LXVIII. THE ENZYMIC HYDROLYSIS OF PHOSPHATIDES.

III. THE HYDROLYSIS OF NATURAL AND SYNTHETIC PHOSPHATIDES.

By EARL JUDSON KING.

*From the Department of Medical Research, Banting Institute,
University of Toronto.*

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It has previously been shown [King, 1931] that an enzyme is contained in the kidney and intestinal mucosa which is capable of hydrolysing lecithin to liberate inorganic phosphate at an optimum p_H of 7.5. The enzyme appears to be present in other tissues to only a slight extent. Bone, which is very rich in an enzyme hydrolysing primary esters of phosphoric acid, is relatively feeble in its action against lecithin. Lecithinase appears to act most rapidly at body temperature (Fig. 1). It is fairly stable at a neutral reaction but is rapidly destroyed in an alkaline or acid medium. The concentration of substrate (Fig. 2) appears to

Fig. 1. Effect of temperature on the hydrolysis of lecithin:

Fig. 2. Effect of substrate concentration on lecithinase hydrolysis:

10 ml. of lecithin emulsion at p_H 7.5 + 2 ml. of dialysed extract of intestinal mucosa.

affect the hydrolysis in a manner similar to that indicated by Michaelis and Menten [1913]. Owing to the slowness with which lecithin is broken down by the enzyme, it has been impossible to obtain any accurate measure of the initial velocity of hydrolysis. The results appear to indicate, however, that the substrate concentration giving half the maximum velocity corresponds to about 0.002 M ($-\log S$ 2.7), which is nearly the same as that found by Martland and

Robison [1927] for phosphatase, *i.e.* $<0.003 M$ [cited by Haldane, 1930]. It is the purpose of the present paper to record the results of a study of the action of this enzyme on other phosphatides, both natural and synthetic.

Lecithin, kephalin and phosphatidic acid.

Liver-lecithin behaved much like egg-lecithin, while kephalin showed a lower rate of hydrolysis with not so marked a dependence on hydrogen ion concentration. A sample of phosphatidic acid from cabbage leaf [Chibnall and Channon, 1927] was kindly supplied to us by Prof. A. C. Chibnall. The formula of phosphatidic acid is represented as corresponding with that of lecithin with the choline absent. It showed a fairly high rate of hydrolysis, but the relation of the amount of hydrolysis to the p_H was not definite as in the case of lecithin (see Table I).

Table I. *Hydrolysis of natural phosphatides by extract of the small intestine.*

(mg. inorganic phosphorus.)

5 ml. borax-boric acid or borax-succinic acid buffer, 5 ml. 0.25 % emulsion of phosphatide and 2 ml. enzyme solution. The hydrolysis was carried out in the presence of chloroform and toluene for 12 hours at 37.5° and was stopped by the addition of 2 ml. 25 % trichloroacetic acid. The inorganic phosphate in the filtrates was estimated by the colorimetric method.

	p_H	Control (1); buffer + phosphatide	Control (2); buffer + enzyme	Test; buffer + phosphatide + enzyme	Phosphate hydrolysed
Egg-lecithin	5.5	0.000	0.041	0.058	0.017
	6.5	0.000	0.037	0.082	0.045
	7.1	0.000	0.038	0.109	0.071
	7.5	0.000	0.039	0.119	0.080
	8.1	0.000	0.039	0.112	0.073
	8.9	0.000	0.038	0.091	0.053
	9.2	0.000	0.037	0.068	0.031
Liver-lecithin	5.6	0.000	0.040	0.056	0.016
	6.5	0.000	0.037	0.067	0.030
	7.1	0.000	0.038	0.091	0.053
	7.6	0.000	0.039	0.096	0.057
	8.0	0.000	0.039	0.095	0.056
	8.9	0.000	0.038	0.066	0.028
	9.2	0.000	0.037	0.056	0.019
Kephalin	4.3	0.020	0.035	0.069	0.014
	4.8	0.020	0.034	0.070	0.016
	5.0	0.021	0.034	0.083	0.028
	5.5	0.020	0.041	0.103	0.042
	6.5	0.021	0.037	0.103	0.045
	7.1	0.020	0.038	0.101	0.043
	7.6	0.020	0.039	0.101	0.042
	8.3	0.019	0.039	0.107	0.049
	8.9	0.020	0.038	0.090	0.032
Phosphatidic acid	9.2	0.021	0.036	0.089	0.032
	5.6	0.068	0.040	0.191	0.083
	6.6	0.069	0.037	0.193	0.087
	7.0	0.067	0.038	0.187	0.082
	7.5	0.067	0.039	0.185	0.079
	8.0	0.070	0.038	0.185	0.077
	8.9	0.073	0.038	0.185	0.074
	9.2	0.069	0.037	0.180	0.074

Lysolecithin.

Lysolecithin was prepared by the action of the venom of the fer de lance on the lecithin contained in egg-yolk [King and Dolan, 1933]. The material so prepared has marked haemolytic properties. The behaviour of lysolecithin with

both the intestinal and bone enzymes was investigated. The amounts of hydrolysis at different hydrogen ion concentrations and at different times, when lysolecithin was subjected to the action of an aqueous extract of rabbit intestinal mucosa, indicated that the maximum hydrolysis occurred at about p_H 7.8, which is a little higher than the corresponding value for lecithin, and that this relatively simpler phosphatide was hydrolysed about twice as fast as the parent lecithin.

Bromolecithin.

Bromolecithin was prepared by passing bromine vapour in a stream of carbon dioxide into a mixed ether and light petroleum solution of egg-lecithin and liver-lecithin [Levene and Rolf, 1926]. The precipitated material was purified by solution in ether and reprecipitation by light petroleum.

Analysis for phosphorus was made by the perchloric acid colorimetric procedure [King, 1932], for nitrogen by micro-Kjeldahl, and for bromine by the potash fusion method of Fendler and Stüber [1914], the silver bromide being collected by means of a porcelain filter stick. (Found: P, 2.02; N, 1.04; Br, 41.3 %. Calculated: for octabromoarachidyl-stearyl-lecithin: P, 2.12; N, 0.96; Br, 43.7 %; for octabromoarachidyl-palmityl-lecithin: P, 2.16; N, 0.97; Br, 44.6 %; for hexabromostearyl-stearyl-lecithin: P, 2.42; N, 1.09; Br, 37.5 %.)

The behaviour of bromolecithin in the presence of phosphate-liberating enzymes was in marked contrast to that of the lecithin from which it had been made. With a variety of enzyme preparations from several sources, bromolecithin was practically completely hydrolysed in 24 hours, while lecithin was attacked only to the extent of 20 % in the most favourable instance. This peculiar property of greatly increased susceptibility to enzymic hydrolysis resulting from bromination of lecithin is probably related to an increased affinity of the enzyme for the phosphatide, due to the change in the fatty acid side-chain. The effect of p_H on the enzymic hydrolysis of bromolecithin is shown in Fig. 3.

Fig. 3. Effect of p_H on hydrolysis of bromolecithin by cat-kidney extract. Each test contained 0.16 mg. of phosphatide-P, and 0.5 ml. of enzyme. Veronal-acetate buffer; hydrolysis at 38° for 12 hours.

The rates of hydrolysis of bromolecithin, lysolecithin and the parent lecithin are given in Table II, the hydrolysis constant being calculated from $k = \frac{1}{t} \log \frac{a}{a-x}$. The velocity of hydrolysis for bromolecithin was about 10 times as great as for

lecithin in the case of the intestinal enzyme, and 100 times as great with bone-phosphatase. The bone-enzyme, moreover, gave with bromolecithin about twice the velocity of hydrolysis that was obtained with the intestinal enzyme. This is in marked contrast to the action of the two enzyme preparations on lecithin. The velocities for bromolecithin were in about the same ratio as the phosphatase activities of the two preparations measured against glycerophosphate.

Table II. *Comparative rates of enzymic hydrolysis of phosphatides.*

Hours	Lecithin		Lysolecithin		Bromolecithin	
	% hydro- lysis	<i>k</i>	% hydro- lysis	<i>k</i>	% hydro- lysis	<i>k</i>
(1) Rabbit intestinal extract.						
$\frac{1}{2}$	1.0	0.0072	1.6	0.0138	9.1	0.0834
1	1.2	0.0054	2.0	0.0090	17.9	0.0860
2	2.4	0.0054	2.4	0.0054	29.0	0.0744
4	3.2	0.0036	4.8	0.0054	44.8	0.0645
7	4.8	0.0031	7.9	0.0052	59.9	0.0567
Average		0.0049		0.0078		0.0750
(2) Rat bone extract (phosphatase).						
$\frac{1}{2}$	—	—	0.7	0.0070	15.1	0.1424
1	0.4	0.0018	0.8	0.0036	28.6	0.1461
2	0.8	0.0018	1.1	0.0025	50.0	0.1505
5	1.2	0.0010	1.3	0.0012	65.9	0.0934
7	1.5	0.0009	1.6	0.0010	70.2	0.0751
Average		0.0014		0.0031		0.1215

Synthetic phosphatides.

Through the kindness of Dr Adolf Grün, of the Hoffman-La Roche Company, Grenzach, Germany, we were able to compare samples of the synthetic phosphatides described by Grün and Limpächer [1927]. Synthetic distearyllecithin did not show the same general effect on enzymic hydrolysis as did the natural lecithin (Table III). The rate of hydrolysis was not so great and the p_H effect

Table III. *Influence of p_H on the enzymic hydrolysis of natural and synthetic phosphatides.*

(1) Natural egg-lecithin		(2) Synthetic lecithin (Grün)		(3) Distearyl phosphate (Grün)	
p_H	mg. P liberated	p_H	mg. P liberated	p_H	mg. P liberated
3.6	0.145	5.7	0.121	4.5	0.183
4.3	0.158	6.2	0.115	5.9	0.193
5.9	0.186	6.9	0.119	6.6	0.176
6.9	0.221	7.3	0.121	6.9	0.178
7.1	0.220	7.5	0.118	7.4	0.174
7.3	0.222	8.0	0.111	7.8	0.176
7.5	0.205	8.8	0.127	8.2	0.170
7.8	0.192			9.5	0.145
8.1	0.170				

was absent. It was thought that this difference might be due to the unsaturation and greater solubility of the natural product. This explanation was found to be untenable, however, by the discovery that hydrogenated lecithin has almost the same rate of enzymic hydrolysis as has the lecithin from which it is made (Table IV).

Synthetic distearyl phosphate showed very little relation between the p_H and the amount of hydrolysis. Since it is a primary ester of phosphoric acid,

Table IV. *Enzymic hydrolysis of hydrolecithin and lecithin.*
(mg. P liberated.)

Tests consisted of 2 ml. of enzyme (rat-intestinal extract), 5 ml. of veronal buffer and 5 ml. of emulsified phosphatide containing 0.086 mg. of hydrolecithin-P per ml. and 0.088 mg. of lecithin-P per ml. The hydrolysis took place at 38° for 48 hours.

p_H	Hydrolecithin*	Lecithin	p_H	Hydrolecithin*	Lecithin
5.8	0.114	0.114	8.0	0.203	0.194
6.5	0.151	0.158	8.5	0.187	0.188
7.1	0.208	0.206	8.9	0.161	0.170
7.5	0.201	0.202	9.4	0.052	0.082

* This sample of hydrolecithin was kindly given to us by Dr P. A. Levene. Other samples prepared in this laboratory by hydrogenation of egg- and liver-lecithins in the presence of palladium gave very similar results.

it might have been expected to behave like glycerophosphoric acid or acetonylecithin, which are hydrolysed at an optimum p_H of 8.9, whereas lecithin, a secondary ester of phosphoric acid, is hydrolysed at an optimum p_H of 7.4 (diphenyl phosphate and diethyl phosphate are hydrolysed at a slightly lower p_H optimum). Comparative figures for the hydrolysis of natural and synthetic lecithin and distearyl-glycerophosphoric acid were obtained by incubation of 5 ml. of 0.25 % emulsion of the phosphatide mixed with an equal volume of buffer at several p_H values and 2 ml. of the enzyme solution for 48 hours. The amount of phosphate liberated from the egg-lecithin was considerably greater than that from the distearyl phosphoric acid and at the optimum p_H was almost double that liberated from the synthetic lecithin.

DISCUSSION.

The extraordinary change induced in lecithin by bromination is not easily explained. It is a more common experience to find that a large increase in molecular weight (that of bromolecithin is almost double that of lecithin) leads to a decrease rather than an increase in reactivity. A possible explanation of this anomalous result of greatly increased susceptibility to enzymic hydrolysis may lie in a greater negativity of the alcohol part of the ester due to introduction of several bromine atoms into the side-chain. Among the simple primary esters of phosphoric acid those containing the more negative alcohol groups are most readily attacked by phosphatase; thus phenyl phosphate is much more rapidly hydrolysed than tolyl phosphate, and tolyl phosphate very much more rapidly than ethyl phosphate.

In the case of phosphatidic acid, the p_H effect was almost absent, and since phosphatidic acid is a primary phosphoric ester, it—as also distearyl phosphate—might have been expected to be hydrolysed optimally at p_H 8.9. Since, however, the phosphate-liberating enzyme is relatively unstable above p_H 8, while it is quite stable in more neutral solutions, the discrepancy may be explicable on the basis of destruction of the enzyme during the course of hydrolysis. If this be so, the length of the fatty acid chains may be the determining factor, since it probably conditions the solubility of the phosphatide and its consequent availability for enzymic hydrolysis. The shorter the carbon chains of the substituent groups, the more soluble the substance should be and the more it should behave like a simple primary phosphoric ester with a p_H optimum of 8.9. It is also possible that the nature of the fatty acid radicals may affect, through some mechanism akin to steric hindrance, the union of substrate and enzyme.

Thus the presence of the fatty acids in phosphatidic acid may hinder its union with the enzyme, giving a lower rate than would be obtained with an unsubstituted glycerophosphate, or with a glycerophosphate substituted with less complex groups than stearic and oleic. This, combined with destruction of the enzyme, might account for the failure of this product to be hydrolysed at the optimum p_H of 8.9.

If the difference between the behaviours of natural and synthetic phosphatides on enzymic hydrolysis were due to the unsaturation of the natural product, then phosphatidic acid, which is more unsaturated than egg-lecithin, might have been expected to show a higher rate of hydrolysis. Also, hydrolecithin, which ought to be very similar in chemical behaviour to the synthetic product if existing theory as to the structure of these substances be correct, might have been expected to behave like synthetic lecithin, instead of which it showed an enzymic hydrolysis almost identical with that of its parent lecithin. These facts would appear to cast some doubt on the similarity of the chemical configuration of the synthetic lecithin of Grün and Limpächer with the naturally occurring phosphatide; and further, if the chemical formula assigned by Grün and Limpächer to the synthetic product be correct, then the formula commonly used to represent the chemical structure of lecithin may be in error. The asymmetric formula which appears in most of the textbooks and represents lecithin as always being a derivative of α -glycerophosphoric acid, has been shown to be incorrect [Rae, 1934], and it is possible that a more drastic revision of the manner of representing the formula for lecithin may yet be necessary.

SUMMARY.

An enzyme is contained in kidney and intestinal mucosa which hydrolyses lecithin from several sources with the liberation of phosphoric acid. Hydrolecithin is attacked as fast as its parent lecithin, kephalin and phosphatidic acid somewhat more slowly, the latter without showing any marked dependence on p_H . Synthetic lecithin and distearyl phosphate are attacked by the enzyme at a lower rate and without showing any p_H optima. Lysolecithin is hydrolysed about twice as fast as lecithin. Brominated lecithin is hydrolysed at a very much greater rate than is lecithin.

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