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Received September 26, 1966. P.S.E.B.M., 1967, v125.

Studies with Clearing Factor V. State of Tissue Lipases After Injection of Heparin.* (32045)

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Lipoprotein lipase (Clearing Factor) is a lipolytic enzyme (EC 3. 1. 1. 3.) which appears in the plasma after intravenous injection of heparin. The presence in the normal rat heart of an enzyme identical with Clearing Factor has been demonstrated(1). It was shown that the increase in the Clearing Factor concentration of plasma after heparin injection was correlated with the depletion of this enzyme in the heart tissue(1).

Lipolytic activity similar to lipoprotein lipase was also reported in kidney(2), liver (3), lung(4), adipose tissue(5) and lactating mammary gland(13). In the plasma the presence of an endogenous lipoprotein lipase was reported in rats(7) and in a small percentage of people(12); a heat stable monoglyceridase(8) was also reported. A hormone sensitive β-monoglyceridase in adipose tissue was reported by Steinberg *et al*(6). The state of the tissue lipases after heparin injection, however, has not been investigated.

* This research was supported by USPHS grant RO1 HE 02823-10.

The work presented in this paper demonstrates the relationship of the tissue lipases to the plasma Clearing Factor that appears after injection of heparin. Evidence is also presented that all tissue lipases and lipoprotein lipases are the source of the postheparin plasma Clearing Factor enzymes. Particle bound β-monoglyceridase of adipose tissue is demonstrated to be markedly affected by heparin injections.

Methods and materials. Rat tissues were prepared from Holtzman rats (150-250 g) which were killed by etherization. Five rats were sacrificed for each assay and the tissues were washed thoroughly with cold physiological saline to remove the blood. Pooled tissues were homogenized in a Waring blendor for 3 minutes with 150 ml of acetone at −15°C. The contents were vacuum filtered and acetone powders were stored under vacuum at −15°C.

The enzyme was prepared by extracting tissue acetone powders (50 mg per ml in the final incubation mixture) with 0.25 M am-

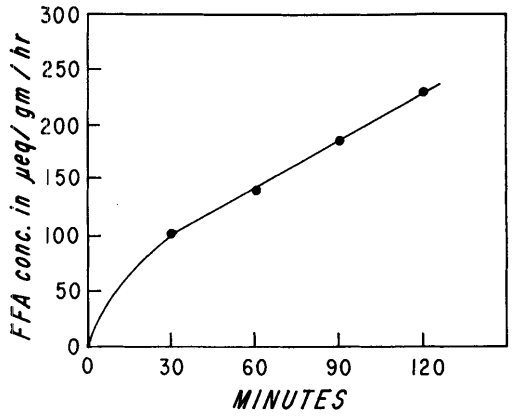

FIG. 1. Time curve of lipase activity of liver extracts. Assay conditions were as described in *Methods*.

monium chloride buffer at pH 8.5 at 0°C by 3 minutes sonication on a biosonic sonicator(11). The clear supernatant used in these experiments was obtained by centrifugation of this homogenate at 20,000 rpm for 15 min.

All tissue lipase activities were totally extractable with ammonium chloride buffer 0.25 M, at pH 8.5. Both total homogenates and/or extracts gave a linear enzyme concentration curve up to 4 hours incubation period. No lipolytic activity was detectable with liver homogenates. Its extracts, however, produced the activity curve shown on Fig. 1, suggesting a particle bound inhibitor in this tissue(18).

Lipoprotein lipase activity was determined by a small modification of the method described by Korn(1). The incubation mixture consisted of 4 ml 10% bovine serum albumin at pH 8.5, 0.2 ml of 0.1 M $CaCl_2$ solution, 0.1 ml of heparin solution (10 μg heparin), 2 ml of 4% coconut oil emulsion (Ediol), and 5.7 ml of the enzyme. The temperature of incubation was 37°C.

Fatty acids were determined, by Dole's method(9), at 0-, 1-, and 2-hour intervals and titration was performed on a Syringe type Microburet Model No. SB2 (Micrometric Co., Cleveland, Ohio). Variations between duplicates were reduced to less than 3%. Activity was recorded as μeq free fatty acids (FFA)/g of protein/hr. The protein was determined by UV spectrophotometric method (10).

Assay conditions for β-monoglyceridase determination(6) were as follows: 2 ml of 10% bovine serum albumin at pH 7.1, 1 ml substrate solution prepared as described below (15 mg β-monomyristin), 0.2 ml 0.1 M $CaCl_2$, 0.5 ml 0.6 M phosphate buffer at pH 7.1, 50 mg of tissue or 2 ml plasma. This mixture was incubated at 37° for 20 minutes. The assay of fatty acids was identical to the above procedure. Activity of β-monoglyceridase in the tissues and plasma was expressed as μeq free fatty acids (FFA)/g of acetone powder or ml of plasma/hr, because this enzyme was particle bound.

Trymyristin was purchased from Mann Biochemical Laboratories. Pancreatic lipase 448 (Porcine) was purchased from Nutritional Biochemicals Corp.

β-Monomyristin was prepared as follows (19): 2.6 g of trimyristin was emulsified in 90 ml distilled water on a Virtis-45 homogenizer, together with 10 ml 0.1 M $CaCl_2$, 50 ml 10% bovine serum albumin at pH 8.5 at 37°C and incubated with pancreatic lipase (0.2 mg/ml) overnight. To this emulsion 0.76 g of sodium metaperiodate was added and incubation was continued for an hour more at room temperature. Then glycerides were extracted with 1 liter of methanol-ether (3:1). Solvents were then evaporated in a flash evaporator at 35°C. β-Monoglycerides were separated from the rest of the glycerides by thin layer chromatography on 1 mm silica gel G plates with petroleum ether, ether, acetic acid (80:20:1) as solvent. The yield was 0.66 g of β-monomyristin. A stable emulsion of the β-monomyristin in a 5% bovine serum albumin solution of pH 7.1 was obtained by treatment for 10 minutes on a biosonic sonicator(11) yielding a 15 mg/ml monoglyceride concentration. The anticoagulant activity per mg of heparin was 100 units (Eli Lilly). Fifteen, 30, 45, 60, 120, 180 minutes and 3 days prior to sacrifice, 2 mg heparin/kg of rat in 0.1 ml saline was injected by the tail vein. Ten ml of blood was withdrawn from the heart of each rat before the removal of the tissues. Five ml of 2% sodium oxalate was added to prevent clotting. Blood collected was centrifuged at 15,000 rpm

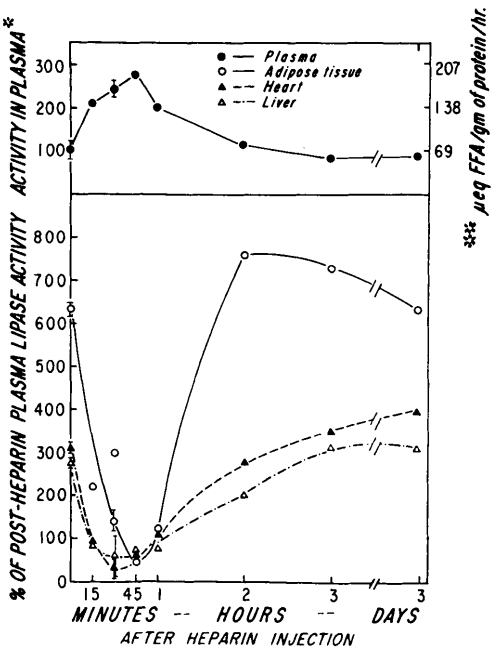

FIG. 2. Levels of plasma and tissue lipases of rats before and after injection of heparin. Rats were sacrificed before and 15, 30, 45, 60 min, 2 and 3 hrs, and 3 days after heparin (2 mg/kg) injection by the tail vein. Preparation of the acetone powders of the tissues, extraction procedure, incubation and assay of free fatty acids are described in *Methods*. To obtain the points, before and after heparin injection, pooled tissues of 5 rats were used. Variability in 2 groups is marked on perpendicular lines. Lipase activity of the tissues was expressed as percent activity of the lipase observed in the plasma in the same group of rats at the same period. The activity of any individual tissue at any time can be calculated when compared to the plasma activity.

*Lipase activity in the plasma, before injection, is taken as 100%.

●-Plasma ○-Adipose Tissue ▲-Heart Δ-Liver

**FFA - free fatty acids.

for 30 min. and the plasma was separated and stored at −5°C.

Results and discussion. It will be seen in Fig. 2 that rat plasma had some lipase activity prior to heparin injection(7). Prior to heparin injection lipase activity of the tissues was higher than plasma activity of the same animal groups, *i.e.*, adipose tissue—6 times, kidney, heart, and liver—3 times as high. Female rats had consistently higher levels of lipase activity in all tissues (Table I). This is in agreement with previous reports(14). In Fig. 2 and Table II, the levels of plasma lipoprotein lipase are shown as μeq free fatty acids (FFA)/g protein/hr. In the same animal groups the lipase activity of the tissues was expressed as percent of the plasma lipase activity before and after heparin injection. There was a decrease of lipase activity in the tissues at the end of 30 to 40 minutes, and the recovery of enzyme levels was observed after 2 to 3 hours. The highest plasma Clearing Factor activity corresponded to the lowest concentration of the enzyme in the tissues (Fig. 2).

The lipase activity of plasma and tissues was inhibited by 0.2 mg/ml of protamine sulfate and 1 M NaCl in varying degrees (Table III). The inhibitory effect of protamine was lower than that obtained with 1 M NaCl in the tissue extracts of kidney, liver and lung, while heart, adipose tissue and plasma enzymes were inhibited to an identical degree by both. This difference remained the same with the enzyme activities observed in the tissue extracts that were obtained 30 minutes after heparin injection. The degree

TABLE I. Comparison of Clearing Factor Activity of Male and Female Rat Plasma and Tissues.

Tissues	Females	Males
Heart	239 ± 22	55 ±10
Adipose tissue	498 ± 65	67 ± 4
Kidney	148 ± 48	43 ± 7
Liver	183 ± 7	67 ± 9
Lung	54 ± 7	27 ± 6
Plasma	48 ± 21	18 ± 4

Results are expressed as μeq free fatty acids (FFA)/g protein/hr. See text for assay conditions. Females were average of 2 groups and males were average of 5 groups of rats. Each group consisted of 5 animals.

TABLE II. Lipase Activity of Kidney and Lung Expressed as Per Cent Activity of Plasma Lipase Activity Before and After Heparin Injection.

	Min				Hr			Days
Tissues	0'*	15'	30'	45'	1	2	3	3
Kidney	285	79	39	34	74	167	444	232
Lung	90	37	20	32	37	57	160	105
Plasma	100†	202	244	269	195	110	77	81

* No heparin was injected.

† 69 μeq free fatty acids (FFA)/g protein/hr.

Lipase activity of kidney and lung were expressed as percentage activity of plasma lipase in the same animal group. Plasma lipase activities after heparin injection were expressed as percentage of initial lipase activity. For details see the legend to Fig. 1.

TABLE III. % Inhibition of Clearing Factor with NaCl and Protamine Sulphate.

Tissues	Before heparin injection		30 min after heparin injection	
	Protamine	NaCl	Protamine	NaCl
Heart	26	24	55	63
Adipose tissue	36	38	60	71
Kidney	16	53	0	12
Liver	10	41	37	56
Lung	22	39	30	46
Plasma	37	37	53	58

Acetone powders of the tissues were prepared from the rats before and 30 min after heparin injections. Assay conditions are described in *Methods*. Inhibitors were added to obtain a final concentration of 0.2 mg protamine sulfate/ml and 1 M NaCl. Controls without inhibitors for each tissue were also incubated in the test.

of inhibition by protamine sulfate and 1 M NaCl before and after heparin injection did not remain constant, but it was consistently increased in the heart, adipose tissue, liver, lung and plasma. The kidney lipase was protamine-resistant and there was little inhibition with NaCl after heparin injection. This indicated that the injection of heparin released both lipases inhibited by lipoprotein-lipase inhibitors and lipases not inhibited by them. It is evident that the fraction of the protamine-inhibited lipase was increased in the tissues, while total quantity decreased.

β-Monoglyceridase activity assayed with β-monomyristin as the substrate in adipose tissue, heart, and kidney was increased several fold after heparin injection but remained relatively unchanged in liver and lung (Table IV). The plasma level of this enzyme was insignificant before or after heparin injection. This enzyme was particle bound and was assayed in total acetone powder homogenates.

TABLE IV. Increase of β-Monoglyceridase Activity in Rat Tissues 30 Minutes After Heparin Injection.

Tissues	μeq fatty acid/g tissue acetone powder or ml plasma/hr		
	No heparin	30 min after heparin injection	% Increase
Heart	30	83	176
Adipose tissue	120	758	531
Kidney	00	120	—
Liver	83	97	16
Lung	30	40	33
Plasma	.5	.8	60

β-Monoglyceridase was assayed as described in *Methods*. Substrate used was β-monomyristin (15 mg/ml). Controls without substrate for endogenous lipid hydrolysis were substrated from β-monoglyceridase activity. Lipase activity was assayed on pooled tissues, obtained before and after heparin injection of group of 5 rats.

Tissues obtained from rats, before heparin treatment, showed lipase activity that was stimulated by epinephrine compared to the controls without epinephrine(6). The epinephrine stimulation was observed in all tissues tested to the extent of 1.5 to 2 times the original levels (Table V). The stimulation of the lipase by epinephrine was observed only with preincubated whole tissues. Only a small rise of lipase activity was observed in the acetone powders and fresh tissue homogenates.

Activation of the lipases was nil in the tissues obtained from the rats sacrificed 30 minutes after heparin injection (Table V). Possibly, the effect of epinephrine was either inhibited or substituted by heparin.

In post-heparin plasma another lipase activity, possibly an α-monoglyceridase was reported by Shore and Shore(8). Similarly, it was a protamine-resistant lipase and was not inactivated by heating at 54°C for 15 minutes, in contrast to lipoprotein lipase. Tributyrinase(15), esterase(16) and postheparin-lecithinase(17) were also increased under the same conditions. These lipases also did not have the characteristics of lipoprotein lipase. There is no available report concerning the discharge of enzymes by heparinoids from their storage places other than lipases.

Summary. Injection of heparin into rats induced an increase in lipase activity of the plasma and a decrease in lipase activity of the tissues. The recovery of lipase levels in the tissues was observed after 2 to 3 hours. Before and after heparin injection the ratio of lipase activity inhibited by protamine sulfate and NaCl to lipase activity resistant to these inhibitors was not constant. After heparin injection increased activity of β-monoglyceridase in the tissues was observed. The tissues (of the No-Heparin-Injected rats) treated with epinephrine exhibited significant increases in lipase activity. However, no marked increase in lipase activity was observed in the tissues (of Heparin-Injected rats) treated with epinephrine.

TABLE V. Levels of Epinephrine-Stimulated Lipase in Tissues of Rats Before and 30 Minutes After Heparin Injection.

	μeq fatty acid/g of wet tissue or ml plasma/hr			
	Before heparin injection		30 min after heparin injection	
Tissues	No epinephrine	Δ Increase due to epinephrine	No epinephrine	Δ Increase due to epinephrine
Heart	31.1 ± 3.8	14.8 ± 3.3	33.0 ± 9.6	.07 ± 2.0
Adipose tissue	26.1 ± 4.3	19.2 ± 3.3	23.0 ± 7.6	1.1 ± 1.9
Kidney	37.9 ± 9.0	30.4 ± 7.0	42.0 ± 4.6	2.8 ± 2.4
Liver	30.6 ±10.3	23.4 ± 8.3	35.0 ± 4.3	4.4 ± 2.7
Lung	24.3 ± 4.3	15.3 ± 9.3	38.0 ± 9.0	.1 ± 1.4
Plasma	2.3 ± 1.9	2.7 ± .9	6.6 ± 1.3	−.5 ± .015

Rats were sacrificed before and after injection of heparin. Tissues were removed, divided into 2 halves, weighed and placed in a solution containing 2 ml of 0.06 M phosphate buffer at pH 7.1 and 1 ml of 10% bovine serum albumin adjusted to pH 7.1 and incubated for 3 hr at 37°C. One half of the tissue was stimulated by addition of 30 μg epinephrine (final concentration 10 μg/ml) to the incubation mixture and was incubated 10 min longer. The other half of the tissue served as a control. Tissues were then homogenized in a suitable volume of 0.06 M of phosphate buffer at pH 7.1 to give a concentration of 50 mg/ml of wet tissue in the final incubation mixture described for assay of the β-monoglyceridase(6). The substrate used in these experiments was Ediol (4 mg coconut oil/ml of incubation mixture). Incubation period after addition of the substrate was 20 min. Free fatty acids were assayed as described in text(4). Data presented above are mean values of 3 experiments, and ± stands for variation from the mean.

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Received October 26, 1966. P.S.E.B.M., 1967, v125.

DNA in Human Pituitary Glands.* (32046)

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The cellular mechanism by which pituitaries become substantially different in weight is not fully known. There appear to be several possible mechanisms; one is hyperplasia, another is hypertrophy and still another may be a shift of one cell type to another. The ex-

* This research was supported in part by USPHS Grant AM-07430 from Nat. Inst. of Arthritis & Metab. Dis. and by United Health Foundation of Western New York.