, employing glycerol media and phosphate buffers at

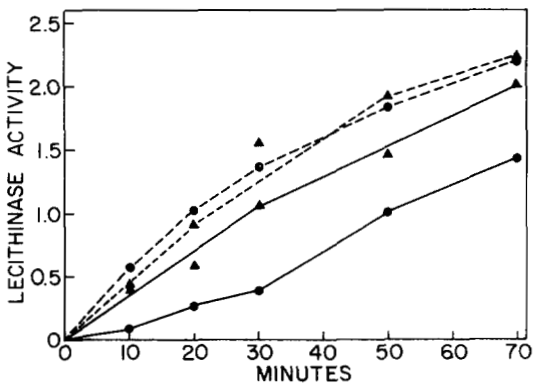

FIG. 2. Ionic strength and lecithin hydrolysis in sonicated and unsonicated lecithin–oleate mixtures.

●——● Unsonicated } Ionic strength
● - - - ● Sonicated } μ = 0.10
▲——▲ Unsonicated } Ionic strength
▲ - - - ▲ Sonicated } μ = 0.48

All other experimental conditions as given under Methods.

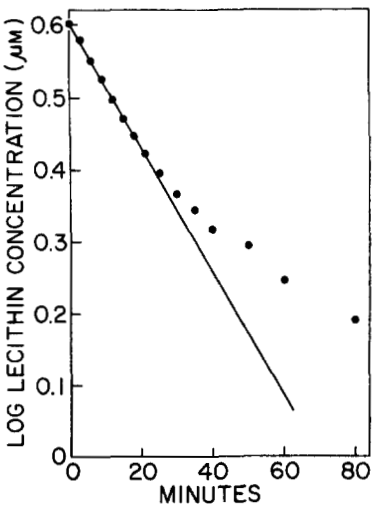

FIG. 3. Kinetics of lecithin hydrolysis by intestinal mucosa preparation. Decline from first-order kinetics after 25% hydrolysis.

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μ = 0.1, is between 6.2 and 6.4 and is somewhat lower than the value of 6.5 given by Epstein and Shapiro (2); it is possible that differences in composition of the reaction media and the procedure for preparation of both lecithinase and substrate might account for the discrepancy.

A fourfold increase in the concentration of either cysteamine or EDTA did not affect the reaction rate. Raising glycerol concentration in the final reaction mixture from 8.3% to 16.6% and higher had a definite inhibitory effect on reaction rate. On the other hand, the higher concentrations of glycerol were found to enhance stability of the enzyme in regard to temperature and storage as mentioned in section A. Since no difference in reaction rate was observed when the glycerol content in the final reaction mixture was lowered below 8.3%, this value was adopted as a standard condition for lecithinase assay and the enzyme preparations were adjusted to 12.5% glycerol prior to the addition of 0.5 volumes of lecithin–oleate suspension in the reaction vessels. The final procedure for estimation of lecithinase developed on the basis of the foregoing results have been summarized under Methods. The applicability of the procedure for routine use was tested in an investigation of the distribution of lecithinase in subcellular fractions. These studies are presented in the next section.

Fractionation of Mucosal Homogenates and Partial Purification of Lecithinase

In this series of experiments sucrose and glycerol solutions of different densities were tested as fractionation media. The results showed that the highest and most consistent recoveries of lecithinase, approximating 100% of the activity of the initial homogenate, were obtained with 12.5 and 25% aqueous glycerol and 2×10^{-3} M

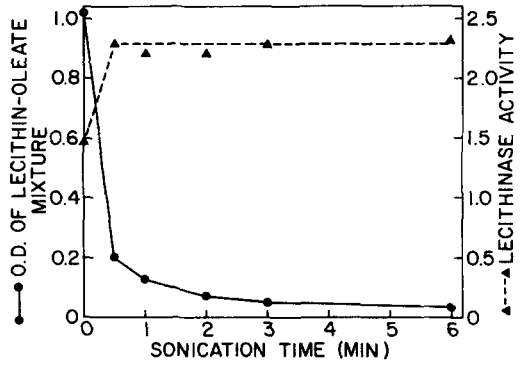

Fig. 4. Effect of sonication time on dispersion of lecithin–oleate mixtures and rate of hydrolysis by mucosal enzyme. Lecithin–oleate samples, withdrawn at specified intervals from the sonication vessel, were used for optical density (●——●) determination. Lecithinase assay (▲- - -▲) as under Methods.

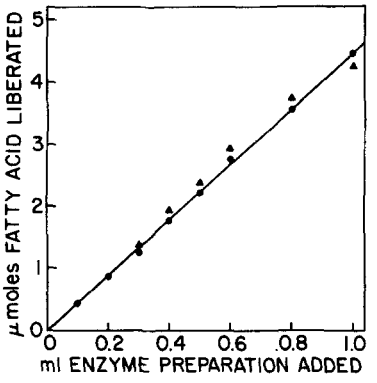

Fig. 5. Proportionality between enzyme concentration and lecithin hydrolysis. Fatty acid liberation estimated by Dole's ●——● and Stern's method ▲——▲. Enzyme preparation: 1 ml is equivalent to 10 mg wet weight mucosa.

cysteamine and EDTA. Results were less consistent and recoveries generally lower when cysteamine and EDTA were added to hypertonic sucrose, and in similarly fortified isotonic sucrose losses of activity were quite marked, especially when repeated homogenization and re-extraction of pellets were necessary.

For the purpose of comparing the distribution of lecithinase in the various subcellular fractions, hypertonic sucrose and 11.45% glycerol were selected. Cysteamine was present, but EDTA was omitted from the homogenization medium to avoid its possible reaction with subcellular particles. The procedure adopted in these experiments is essentially the one developed by Palade and Siekevitz (17) for liver homogenates with the addition that one or two washings of the first sediment at 20,000 × *g* were pooled with the original supernatant solution prior to high-speed centrifugation. The data from two such experiments which gave good recovery values are presented in Tables 1 and 2.

It can be seen from Table 1 that in hypertonic sucrose practically all the activity is recovered in the post-mitochondrial supernatant fraction: further fractionation of this preparation at 100,000 × *g* for 1 and 3 hr leads to separation in the sedimented microsomal pellets

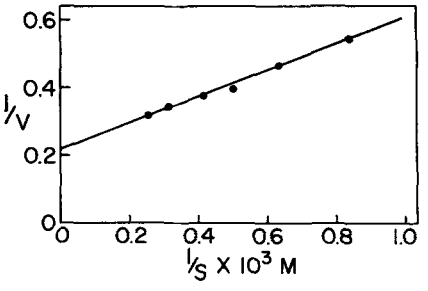

Fig. 6. Lineweaver-Burk plot of rates of lecithin hydrolysis vs. substrate concentration.

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TABLE 1 FRACTIONATION OF MUCOSAL HOMOGENATES IN 30% SUCROSE

Procedure	Homogenate Fraction	% Activity	
	Total	100	
30 min, 20,000 × *g*	→Pellet (nuclei, mitochondria)	12	94
	→Mitochondrial supernatant fraction	82	
a) 60 min, 100,000 × *g*	→Microsomal pellet + "fluffy layer"	16	74
	→Microsomal supernatant fraction	58	
b) 180 min, 100,000 × *g*	→Microsomal pellet and first post-microsomal fraction	37	74
	→Supernatant solution	37	

Homogenization medium contained 2×10^{-3} M cysteamine. All fractions were suitably diluted with medium containing phosphate buffer, glycerol, cysteamine, and EDTA, to ensure final assay conditions as given under Methods.

TABLE 2 FRACTIONATION OF MUCOSAL HOMOGENATE IN 11.45% GLYCEROL

Procedure	Homogenate Fraction	% Activity	
	Total	100	
15 min, 20,000 × *g*	→Nuclear and mitochondrial pellet, washed twice	8	93
	→Mitochondrial supernatant fraction	85	
a) 30 min, 100,000 × *g*	→Microsomal pellet	16	87
	→Microsomal supernatant fraction + "fluffy layer"	71	
b) 180 min, 100,000 × *g*	→Microsomal and first post-microsomal pellets	49	79
	→Supernatant solution	30	

Homogenization medium contained 2×10^{-3} M cysteamine. Reaction mixture composition for assay as given in Methods except for slightly lower final concentration of glycerol (7.6%).

TABLE 3 FRACTIONATION OF MUCOSAL HOMOGENATES BY COMBINED CENTRIFUGATION AND POLYMIXIN PRECIPITATION

Homogenate Fraction	Procedure	% Activity	Specific Activity Ratio*
Total	12 min, 12,000 × *g*	100	1.00
Supernatant solution	Polymixin 0.60 vol + 2 vol H_2O		
	12 min, 12,000 × *g*	74	1.97
Pellet		74	5.40

* Ratios of the specific activities of each fraction to that of the total homogenate. Specific activity was calculated on the basis of the acid-insoluble protein content for each fraction.

of 20 and 50% respectively of the total recoverable activity. These data indicate that a major part, at least, of the lecithinase is bound to particles in the microsomal–ribosomal range, with a larger proportion bound to particles of diameter less than 35 mμ. The same conclusion is reached by considering (Table 2) the results of fractionation in 11.45% glycerol, a medium of density and viscosity closely approximating those of isotonic sucrose.

Supporting evidence came from experiments employing precipitation of the nucleic acid by bases or by acidification. The bases tested were polymixin, protamine, streptomycin, and streptidine. All of these produced at least partial precipitation of nucleic acid and lecithinase activity from mitochondrial and microsomal fractions: polymixin gave quantitative precipitation and recovery of the lecithinase and was used in subsequent studies for the purpose of separating the activity from soluble matter of the cell without recourse to prolonged centrifugation. As can be seen from Table 3, fivefold purification of the lecithinase activity can be achieved by this method. Acidification of the 12,000 or 100,000 × *g* supernatant solution to pH 4.9–5.0 precipitated a fraction "enriched" in both lecithinase and nucleic acid. Similar (unpublished) results have been obtained by chromatography on Sephadex G200.

DISCUSSION

Most of the relevant observations about the procedure for the estimation have already been discussed. Some comment is necessary about the substrate concentration used in the assay. An arbitrary value had to be selected because the use of substrate levels corresponding to the extrapolated V_{max} was ruled out by solubility problems. The concentration adopted here (4×10^{-3} M) offers the advantage of allowing measurements of reaction rates not too far from V_{max} and without the appearance of early inhibition due to accumulation of reaction products. At this substrate level the rate of the reaction is practically linear up to 25% hydrolysis of the lecithin and is proportional to the amount of added tissue preparation. The initial and final concentrations of fatty acids are within the limits which ensure total

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extraction in the organic phase and a reliable titration when the Dole procedure is used. Thus the requirements for a quantitative method seem to be satisfied.

The studies on fractionation of mucosal homogenates have tested the applicability of the method to intestinal mucosa in a variety of conditions. The results show a close association of the lecithinase with particulate material exhibiting the sedimentation characteristics of ribosomes. This association is also suggested by the results of fractionation with polymixin and with pH change.

Application of the method to other tissues might possibly reveal differences in the properties of lecithinases from other sources.

The author is greatly indebted to Dr. F. Bernheim for discussion and much helpful advice during the course of this work.

This study was supported by grant No. CA 04 605 from the National Institutes of Health and grant No. AT-(40-1)-2919 from the U. S. Atomic Energy Commission.

Manuscript received March 2, 1964; accepted April 22, 1964.

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