Biochemical Pharmacology, Vol. 22, pp. 2165–2170. Pergamon Press, 1973. Printed in Great Britain.

SOME PROPERTIES OF A PROTAMINE-STIMULATED TRIGLYCERIDE-HYDROLYZING ENZYME FROM RAT VENTRICLE*

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(*Received* 26 *December* 1972; *accepted* 20 *February* 1973)

Abstract—Ventricle from fed or fasted rat has been shown to contain enzymatic activity which hydrolyzes rat adipose tissue triglycerides and which is stimulated by protamine sulfate. Ten-fold purification of this enzyme was achieved by discontinuous sucrose gradient centrifugation at 100,000 *g*. The enzyme thus obtained shows the following characteristics: it hydrolyzes triglyceride extracted from rat adipose tissue; has a pH optimum between 7·2 and 7·5 in Tris or phosphate buffer; has a requirement for albumin, but not of serum, for maximal activity; is stimulated by protamine sulfate, but is unaffected by heparin, NaCl and NaF; is thermolabile, 5 min at 60° being sufficient to reduce activity by almost 100 per cent. The hydrolytic activity of a 40% sucrose gradient preparation was not limited to triglycerides; both monoglycerides and methyl esters of fatty acids were also hydrolyzed, although the stimulant effect of protamine was restricted essentially to the triglycerides.

INFORMATION about lipolytic enzymes in cardiac tissue is very limited. Several observations of such activity have been reported: (1) Lipoprotein lipase, described by Korn,[1] which hydrolyzes chylomicrons or artificial triglyceride substrate in the presence of serum, has an optimum pH of 8·5 and is inhibited by protamine sulfate or sodium chloride. (2) An enzyme described by Björntorp and Furman,[2] which hydrolyzes the esters in ediol, has a pH optimum of 6·8, but, unlike lipoprotein lipase, is not inhibited by NaCl or protamine sulfate. It is, however, strongly inhibited by NaF. (3) Activity reported by Yamamoto and Drummond,[3] which appears to hydrolyze monoglycerides rather than triglycerides. Both of these authors[2,3] used ediol as substrate, which consists of a mixture of medium chain fatty-acid-containing trigylcerides, monoglyceride and Tween 80. In the case of the latter authors,[3] no hydrolysis of ^{14}C-triolein added to the ediol could be found.

While examining the activities of lipases from different sources, including rat ventricle, in a system used for assay of lipoprotein lipase, we observed that the addition of protamine sulfate significantly stimulated the hydrolysis of emulsions of triglycerides extracted from rat fat. Björntorp and Furman[2] also noted some stimulation of their enzyme activity at pH 6·8 in the presence of protamine sulfate as did Rubinstein *et al.*[4] for an adipose tissue lipase. The present report deals with partial purification and characterization of this enzyme.

* Supported by United States Public Health Service Grant HE 07564 and American Heart Grant 72–766.

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METHODS AND MATERIALS

Male Sprague–Dawley rats (250–275 g), either fed *ad lib* or fasted for 36 hr, were sacrificed by decapitation. The hearts were removed and washed in cold 0·05 M sodium phosphate buffer, pH 7·2. The venticles were minced, then homogenized in 3 vol. of 0·3 M sucrose with a glass homogenizer. The homogenate was centrifuged at 12,000 *g* for 10 min at 0°. The particulate fraction was saved in some experiments for the assay of lipoprotin lipase activity. The supernatant fraction was layered on a discontinuous sucrose gradient consisting of 40 and 60% sucrose solutions and centrifuged at 100,000 *g* for 30 min. The different fractions were then carefully removed for assay of lipolytic activity.

The natural triglycerides, used as substrate, were extracted from the perinephric and ovarian fat bodies of Holtzman retired breeders and separated from the di- and monoglycerides by the method of Borgström[5] (separation verified by thin-layer chromatography). Emulsions of substrates were freshly prepared daily as follows: sufficient triglyceride or other substrate for 10 ml of 0·1 M (assuming the molecular weight of tripalmitin for the triglyceride) was weighed, 2·5 ml of 20% albumin added and, whenever necessary, heated slightly to melt the substrates. The mixture was sonicated with a Branson Sonifier Cell Disruptor model S 125 at a setting of 8 for 2 min at room temperature. The resultant emulsion was diluted to a final volume of 10 ml with 0·05 M phosphate buffer, pH 7·2. Emulsions prepared in this way remained suspended for several hours. No spontaneous hydrolysis could be detected in such emulsions during the period they were employed in this study. Protein was determined by the method of Lowry *et al.*[6] Incubations were carried out in a Dubnoff metabolic shaker and terminated by the addition of Dole's extraction mixture.[7] The free fatty acids produced during incubation were titrated by the method of Dole and Meinertz.[7]

The materials used in this study were obtained as follows: bovine serum albumin, Armour Pharmaceutical Company, Chicago, Ill.; protamine sulfate (essentially histone free), tributyrin, trilaurin, triolein, 1-monoolein, methylmyristate and methylpalmitate (all 99 per cent pure), Sigma Chemical Company, St. Louis, Mo.; heparin, sodium salt, Nutritional Biochemical Company, Cleveland, Ohio; sodium fluoride (certified grade), Fisher Scientific Company, Chicago, Ill.; monobutyrin and methyl butyrate, Eastman Organic Chemicals, Rochester, N.Y.; and 1-monolaurin (90 per cent pure), C. P. Hall Company, Chicago, Ill. All other chemicals were of the highest purity commercially available. Substrate purity was checked by thin-layer chromatography.

RESULTS

Partial purification of the enzyme from rat ventricle. The results of five experiments (mean ± S.E.) of the purification procedure for the enzyme obtained from the ventricles of fed rats are shown in Table 1. As can be seen, when 500 μg/ml of protamine or 100 μg/ml of heparin was added to the homogenate, increases in hydrolytic activity were observed. In the other two steps, the increase in activity produced by protamine was statistically significant ($P < 0·05$), while the slight decrease seen in the presence of heparin was not significant. The procedures produced about a 12-fold increase in specific activity in the control state (also seen in the protamine addition). About 88 per cent of the total activity in the homogenate was recovered in the 12,000 *g*

TABLE 1. SPECIFIC ACTIVITIES OF THE FRACTIONS OBTAINED IN THE PURIFICATION OF PROTAMINE-STIMULATED LIPASE ACTIVITY FROM RAT VENTRICLE*

Fraction	Specific activity (μ-equiv/mg protein/hr)†		
	Control	+ Protamine (500 μg/ml)	+ Heparin (100 μg/ml)
Homogenate	0·10 ± 0·003	0·17 ± 0·03	0·14 ± 0·03
12,000 *g* Supernatent	0·20 ± 0·02	0·32 ± 0·01	0·17 ± 0·02
40% Sucrose layer (100,000 *g*)	1·32 ± 0·14	1·82 ± 0·13	0·98 ± 0·14

* Reaction mixture for the enzyme assays consisted of 0·05 M phosphate buffer, pH 7·2; 4% albumin; 0·1 ml of freshly prepared rat serum; and 0·01 M extracted rat fat triglyceride. Incubation time was 30 min at 37°.

† All activities are expressed as the net release of FFA: 60-min value minus the 0-time value. Values are mean ± S.E. for five experiments.

supernatant fraction and 57 per cent in the 40% sucrose fraction (percentages based on original homogenate activity in the absence of added drug).

In order to utilize the 40% sucrose preparation for further studies of the properties of the enzyme, an examination of the linearity of the activity with both time and enzyme protein concentration was made. It was found that under the assay conditions employed the enzyme activity of either 0·1 or 0·2 mg protein was linear for 60 min, either alone or in the presence of added protamine or heparin. All assays were therefore performed utilizing 0·1 mg protein and an incubation time of 30 min.

The disappearance of the heparin-stimulated lipolytic acitivity from the 12,000 *g* supernatant prompted the investigation of the lipolytic activity present in the 12,000 *g* precipitate. It was found that addition of heparin produced a significant increase in the lipolytic activity of the 12,000 *g* precipitate, and the addition of protamine caused inhibition of the lipolytic activity in either the presence or absence of heparin. NaCl, 0·5 M, also inhibited the heparin-stimulated activity.

TABLE 2. EFFECT OF ALBUMIN AND/OR SERUM UPON TRIGLYCERIDE HYDROLYSIS BY A PROTAMINE SULFATE-STIMULATED ENZYME FROM VENTRICLE OF FED RATS*

Addition	Specific activity (μ-equiv FFA/mg protein/hr)		
	Control	Protamine sulfate (500 μg/ml)	Heparin (100 μg/ml)
None	0·27 ± 0·04	0·50 ± 0·07	0·40 ± 0·05
Bovine albumin (4%)	0·63 ± 0·11	1·11 ± 0·09	0·84 ± 0·13
Rat serum (0·1 ml/ml)	0·36 ± 0·03	0·83 ± 0·07	0·52 ± 0·10
Albumin and serum	0·77 ± 0·08	1·26 ± 0·12	0·84 ± 0·14

* The incubation medium contained: 0·05 M phosphate buffer, pH 8·0; 0·01 M extracted rat fat triglyceride as substrate; and 0·1 mg protein from 40% sucrose fraction. Incubation time was 30 min at 37°. All values are the mean of five experiments ± S.E.

Table 2 shows the effect of the presence of albumin or serum, or of both, on the lipolytic activity of the 40% sucrose layer. In this set of experiments, the enzyme was incubated for 1 hr in 0·05 M sodium phosphate buffer at pH 8·0 containing the indicated concentrations of albumin or serum or both. It is clear that the addition of albumin increased the lipolytic activity about 2-fold in the control experiments. On the other hand, serum had no stimulatory effect alone, nor did it increase the stimulatory effect of added albumin. In all experiments which utilized this fraction, with or without albumin or serum in the incubation medium, protamine sulfate increased lipolysis over the control level (significant at $P < 0{\cdot}05$ or less). The changes in lipolytic activity from control when heparin was present were found to be not statistically significant.

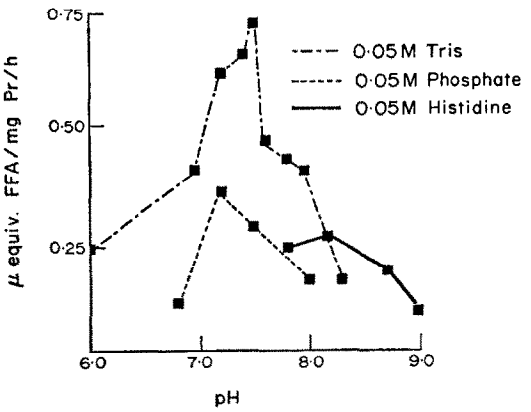

FIG. 1. Effect of pH on the lipolytic acitivity of the protamine-stimulated enzyme from venticle of fed rat (40% sucrose fraction). The incubation medium contained: 0·05 M specific buffer; 0·01 M extracted rat fat triglyceride; 4% albumin and 0·1 mg protein present in 40% sucrose fraction. The pH of each medium was checked immediately before incubation and again at the end of the 30-min incubation. No changes in pH were observed during incubation. All values are the mean of two experiments.

Figure 1 shows the effect of pH upon the lipolytic activity of the 40% sucrose fraction. It can be seen that in Tris buffer, the lipolytic activity was maximal at pH 7·5 with a sharp decrease on either side. A similar result was observed with phosphate buffer, maximum activity being seen at pH 7·3. The activity was measured with histidine buffer, principally because of its high pK for satisfactory pH maintenance above 8. Very low activity was seen at this pH.

Dose–response curves to protamine or heparin were run using the 40% sucrose fraction. In five experiments, a dose-related increase in activity was produced by protamine (500 μg producing the maximal effect). Heparin in doses of 50 through 250 μg/ml produced a slight, non-dose-related, not statistically significant increase in lipolytic activity.

The enzyme activity of the 40% sucrose fraction was very heat labile, being essentially completely destroyed when held at 60° for 5 min.

Utilizing the 40% sucrose layer preparation, three different types of glyceride were tested as substrates. The results are presented in Table 3. The four triglycerides tested

were hydrolyzed by the enzyme preparation and all of them were hydrolyzed more rapidly in the presence of protamine than in its absence. Heparin, in contrast, stimulated the hydrolysis of only tributyrin. The enzyme preparation was also capable of hydrolyzing the three monoglyceride substrates, but protamine significantly stimulated the hydrolysis of only monobutyrin, while heparin had no significant effect on the hydrolysis of the monoglycerides. The methyl esters of three fatty acids were also capable of being hydrolyzed by this fraction but, with the exception of methyl butyrate, no significant effect of either protamine or heparin was observed.

TABLE 3. HYDROLYSIS OF DIFFERENT SUBSTRATES BY THE PROTAMINE-STIMULATED LIPASE OF RAT VENTRICLE*

Substrate	Specific activity (μ-equiv FFA released/mg protein/hr)†		
	Control	+ Protamine (500 μg/ml)	+ Heparin (100 μg/ml)
Triglycerides			
Natural	1·28 ± 0·17	1·79 ± 0·15‡	0·88 ± 0·44
Tributyrin	1·47 ± 0·20	2·22 ± 0·09‡	2·08 ± 0·07‡
Triolein	0·35 ± 0·06	0·51 ± 0·04‡	0·34 ± 0·12
Trilaurin	0·45 ± 0·04	0·66 ± 0·08‡	0·55 ± 0·08
Monoglycerides			
1-Monobutyrin	0·83 ± 0·16	1·19 ± 0·13‡	0·99 ± 0·09
1-Monoolein	2·86 ± 0·69	3·57 ± 0·39	2·98 ± 0·26
1-Monolaurin	3·56 ± 0·29	4·44 ± 0·49	4·24 ± 0·34
Methyl esters			
Methylbutyrate	0·62 ± 0·03	1·19 ± 0·05‡	0·86 ± 0·13
Methylmyristate	1·37 ± 0·30	1·59 ± 0·16	1·47 ± 0·07
Methylpalmitate	0·97 ± 0·27	1·26 ± 0·15	1·19 ± 0·14

* The incubation medium contained: 0·05 M phosphate buffer, pH 7·2; 4% albumin; 0·01 M indicated substrate; 40% sucrose fraction as enzyme source. Incubation was for 30 min at 37°.
† Means of five experiments ± S.E.
‡ Significantly different from control.

In a set of experiments designed to test the effect of fasting on the lipolytic content of rat ventricle, no change in hydrolytic activity was observed after the fasting either in the homogenate or in the two centrifugal fractions used.

DISCUSSION

It is well established that heart muscle is a consumer of lipids as a source of energy for contraction. The source of such lipids is largely the blood where both free fatty acids (carried by plasma albumin) and glycerol esters (in the form of lipoproteins) are available for utilization, although it has been shown that endogenous triglyceride is utilized in the isolated heart perfused in the absence of added substrate.[8]

The early work of Korn[1] indicated that lipoprotein lipase (LPL) was present in the heart and hence seemed responsible for making circulating triglyceride available to the

heart. The protamine-stimulated lipase herein described differs markedly from LPL in that the pH optimum is at least 1 unit lower, it has no requirement for the presence of serum for maximal activity, it is stimulated by protamine and not by heparin (which may be slightly inhibitory) and can be separated from it by differential centrifugation (LPL being precipitated at 12,000 *g*, the protamine-stimulated enzyme remaining in the supernatant).

The relationship of the protamine-stimulated lipase to the two other lipases described in heart is somewhat difficult to evaluate. The "tissue lipase" of Björntorp and Furman[2] was capable of hydrolyzing ediol, but since this contains mixed triglycerides and monoglycerides as well as Tween 80, it is somewhat difficult to know which substrate was being hydrolyzed. Indeed, Yamamoto and Drummond[3] found the heart lipolytic activity capable of acting upon ediol but not on added ^{14}C-triolein, leading them to conclude that the enzyme had principally monoglyceride-hydrolyzing activity. Triolein, however, is a relatively poorly hydrolyzed triglyceride (Table 3) and has been shown, using adipose tissue lipase, to be much more poorly hydrolyzed than is natural substrate.[9] Moreover, it is of interest that Björntorp and Furman[2] observed a small stimulatory effect of protamine in their preparation.

It is certain that monoglycerides (and methyl esters of fatty acids) are hydrolyzed by the protamine-stimulated enzyme preparation, although in the face of the relatively low degree of purification attained it is possible that multiple activities are associated in some manner, as has been noted by Huttunen *et al.*[10] for the hormone-sensitive lipase from adipose tissue.

Thus, on balance, the protamine-stimulated enzyme seems to be quite a distinct entity, although at present it is only incompletely purified, with monoglyceride- and methyl ester-hydrolyzing activity still present. The function which protamine plays as a stimulant of the enzyme activity is of considerable theoretical interest though of no obvious physiological moment at present.

The experiments recently reported by Tao[11] are of interest in relation to the present results and may explain the mechanism whereby protamine produces its stimulant effect. Tao[11] indicates that, in the red blood cell of the rabbit, protamine appears to be able to substitute for cAMP on the regulatory subunit of protein kinase. Experiments utilizing the protein kinase of rat heart are presently underway to test this hypothesis.

Acknowledgements—We thank Mrs. Patricia Wallace and Mrs. Patricia Cerutti for their technical assistance.

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