SUMMARY

Ribonuclease is spontaneously inactivated in the presence of atmospheric oxygen. This inactivation can be suppressed by reduced glutathione or cysteine.

New forms of the protein appear during the oxidation process. These forms, which are separable by chromatography, have low enzymic activity; they can be activated by reducing agents.

The oxidizability of the samples is very variable and apparently depends on their inital state.

The presence of reduced glutathione or gelatin prevents oxidation, dilution of the solution favours it.

The results confirm the hypothesis that RNase contains sluggish S^- groups, essential for an important part of the enzymic activity.

BIBLIOGRAPHIE

1 L. LEDOUX, *Biochim. Biophys. Acta*, 13 (1954) 121.
2 L. LEDOUX, *Arch. intern. physiol.*, 61 (1953) 428.
3 L. LEDOUX, *Biochim. Biophys. Acta*, 11 (1953) 517.
4 L. LEDOUX, *Biochim. Biophys. Acta*, 14 (1954) 267.
5 L. WEIL ET T. S. SEIBLES, *Arch. Biochem. Biophys.*, 54 (1955) 368.
6 M. RABINOVITCH ET G. E. S. BARRON, *Biochim. Biophys. Acta*, 18 (1955) 316.
7 O. GAWRON, J. KEIL ET A. J. GLAID, *Biochim. Biophys. Acta*, 19 (1956) 170.
8 C. TANFORD ET J. D. HAUENSTEIN, *Biochim. Biophys. Acta*, 19 (1956) 535.
9 C. H. W. HIRS, S. MOORE ET W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
10 L. LEDOUX ET J. BRACHET, *Biochim. Biophys. Acta*, 16 (1955) 290.
11 G. E. S. BARRON, *Advances in Enzymology*, 11 (1951) 201.
12 G. KALNITSKY ET E. E. ANDERSON, *Biochim. Biophys. Acta*, 16 (1955) 302.
13 S. M. KALMAN, K. LINDERSTRØM-LANG, M. OTTESEN ET F. M. RICHARDS, *Biochim. Biophys. Acta*, 16 (1955) 297.
14 C. B. ANFINSEN, W. F. HARRINGTON, AA. HVIDT, K. LINDERSTRØM-LANG, M. OTTESEN ET J. SCHELLMAN, *Biochim. Biophys. Acta*, 17 (1955) 141.
15 C. H. W. HIRS, W. H. STEIN ET S. MOORE, *J. Biol. Chem.*, 211 (1954) 941.

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ENZYMIC HYDROLYSIS OF CEPHALIN IN RAT INTESTINAL MUCOSA*

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In contrast to the enzymic hydrolysis of lecithin, very little information is available concerning the enzyme systems involved in the hydrolysis of the cephalin fraction in tissues of higher animals. In an earlier paper from this laboratory[1], the rapid disappearance of lipide phosphorus during the incubation of minced mucosa of the small intestines of the rat was reported. Aqueous emulsions of brain cephalin are rapidly transformed to water-soluble phosphorus compounds by this material. The results of the experiments reported in this paper show that the enzyme system which catalyzes this transformation is closely associated with a particle fraction of the mucosa, and that

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the phosphorus-containing hydrolysis products consist almost entirely of glycerophosphorylesters. In contrast to the phosphorus groups of phosphatidylethanolamine and phosphatidylserine, those of the acetalphospholipides which account for a considerable portion of all preparations of brain cephalins are not transformed to acid-soluble hydrolysis products by the intestinal enzyme.

MATERIAL AND METHODS

The total cephalin fraction of beef brain prepared according to FOLCH[2] was used as substrate in most of the experiments. It was dissolved in ether and stored in a brown bottle at —10°. Samples stored under these conditions could be used as substrate for approximately one month.

Lecithin was prepared from egg yolk according to HANAHAN AND JAYKO[3].

For enzyme experiments, suitable portions of the ethereal stock solutions of cephalin or lecithin were evaporated to dryness under reduced pressure at a bath temperature of 40° on the day of the experiment. The phospholipides were emulsified with water, and the emulsions were transferred to a homogenizing vessel of the Potter-Elvehjem type. Portions of the homogenized emulsions were pipetted into the enzyme-buffer mixture in the proportions indicated in the individual experiments.

L-*a*-Glycerylphosphorylethanolamine was prepared from acetalphospholipides according to THANNHAUSER, BONCODDO AND SCHMIDT[4].

Synthetic DL-glycerol monostearate was purchased from Matheson Co., Inc. It was purified by extracting solutions containing 5 g in 200 ml of warm ether with 500 ml of water several times. When aqueous emulsions of the purified material were precipitated with trichloroacetic acid, the filtrates showed no measurable consumption of periodate.

Sodium cetylsulfate was synthesized according to DREGER *et la.*[5].

Butyric, caproic, caprylic, capric and lauric acids were purchased from Schering-Kahlbaum A.-G., Berlin, propionic, valeric, heptanoic, and undecylic acids from Matheson Co., Inc., nonanoic acid from Eastman Kodak Co., Inc., and sodium oleate from Merck Co., Inc. Sodium cholate and deoxycholate were purchased from Fisher Scientific Co, Inc., sodium taurocholate was obtained from Difco Laboratories, Inc.

Prostatic phosphatase was prepared according to SCHMIDT *et al.*[6].

Phosphate was determined according to FISKE AND SUBBAROW[7], serine and ethanolamine according to ARTOM[8], choline according to SCHMIDT *et al.*[1], glycerophosphorylesters according to SCHMIDT *et al.*[9].

Alkaline phosphatase was determined according to SCHMIDT AND THANNHAUSER[10].

Assay of the enzymic hydrolysis of DL-glycerolmonostearate: Since the alkalimetric assay methods for lipases were not sufficiently sensitive for the purposes of this study, the rate of the enzymic hydrolysis of the model substrate glycerol monostearate was estimated on the basis of glycerol determinations in the trichloroacetic acid filtrate of the digest by means of periodate oxidation. 1 ml of the enzyme preparation equivalent to 500 mg of fresh tissue was incubated with a substrate-buffer mixture consisting of 2 ml of a 0.1 *M* emulsion of glycerol monostearate in 5 m*M* sodium deoxycholate and 0.3 ml of *N* ammonium chloride buffer of pH 9. The digests and control samples containing comparable amounts of enzyme and substrate respectively were incubated for one hour at 37° and precipitated with 3 ml of 15% trichloroacetic acid. For the glycerol determination, measured samples of the water-clear filtrates were neutralized to pH 5.5 with sodium hydroxide and mixed with an exactly measured volume of 0.01 *N* sodium periodate solution. After standing for 30 minutes at room temperature, the excess of periodate was titrated with arsenious acid. The amounts of glycerol were calculated from those of the periodate consumed.

Proportionality between activity and enzyme concentration was observed up to 5% hydrolysis of the substrate. When measured at higher degrees of hydrolysis the slopes of the enyzme activity as plotted against enzyme concentration rapidly decreased.

General technique of enzyme experiments

All incubation of enzymic digests were carried out in 20 ml Erlenmeyer flasks which were closed with rubber stoppers and placed in a shaking incubator at 37°. Control samples containing only substrate and enzyme respectively were incubated under otherwise comparable conditions simultaneously with the enzyme-substrate mixtures. All figures obtained by the analyses of the latter were corrected by subtracting the corresponding control values.

Owing to the lability of the aqueous suspensions of cephalin phospholipase toward increases of temperature (Fig. 7), prewarming of the separate ingredients prior to the incubation of the enzyme-substrate mixtures had to be omitted. This necessity prevented kinetic studies requiring measurements of initial hydrolysis rates under accurately defined temperature conditions.

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Paper electrophoresis was carried out in an apparatus similar to that of MARKHAM AND SMITH[11] on strips of Whatman No. 3 paper. Chromatograms were developed with a 0.1 % solution of ninhydrin in *n*-butanol.

EXPERIMENTAL

1. *Preparation of enzyme; general conditions of activity*

So far, active enzyme preparations were only obtained from rat intestines. Suspensions of the intestinal mucosa of rabbit, dog or calf had only negligible activity toward added cephalin emulsions.

The small intestines of one or several adult rats of the Wistar strain were excised from the animals in ether anesthesia and rinsed with a 0.85% sodium chloride solution. The mucosa was squeezed out by scraping the outside of the intact loops with a wooden spatula.

Since preliminary fractionation studies demonstrated the close association of the cephalin-splitting enzyme with insoluble particles, suspensions of intestinal mucosa were invariably used as starting preparations. The potency of suspensions obtained by homogenizing the minced mucosa with water varied greatly, and large losses (presumably by denaturation) occurred usually during the washing of the centrifuged particles with water. It was found, however, that enzyme preparations of reproducible high potency were obtained when the minced mucosa was suspended in an equal volume of ice-cold 0.85% sodium chloride solution containing one-tenth volume of *N* sodium acetate buffer of pH 6.7. The use of potassium salts had no advantage over that of sodium salts. The suspension was centrifuged at 1° for 5 minutes at 24,000 × *g* in the high-speed attachment of an International Refrigerated Centrifuge, Model PR-1. The supernatant fluid was discarded, and the residue was homogenized in a volume of the buffered sodium chloride solution equal to the original tissue weight. This preparation had practically the full activity of the original suspension, but contained only relatively small amounts of acid-soluble phosphates and of mucins. While some experiments regarding the enzymic hydrolysis of cephalin were carried out with this preparation which will be referred to in the following as "crude enzyme", most of the observations were obtained with preparations which had been further purified by fractional centrifugation[12].

For this purpose, the sediment obtained from the high speed centrifugation of the minced mucosa was homogenized in a Potter-Elvehjem Teflon apparatus (A. H. Thomas Co., Philadelphia, Pennsylvania) in 1 ml of a 0.25 *M* sucrose solution per gram of fresh mucosa. After centrifugation at 700 × *g* for 10 minutes, the upper layer was pipetted off and centrifuged at 24,000 × *g* for 10 minutes. The supernatant fluid was discarded, and the sediment was dispersed by gentle homogenization in 1 ml of the salt-buffer mixture described above for each gram of fresh mucosa when it was to be used for immediate enzyme experiments. A more stable enzyme preparation was obtained by lyophilizing the sediment. 20 mg of lyophilized material was usually obtained from 1 g of moist mucosa. The approximate yield of activity was 75% of that obtained under comparable conditions with the fresh mitochondrial fraction. The lyophilized samples could be stored for 10 days at —10° with very little loss of activity. A suspension of the mitochondria in the salt-buffer mixture could be dialyzed for 3 hours against this mixture with a recovery of 86% of the activity.

Phosphorus and nitrogen content of mitochondrial fraction. 1 ml of the mitochondrial

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fraction (corresponding to 0.5 g of minced mucosa) contained 1.13 mg of total N, 0.107 mg of total P, 0.024 mg of lipide P, and 2.8 mg of total lipides. The ratio between lipide phosphorus and total nitrogen of intestinal mitochondria was thus about ten times smaller than the corresponding ratio in similar fractions of other tissues such as liver, kidney[13] and heart[14]. Very possibly, the mitochondrial phospholipides were enzymically hydrolyzed during the time required for their isolation. The low phospholipide content of the enzyme preparations as compared with the amounts of acid-soluble phosphorus compounds liberated in the presence of added cephalin shows that only insignificant quantities of these compounds could have been contributed by the autodigestion of the mitochondria.

pH Optimum. Activity determinations at different pH values with both the "crude enzyme" and with a suspension of mitochondria showed a narrow optimal range of activity in the region of pH 6.7 (Fig. 1). Since the use of phosphate buffers was not feasible for analytical reasons, acetate buffers and sodium bicarbonate respectively were employed in the range near pH 7 despite the limited buffer capacity of acetate buffer mixtures in this region.

Time course of enzymic hydrolysis of cephalin; substrate concentration and enzymic activity. The time curve of the action of the phospholipase on brain cephalin (Fig. 2) shows that the hydrolysis of the substrate does not go to completion, but practically ceases when approximately 65% of the phospholipide phosphorus was converted to acid-soluble phosphorus compounds. It will be shown below that this behavior is mainly caused by the presence of acetalphospholipides in brain cephalin.

The rates of hydrolysis increased with increasing substrate concentrations (Fig. 3) in a manner deviating from a Michaelis-Menten function. A detailed study of the

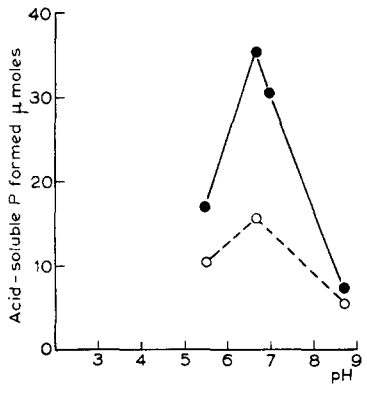

Fig. 1

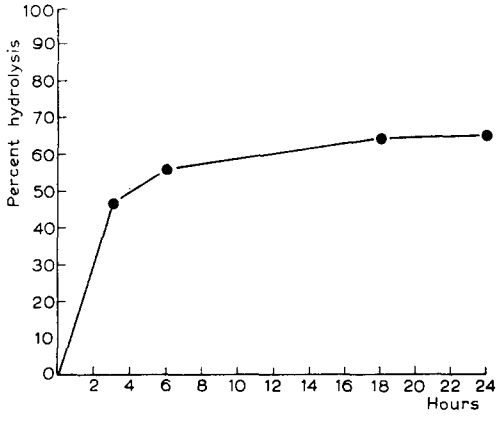

Fig. 2

Fig. 1. pH-activity curves of intestinal cephalin phospholipase. Solid curve: "crude enzyme". Dotted curve: suspension of mitochondria. The incubation mixture contained 1 ml of enzyme equivalent to 0.5 g of fresh tissue, 2 ml of a cephalin emulsion containing 155 µmoles of lipide P and 0.3 ml of the respective buffer solution (pH 5.5 and pH 6.7, *M* sodium acetate buffers; pH 7, 0.5 *M* sodium bicarbonate solution adjusted with carbon dioxide; pH 8.5, *M* ammonium acetate buffer; pH values measured electrometrically in enzyme-substrate-buffer mixtures). The tubes were incubated at 37° for 30 minutes and deproteinized with equal volumes of 15% trichloroacetic acid.

Fig. 2. Time curve of hydrolysis of cephalin by intestinal cephalin phospholipase. 3 ml of homogenate equivalent to 1.5 g of fresh tissue were incubated with 6 ml of a cephalin emulsion containing 250 µmoles of lipide P and 0.9 ml of *M* sodium acetate buffer of pH 6.7. Samples of suitable volumes were deproteinized with trichloroacetic acid at the times indicated, and analyzed for their contents of acid-soluble phosphorus compounds.

kinetics of cephalin phospholipase was not undertaken, however, because a theoretical interpretation of such data would be inconclusive in view of the chemical heterogeneity of the substrate and of the complex physicochemical conditions encountered in a system which contained both enzyme and substrate in the form of particles.

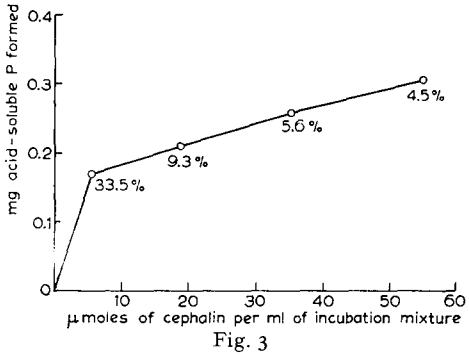

Fig. 3

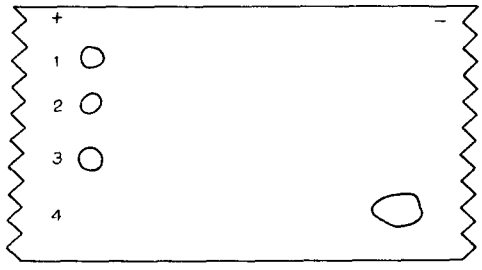

Fig. 4

Fig. 3. Influence of substrate concentration on activity of cephalin phospholipase. 420 mg of lyophilized mitochondria obtained from 14 mg mucosa and suspended in 14 ml of 0.85% sodium chloride solution containing 0.1 volume of *M* sodium acetate buffer of pH 6.7. 1 ml of mitochondrial suspension incubated with 2 ml of cephalin emulsion and 0.3 ml of *M* sodium acetate buffer of pH 6.7 at 37° for 30 minutes. Degrees of hydrolysis indicated below experimental points of curve.

Fig. 4. Tracing of paper electrophoresis strips of neutralized (see legend, Table III) acid filtrate of enzymic cephalin digest. Voltage gradient 8 V per cm at 30 mA; electrode cells filled with 0.05 *M* ammonia-acetic acid mixture of pH 6.5, paper moistened with the same solution, duration of electrophoresis 3 hours. *Spot 1:* reference spot containing 0.2 μmoles of pure L-α-glycerylphosphorylethanolamine. *Spot 2:* filtrate of enzymic cephalin digest containing 0.2 μmoles of organic phosphorus. *Spot 3:* filtrate of enzyme control (aliquot equal to Spot 2) containing no organic phosphorus *Spot 4:* reference containing 0.2 μmoles of ethanolamine hydrochloride. Spots developed with a 0.1% ninhydrin solution in *n*-butanol.

2. *Nature of the acid-soluble hydrolysis products formed by the action of intestinal cephalin phospholipase*

Table I shows the results of a typical analysis of the acid-soluble hydrolysis products obtained from brain cephalin after incubation with the "crude" intestinal enzyme. The first 2 lines show that 89.4% of the acid-soluble phosphorus compounds are organic phosphates. The application of the method of SCHMIDT *et al.*[9] for the determination of phosphoric acid diesters showed that almost the total amount of the organic phosphorus compounds was accounted for by the increase of phosphodiesters in the digest.

Information regarding the nature of the phosphodiesters was obtained by chromatographic and electrophoretic identification of their nitrogenous groups. These experiments were carried out with the purified mitochondrial fraction for the purpose of eliminating preformed acid-soluble phosphorus compounds and phospholipides of the mucosa. It was shown in earlier investigations[1,15] that the glycerylphosphorylesters of choline and ethanolamine differ sharply from the corresponding phosphomonesters by the extraordinary acid-lability of the ester bond of the basic alcohols in the phosphodiesters. The chromatograms obtained with the protein-free filtrates of the enzymic digests were therefore compared with those of their hydrolysates obtained by treatment with *N* hydrochloric acid at 100° for 20 minutes. Ethanolamine which was practically absent in the unhydrolyzed digests (Fig. 4) was identified in the hydrolysates

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TABLE I

PHOSPHODIESTER DETERMINATION ON THE ACID-SOLUBLE FRACTION OF ENZYMIC CEPHALIN DIGEST*

Line	*P-fraction*	*Acid-soluble phosphorus fractions formed μmoles in total digest*
1	Total acid-soluble P	132
2	Inorganic P	14
3	Inorganic P after phosphatase	16
4	Inorganic P after a 20′ acid hydrolysis in *N* HCl	12
5	Inorganic P after acid hydrolysis, and incubation with phosphatase	130
6	Phosphodiester P (line 5 minus line 3)	114

* The incubation mixture consisted of 5 ml of a homogenate containing 2.5 g of fresh mucosa, 10 ml of a cephalin emulsion containing 775 μmoles of lipide P, and 1.5 ml of 1 *N* sodium acetate buffer of pH 6.7. The reaction was stopped after 30 minutes by the addition of 1 ml of 6 *N* hydrochloric acid and 5 ml of 0.2 *M* mercuric chloride. After filtration and removal of mercuric ions with hydrogen sulfide, the various analyses were carried out on the filtrate (see text).

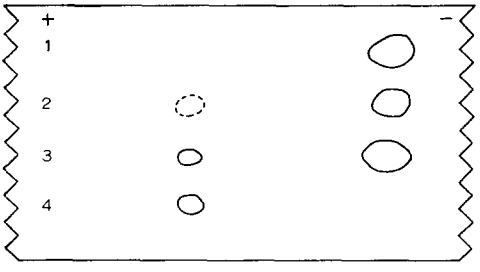

Fig. 5

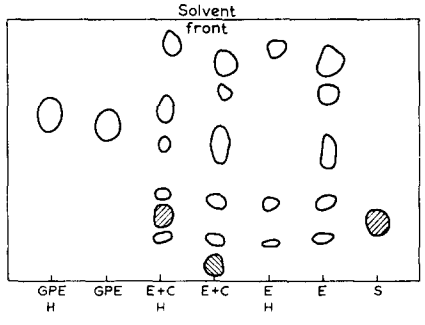

Fig. 6

Fig. 5. Tracing of paper electrophoresis strips of the filtrate used in the experiment of Fig. 4 after a 20-minute hydrolysis in *N* hydrochloric acid at 100° (neutralization as described in legend to Table III). Conditions of electrophoresis as indicated in legend to Fig. 4. *Spot 1:* Reference spot of ethanolamine hydrochloride containing 0.2 μmoles. *Spot 2:* Reference spot of hydrolysate of pure glycerylphosphorylethanolamine (1 *N* hydrochloric acid, 100°, 20′), containing 0.2 μmoles of phosphorus. *Spot 3:* Hydrolysate of acid-soluble fraction of enzymic cephalin digest containing 0.2 μmoles of phosphorus. *Spot 4:* Hydrolysate of acid-soluble fraction of enzyme control.

Fig. 6. Tracing of paper chromatogram of the acid-soluble fraction of enzymic cephalin digest, before and after acid hydrolysis. Solvent: phenol, saturated with water, ascending. Abbreviations: H: hydrolyzed for 20′ in *N* hydrochloric acid at 100°; GPE: L-α-glycerylphosphorylethanolamine. E + C: Enzymic digest of cephalin; E: Enzyme incubated without cephalin; S: DL-serine (reference spot).

by paper electrophoresis (Fig. 5), serine which migrates only very slowly in the electric field, by ascending partition chromatography with water-saturated phenol as solvent (Fig. 6). Fig. 5 shows that the appearance of the ethanolamine spot after hydrolysis is accompanied by the disappearance of a spot which was present in the non-hydrolyzed enzymic digests (Fig. 4), and which migrated at a rate identical with that of a reference spot of L-α-glycerylphosphorylethanolamine. The appearance of the

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serine spot in the partition chromatograms of the acid-hydrolyzed digests was likewise accompanied by the disappearance of a spot found in the chromatograms of the nonhydrolyzed digest (Fig. 6). This spot is presumably that of the serine precursor, possibly glycerylphosphorylserine, which, however, was not available as reference substance.

Further evidence for the presence of easily hydrolyzable compounds of ethanol amine and serine in the acid-soluble fraction of the enzymic cephalin digests was obtained by periodate oxidation of the acid hydrolysates of these digests and subsequent determination of ammonia. The results of Table II demonstrate that the increment of the amounts of ammonia in the cephalin digest over those found in the enzyme control accounts for most of the acid-soluble phosphorus liberated. Separation of the ammonia precursors by adsorption on permutit into the ethanolamine and serine fraction supports the conclusion that glycerylphosphorylethanolamine as well as glycerylphosphorylserine were formed from cephalin during its incubation with intestinal cephalin phospholipase.

TABLE II

ACTION OF PERIODATE ON ACID HYDROLYSATES OF ACID-SOLUBLE FRACTION OF ENZYMIC CEPHALIN DIGESTS

Substances determined		*Acid hydrolysate of acid-soluble fraction*		
		Enzyme control	*Cephalin digest*	*Increment*
Inorganic P	μmoles	0	12.5	12.5
Organic P	μmoles	0	67	67
Ammonia after periodate oxidation:				
Total hydrolysate	μmoles	20.0	87.0	67
Permutit filtrate	μmoles	9.3	51.3	42
Permutit eluate	μmoles	10.7	41.7	31

Suspension of mitochondria from 10 g of mucosa incubated with 20 ml of cephalin emulsion (600 μmoles P) + 3 ml of *N* sodium acetate buffer (pH 6.7) incubated for 20 hours. Digest deproteinized with 30 ml of 4% hydrochloric acid and 30 ml of 5% mercuric chloride solution. Mercuric ions removed by hydrogen sulfide, filtrate aerated, shaken with silver carbonate until pH 6.5 was reached. 20 ml aliquots were heated with 5 ml of 5 *N* hydrochloric acid in a boiling water bath for 20 minutes, cooled and shaken with solid silver carbonate until the pH was 6.5. Aliquots of the filtrates were used for the determinations reported in the table[8].

3. *Specificity of intestinal cephalin phospholipase*

A. *Acetalphospholipides.* The fact that the enzymic hydrolysis of cephalin phospholipase by mitochondria never exceeded a degree of 65% suggested the possibility that at least a part of the non-hydrolyzed fraction consisted of phospholipides which were resistant toward the enzyme because of their specific chemical structures. FEULGEN AND BERSIN[16] found already that acetalphospholipides accompany the cephalin fraction during the isolation of phospholipides, and subsequent investigators confirmed this observation[17].

The question was therefore studied whether the acetalphospholipide fraction of the substrate cephalin decreased during the incubation with intestinal mitochondria. The Schiff reaction which is so far the only basis for the analytical determination of acetalphospholipides could not be applied because it does not permit a differentiation between bound and free lipide aldehydes. Another characteristic property of the acetal-

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phospholipides, namely the resistance of the acetal linkage toward alkali, offered a possibility for obtaining this information. At the end of the incubation, a measured sample of the enzymic digest was mixed with an equal volume of an aqueous *N* sodium hydroxide solution and shaken for 16 hours at 37°. Another sample was treated in the same manner at the beginning of the incubation. Under these conditions, phospholipides containing exclusively fatty acids as lipide groups were quantitatively transformed into water-soluble phosphorus compounds, whereas the phosphoryl groups of sphingomyelin and of the acetalphospholipides retained the solubility properties of lipides. The alkali-resistant phospholipides were quantitatively isolated by precipitation with saturated ammonium sulfate at neutral reaction, according to a procedure which will be published elsewhere. The results reported in Table III show that the alkali-resistant fraction of cephalin which consists mainly of acetalphospholipides did not decrease under conditions resulting in the transformation of 55% of the lipide phosphorus of the substrate into acid-soluble compounds.

TABLE III

RESISTANCE OF NON-SAPONIFIABLE PHOSPHOLIPIDE FRACTION OF CEPHALIN TOWARD ACTION OF INTESTINAL CEPHALIN PHOSPHOLIPASE

*Time of incubation**	*μmoles of P in digest*		
	Saponifiable lipide-P	*Acid-soluble P*	*Non-saponifiable lipide-P*
Initial	1160	17.8	232
21 hours	368	785.0	240

* 385 mg of lyophilized mitochondria suspended in 25 ml of buffered sodium chloride solution. Digestion mixture: 12 ml of mitochondrial suspension + 24 ml of cephalin emulsion (containing 43.2 mg of total P) + 3.6 ml of *N* sodium acetate buffer (pH 6.7).

B. *Lecithin.* The results reported in Table IV demonstrate that emulsions of lecithin prepared according to HANAHAN AND JAYKO[3] were hydrolyzed at much slower rates than cephalin emulsions. The degree of hydrolysis observed after incubation of the mitochondria with a mixture of cephalin and lecithin were appreciably higher than those calculated from the sum of the hydrolysis products formed in the corresponding controls in which each phosphatide fraction was incubated individually with the mitochondria. This finding excludes competitive inhibition between the two substrates, but it does not permit the conclusion that they are hydrolyzed by two different enzymes since for the practical reasons mentioned previously the experiments had to be carried out at cephalin concentrations at which the enzyme was not saturated with substrate (see Fig. 3).

Phospholipides incubated in the form of the supernatant solution obtained by high-speed centrifugation from egg yolk were hydrolyzed by the intestinal enzymes at much slower rates than those observed with emulsions of brain cephalin.

C. DL-*Glycerol monostearate.* Glycerol monostearate was hydrolyzed at considerable rates by suspensions of minced intestinal mucosa. In a typical experiment, 23 μmoles of glycerol were liberated from 200 μmoles of added substrate by a suspension of 500 mg of homogenized mucosa during one hour. (Total volume 3.3 ml, temperature 37°, 0.1 *M* ammonia-ammonium chloride buffer, pH 8.9.) The intracellular distribu-

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TABLE IV

BEHAVIOR OF INTESTINAL PHOSPHOLIPASES TOWARD CEPHALIN, LECITHIN AND EGG YOLK LIPOPROTEINS

Enzyme preparation	*Substrate*	*pH*	*Acid-soluble P formed in total digest*	*Total P of substrate*
			μmoles	
Homogenized mucosa*	Egg yolk	6.7	0.5	115
	Egg yolk	8.0	16.5	115
	Cephalin	6.7	42.5	100
Total mucosa, frozen, powdered	Egg yolk	6.7	0	115
	Cephalin	6.7	36.7	100
Mitochondria	Egg yolk	6.7	2	115
	Cephalin	6.7	20	100
Mitochondria	Lecithin	6.7	4.5	65
Mitochondria	Lecithin	8.0	5.3	65
Mitochondria	Cephalin	6.7	22.0	65
Mitochondria	Lecithin	6.7	5.7	64
Mitochondria	Cephalin	6.7	27.4	64
Mitochondria	Lecithin + Cephalin	6.7	37.2	64 μmoles Lecithin + 64 μmoles Cephalin

* Incubation mixture: 1 ml of enzyme suspension corresponding to 0.5 g of intestinal mucosa, 2 ml of a mixture of equal volumes of egg yolk supernatant solution and 0.85% sodium chloride solution, 0.3 ml of *M* sodium acetate buffer of pH 6.7 or of 0.5 *M* sodium bicarbonate solution. Incubation time: 2 hours. Temperature 37°.

tion of the enzyme catalyzing this hydrolysis, however, differed from that of cephalin phospholipase inasmuch as the mitochondrial fraction contained about 70% of the cephalin phospholipase activity of the tissue, but only about 5% of its activity toward glycerol monostearate. This observation strongly suggests the assumption that both substrates are hydrolyzed by different enzymes.

4. *Alkaline phosphatase activity of intestinal mitochondrial fraction*

The mitochondrial fraction isolated from intestinal mucosa contained considerable amounts of alkaline phosphatase. For example, the amount of mitochondria corresponding to 500 mg of fresh tissue hydrolyzed during 30 minutes 38 μmoles of added cephalin in 0.1 *M* sodium acetate buffer at pH 6.5 and 16 μmoles of glycerophosphate under the assay conditions of SCHMIDT AND THANNHAUSER[10]. The presence of alkaline phosphatase in the mitochondrial fraction is in agreement with earlier observations concerning the association of this enzyme with particles.

5. *Some factors influencing the activity of cephalin phospholipase*

The influence of some factors on the activity of cephalin phospholipase was measured by the ratio between the degrees of hydrolysis in the presence and absence of the factor under otherwise comparable conditions at hydrolysis degrees not exceeding 15% of the added cephalin phosphorus. These ratios, however, are only approximations of the true percentages of inhibition or activation, owing to the fact that the values calculated for the activities per mg of a given enzyme sample showed a decreasing trend with increasing enzyme concentrations. For example, incubation of a cephalin emulsion

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TABLE V

EFFECTS OF DIFFERENT CONCENTRATIONS OF SODIUM CAPRYLATE ON CEPHALINASE ACTIVITY

Sodium caprylate concentration mM	*Acid-soluble phosphorus formed μmoles in total digest*	*% Inhibition*
0	32.0	0
1.16	30.6	4
4.64	27.2	15
23.20	15.1	53

Incubation 90 mintes at 37°.
Other conditions of incubation as described in legend to Fig. 8.

respectively with 30 and 10 mg of lyophilized mitochondria resulted in 15.5 and 6.4% hydrolysis (total volume 3.3 ml, final substrate concentration 47.5 m*M*, pH 6.7, temperature 37°, time of incubation 90'). So far, no conditions permitting a generally valid definition of an enzyme unit for a reasonably large range of enzyme concentrations were found.

A. *Temperature.* The activity of intestinal mitochondria toward cephalin is very sensitive toward temperature increases. The results plotted in Fig. 7 show that standing of the enzyme suspension at 37° for 10' prior to the incubation with the substrate decreased the rate of the subsequent formation of acid-soluble phosphorus compounds

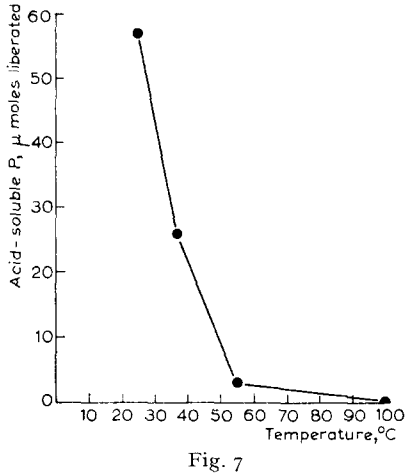

Fig. 7

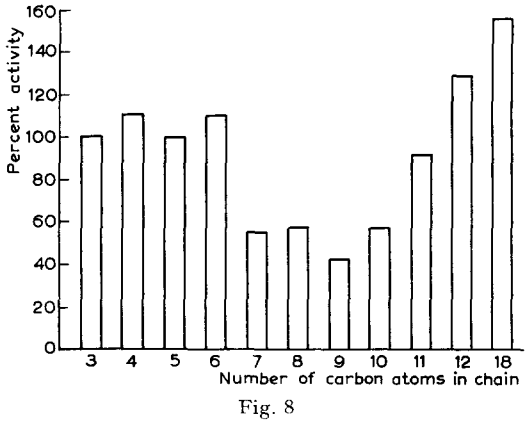

Fig. 8

Fig. 7. Heat inactivation of cephalin phospholipase. Suspension of lyophilized mitochondria from 9 g of fresh mucosa in 9 ml of 0.85 % sodium chloride solution, buffered with 0.1 volume of 1 *N* acetate buffer of pH 6.7; 1 ml portions were heated at the indicated temperatures for 10 minutes and cooled to room temperature. The incubation mixture contained 1 ml of enzyme equivalent to 1 g of fresh tissue, 2 ml of cephalin emulsion containing 100 μmoles of lipide P, and 0.3 ml of 1 *M* acetate buffer of pH 6.7.

Fig. 8. Effect of added soaps on the activity of cephalin phospholipase. Incubation mixture: 1 ml of enzyme suspension corresponding to 1 g of fresh tissue, 2 ml of cephalin emulsion containing 149 μmoles of lipide-P, 1 ml of a 0.1 *M* solution of the sodium salt of the respective fatty acid (adjusted to pH 6.7), and 0.3 ml of 1 *M* sodium acetate buffer of pH 6.7. Incubation time 1 hour, temperature 37°. The amount of acid-soluble phosphorus formed during the incubation of cephalin with the enzyme under comparable conditions without added soaps (23.6 μmoles, degree of hydrolysis: 15.8 %) is designated as 100 % activity.

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from cephalin substrate to approximately 50% of its original value, and that after standing of the enzyme at 55°, only negligible activity remained.

B. *Effects of soaps.* Fig. 8 shows the effects of some soaps of varied chain lengths at 0.023 m*M* concentration on the cephalin phospholipase of intestinal mitochondria. Saturated soaps up to hexanoate had little effect on the enzyme; those ranging between heptanoate and decanoate were inhibitory. Laurate and oleate considerably increased the rate of enzymic cephalin degradation.

C. *Bile salts* which are activators of pancreas lipase had considerable inhibitory effects on cephalin phospholipase (Table VI). A synthetic detergent, sodium cetyl sulfate, was likewise found to inhibit considerably the action of cephalin phospholipase. A comparison between the data reported in Tables V and VI shows that a concentration of caprylate in the range of 20 millimolarity is required for a degree of inhibition close to 50%, whereas some of the bile salts produce an inhibition of this order already at 5 m*M* concentration.

D. *Cyanide* in $1 \cdot 10^{-3}$ *M* concentration had no appreciable influence on cephalin phospholipase.

TABLE VI

EFFECT OF SURFACE-ACTIVITY SUBSTANCES ON CEPHALIN PHOSPHOLIPASE ACTIVITY

Substance	*Concentration mM*	*Acid-soluble phosphorus formed μmoles in total digest*	*% inhibition*
Control		14.6	
Taurocholate	25	6.1	58
Control		24.8	
Deoxycholate	25	3.3	87
Control		13.3	
Deoxycholate	5	7.1	47
Control		14.7	
Cholate	5	7.0	53
Control		21.4	
Cetyl sulfate	5	10.6	50.5

The incubation mixture consisted of 1 ml of a suspension of lyophilized mitochondria equivalent to 1 g of fresh mucosa, 2 ml of a cephalin emulsion containing 140 μmoles of lipide phosphorus, 1 ml of an aqueous solution of the surface-active agent adjusted to pH 6.7 and 0.3 ml of a 1 *N* acetate buffer of pH 6.7. The tubes were incubated at 37° for 60 minutes.

DISCUSSION

The detection in intestinal mucosa of an enzyme transforming the ethanolamine and serine containing cephalins into their respective glycerophosphorylesters furnishes additional evidence suggesting the metabolic significance of these esters. The fact that this enzyme was found to occur exclusively in close association with mitochondrial structures suggests that its function is not involved in the extracellular digestion, but in the intracellular metabolism of cephalin. On the other hand, the possibility that glycerophosphorylesters whose occurrence in considerable concentrations in some tissues was recently demonstrated in several laboratories[1,9,18] might have a role as acceptors of activated fatty acids is so far not supported by direct observations. Only phosphorylcholine has been shown to be incorporated into phospholipides by cell-free

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systems[19,20]. This lack of direct evidence does obviously not exclude the existence of a third pathway of phospholipide biosynthesis involving glycerophosphorylesters as intermediaries. In evaluating the available information, it must be considered that, so far, only L-α-glycerylphosphorylcholine was tested with negative results as acceptor of activated fatty acids, whereas no such experiments were carried out with L-α-glycerylphosphorylethanolamine or its serine analogue.

SUMMARY

1. The phosphatidyl ethanolamine and phosphatidyl serine fractions of crude brain cephalin are hydrolyzed by a phospholipase found in the small intestines of the rat. The optimal pH of the hydrolysis is between 6.5 and 7.0.

2. The enzyme was found exclusively in the mitochondrial particle fraction of intestinal mucosa.

3. The glycerylphosphorylesters of ethanolamine and serine have been identified as the principal products of the action of intestinal phospholipase on cephalin. Only negligible amounts of inorganic phosphate are formed during the incubation of cephalin with preparations of intestinal phospholipase.

4. Cephalin is hydrolyzed at much faster rates than lecithin by the intestinal particle fraction studied in this investigation. The acetal bonds of acetalphospholipides are resistant toward intestinal cephalin phospholipase. At least 95% of the considerable hydrolytic activity of crude intestinal mucosa toward glycerol monostearate was found not to be present in the mitochondrial fraction.

5. The cephalin phospholipase of rat intestinal mucosa is considerably inhibited by bile salts and by salts of fatty acids containing between 7 and 11 carbon atoms in the form of straight chains, but considerably enhanced by soaps of higher chain lengths. It is not affected by cyanide ions.

REFERENCES

[1] G. SCHMIDT, L. HECHT, P. FALLOT, L. M. GREENBAUM AND S. J. THANNHAUSER, *J. Biol. Chem* 197 (1952) 601.
[2] J. FOLCH, *J. Biol. Chem.*, 146 (1942) 35.
[3] D. J. HANAHAN AND M. E. JAYKO, *J. Am. Chem. Soc.*, 74 (1952) 5070.
[4] S. J. THANNHAUSER, N. F. BONCODDO AND G. SCHMIDT, *J. Biol. Chem.*, 183 (1951) 423.
[5] E. E. DREGER, G. I. KEIM, G. D. MILES, L. SPEDLOWSKY AND J. ROSS, *Ind. Eng. Chem.*, 36 (1944) 610.
[6] G. SCHMIDT, R. CUBILES, N. ZÖLLNER, L. HECHT, N. STRICKLER, K. SERAIDARIAN, M. SERAIDARIAN AND S. J. THANNHAUSER, *J. Biol. Chem.*, 192 (1951) 715.
[7] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[8] C. ARTOM, *J. Biol. Chem.*, 157 (1945) 585.
[9] G. SCHMIDT, L. M. GREENBAUM, P. FALLOT, A. C. WALKER AND S. J. THANNHAUSER, *J. Biol. Chem.*, 212 (1954) 887.
[10] G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 149 (1943) 369.
[11] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
[12] W. C. SCHNEIDER AND G. H. HOGEBOOM, *J. Biol. Chem.*, 183 (1950) 123.
[13] W. C. SCHNEIDER, *J. Biol. Chem.*, 165 (1946) 585.
[14] S. W. EDWARDS AND E. BALL, *J. Biol. Chem.*, 209 (1954) 619.
[15] G. SCHMIDT, M. J. BESSMAN AND S. J. THANNHAUSER, *J. Biol. Chem.*, 203 (1953) 849.
[16] R. FEULGEN AND T. BERSIN, *Z. physiol. Chem.*, 160 (1939) 217.
[17] E. KLENK AND H. DEBUCH, *Z. physiol. Chem.*, 296 (1954) 179.
[18] H. H. TALLAN, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 927.
[19] A. KORNBERG AND W. E. PRICER, *Federation Proc.*, 13 (1952) 241.
[20] E. P. KENNEDY AND S. B. WEISS, *J. Biol. Chem.*, 222 (1956) 193.

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