

Response of Plasma Histaminase Activity to Small Doses of Heparin in Normal Subjects and Patients with Hyperlipoproteinemia

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ABSTRACT The release of histaminase activity in plasma after small intravenous doses of heparin was studied in 85 normal subjects and patients. In normal subjects, plasma histaminase activity (basal level, 1.7 ± 0.1 U/ml, mean \pm SEM) increased 1.6 ± 0.2 U/ml after 10 U of heparin/kg, 8.5 ± 2.4 U/ml after 20 U/kg, and 33 ± 4.9 U/ml after 75 U/kg. The extent of the increase varied widely among individuals but in a particular individual the response was constant and dose-dependent. Histaminase activity rose to peak levels within 7–15 min and then declined exponentially with a half-life of 40–120 min. This pattern of response was also observed in two patients with the histaminase-producing tumor, medullary carcinoma of the thyroid. A significantly reduced response was observed, however, in 14 patients with type I hyperlipoproteinemia, a disorder in which high plasma triglyceride levels are associated with low postheparin plasma lipolytic activity. After 10 U heparin/kg, plasma histamine activity increased 0.5 ± 0.2 U/ml, and after 75 U heparin/kg, 10.9 ± 5.6 U/ml. In contrast, in 27 patients with other types of hyperlipoproteinemia in whom postheparin lipolytic activity was normal, the increase (2.4 ± 0.6 U/ml) in plasma histaminase activity after 10 U heparin/kg was not significantly different from that of normal subjects. The reduced response of the plasma histaminase activity to heparin in patients with type I hyperlipoproteinemia did not appear to be due to the presence of lipemia or to an inhibitor of the enzyme in plasma. These findings suggest that many patients with type I hyperlipoproteinemia may have deficient release

of both lipolytic and histaminase activities into plasma after heparin administration.

INTRODUCTION

A variety of enzyme activities appear in human plasma after the administration of heparin. These include enzymes with lipolytic activity such as lipoprotein lipase (glycerol-ester hydrolase, EC 3.1.1.3) (1, 2), monoglyceride hydrolase (2, 3), and phospholipase (4), as well as diamine oxidase (5, 6), an enzyme that catalyzes the deamination of histamine (histaminase activity), and diamines such as putrescine (7). The release of plasma lipolytic activity occurs after the injection of as little as 10 U heparin/kg (2). It was not known whether plasma diamine oxidase activity increases with this dose of heparin, because the changes in diamine oxidase have been studied only after large doses (5, 6). Diamine oxidase activity has been shown to increase in plasma during pregnancy (7, 8), where the maternal placenta is the source of the high enzyme activity (9, 10), and in some patients with medullary thyroid carcinoma, a histaminase-producing tumor (11–13).

In the present study a sensitive radioassay for histaminase activity (14) was utilized to determine if changes in plasma histaminase activity occur after both small and large doses of heparin in normal subjects and in patients with two diseases that might be expected to alter the response of the plasma histaminase activity. These included two patients with medullary carcinoma of the thyroid, 14 patients with fat-induced hypertriglyceridemia (classified as type I hyperlipoproteinemia according to the system of Fredrickson, Levy, and Lees [15]), in whom the release of plasma lipolytic activity

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TABLE I
*Plasma Lipid Values of the 14 Patients
 with Type I Hyperlipoproteinemia*

Patient	Age	Sex	Plasma triglyceride	Plasma cholesterol
	<i>yr</i>		<i>mg/100 ml</i>	<i>mg/100 ml</i>
S. K.	11	F	1683	186
J. K.	21	M	3528	360
R. N.	16	M	2805	187
T. P.*	2½	M	5000	714
J. P.	38	M	528	192
L. P.	34	M	1974	259
P. P.	22	M	785	171
G. S.	9	F	514	128
I. S.	36	M	2057	171
K. S.	10	F	1819	225
D. T.	15	F	800	122
P. W.	25	F	560	168
G. W.	29	F	800	160
L. W.	36	M	4590	364
Normal range (<40 yr)			10-150	120-270

Subjects J. P., P. P., G. S., D. T., P. W., and G. W. were consuming diets containing less than 5 g fat for at least 1 wk before sampling; the other patients were consuming diets unrestricted in fat content. On initial evaluation all patients had significant hyperchylomicronemia with minimal elevation of plasma pre-beta lipoproteins as determined by observation of standing plasma, lipoprotein paper electrophoresis, and plasma cholesterol:triglyceride ratios (<0.19) (15).

* Patient kindly referred by Dr. D. A. Applegarth, Children's Hospital, Vancouver, B.C.

by heparin was subnormal, and 27 patients with other types of hypertriglyceridemia, in whom the release of lipolytic activity after heparin was normal. It was of particular interest to determine if patients with type I hyperlipoproteinemia had an abnormally small increase and patients with medullary thyroid carcinoma an abnormally large increase in plasma histaminase activity after the injection of heparin.

METHODS

Control subjects and patients. Control subjects were 21 women and 21 men, 18-60 yr, who were either healthy normal volunteers hospitalized at the National Institutes of Health, or laboratory personnel who had no known medical problems. Patients included a man (60 yr) and a woman (44 yr) with medullary carcinoma of the thyroid, previously reported as W2 and W6 (11); and 18 females and 23 males with various types of hypertriglyceridemia.

The determination of plasma triglyceride and cholesterol concentrations and the characterization of plasma lipoproteins by paper electrophoresis were performed by standard procedures described previously (15); postheparin plasma lipolytic activity was measured by the assay of Fredrickson et al (15) and by a new and more specific assay of triglyceride lipase activity (16). The description of the latter assay and the values obtained for normal subjects and the patients with hypertriglyceridemia will be the subject of a separate

report.¹ With the data obtained by the above procedures the patients were classified according to the system of Fredrickson et al. (15). 14 patients satisfied the criteria for type I hyperlipoproteinemia; clinical and biochemical data pertaining to these patients are given in Table I. Type I patients had reduced postheparin triglyceride lipase activity as described elsewhere (16).¹ Five of the type I patients, J. P., L. P., P. P., G. W., and L. W., had at least one affected sibling and were thus considered to have familial type I hyperlipoproteinemia (15).

The other patients with hypertriglyceridemia were classified according to the criteria of Fredrickson et al. as type III, IV, or V hyperlipoproteinemia (15). The clinical and biochemical data pertaining to these patients will be reported separately.¹ Postheparin plasma lipolytic activity was normal in all the type III, IV, and V patients included in this study, and thus they served as a hypertriglyceridemic control group for the lipase-deficient type I subjects. None of the patients with hyperlipoproteinemia had clinical or biochemical evidence of hepatic, renal, or thyroid dysfunction.

Six patients with type I hyperlipoproteinemia were children, 2½-16 yr of age. All other patients were adults aged 18-64 yr.

Procedure for heparin injection and collection of samples. Subjects fasted overnight and studies were performed the next morning. The time-course of the response to heparin was studied by injecting heparin intravenously through an indwelling antecubital scalp vein needle over an approximately 5-s period and withdrawing blood at various intervals through the same needle. The needle was flushed with saline after the injection of heparin or withdrawal of blood sample. In studies in which blood was collected at one time point (10 min) after heparin administration, heparin was injected intravenously into one arm and the blood sample was withdrawn from the opposite arm. 10 ml of blood was drawn into syringes containing 0.1 ml (10 U) of heparin solution; plasma was obtained by centrifugation of blood at 150 g for 20 min. Serum samples were obtained from blood taken into nonheparinized syringes; the blood sample was kept ice-cold for 30 min and then centrifuged as above. Plasma and serum samples were stored at -20°C.

Assay procedures. Histaminase activity was assayed by the procedure of Beaven and Jacobsen (14), in which [β -³H]histamine is deaminated by histaminase with the quantitative release of the β -tritium as tritiated water. The assay is specific, because other histamine-metabolizing enzymes do not release the β -tritium (15). 100 μ l of plasma or serum was incubated with 100 μ l of a solution of [β -³H]histamine (0.1 μ Ci, 14.3 pmol) in 0.1 M sodium phosphate buffer, pH 6.8, for 60 min at 37°C. Assay blanks were prepared with serum or plasma and aminoguanidine bicarbonate, 2×10^{-5} M, a specific inhibitor of histaminase activity (17). Water was obtained from the incubation mixture by sublimation in a Thunberg tube and was assayed for tritium by liquid scintillation counting. Enzyme activity was expressed as units per milliliter of serum or plasma where 1 U equals 1 pmol of [β -³H]histamine deaminated/h incubation. The assay was sufficiently sensitive to permit measurement of enzyme activity down to 0.1-0.2 U/ml plasma. Plasma monoamine oxidase activity was assayed by the method of Robinson, Lovenberg, Keiser, and Sjoerdsma (18), in which [α -¹⁴C]benzylamine was used as substrate for the enzyme. Activity was expressed as units per milli-

¹ Krauss, R. M., R. I. Levy, and D. S. Fredrickson. Manuscript in preparation.

liter of serum or plasma where 1 U equals 1 nmol [α - 14 C]-benzaldehyde deaminated/h.

In vitro studies of the effect of inhibitors of lipoprotein lipase and lipemic plasma on plasma histaminase activity. Plasma was obtained from a control subject who had received 75 U/kg heparin i.v. The plasma, 20 μ l, was incubated for 30 min at 37°C in 0.1 M sodium phosphate buffer, pH 6.8, with 80 μ l of a solution of the inhibitor (protamine sulfate, sodium or potassium chloride), or 80 μ l of lipemic plasma obtained from patients with type I hyperlipoproteinemia. The [β - 3 H]histamine solution, 100 μ l, was then added to the incubation mixture, and histaminase activity was determined as described above.

Chemical and drugs. [β - 3 H]histamine was prepared as previously described (14). [α - 14 C]benzylamine was obtained from Nuclear Research Chemicals Inc., Orlando, Fla., and aminoguanidine bicarbonate from Eastman Kodak Chemical Products, Rochester, N. Y. Heparin sodium solution, 1000 U/ml (Upjohn Co., Kalamazoo, Mich) was used undiluted for doses of 75 U/kg or was diluted to 100 U/ml in sterile isotonic saline for doses of 10 and 20 U/kg.

Statistical analysis of results. Values for plasma histaminase activity are reported as the mean \pm SEM. The statistical significance of the differences in the response of plasma histaminase to heparin was determined by the standard Student *t* test (two-tailed).

RESULTS

Changes in plasma histaminase and serum monoamine oxidase activities after heparin. The increases in plasma histaminase activity after the administration of 10, 20, and 75 U heparin/kg in normal subjects are summarized in Table II. As seen from these data, there was a wide variation in the response after all doses of heparin. In a particular individual, however, the increase was constant (Table III) and dose-dependent (Table IV). Individuals with a small response to 10 U heparin/kg also showed a small response to 75 U/kg, and those with a large response to 10 U/kg showed a large response to 75 U/kg (Table IV). Histaminase activity was similar in both serum and heparinized plasma. In four control subjects and one patient with medullary carcinoma of the thyroid, histaminase activity in serum was 2.2, 1.3, 1.3, 0.6, and

TABLE II
Increase in Plasma Histaminase Activity in Control Subjects in Response to Various Doses of Heparin

Heparin dose	No. of subjects tested	Basal plasma histaminase activity	Increase in plasma histaminase activity*
<i>U/kg i.v.</i>			<i>U/ml</i>
10	35	1.7 \pm 0.1	1.6 \pm 0.2 (0-4.5)
20	5	2.0 \pm 0.4	8.5 \pm 2.4 (3-16)
75	15	1.9 \pm 0.3	33 \pm 4.9 (7-70)

Values show mean \pm SEM and, in parentheses, range of increases.

* 10 min after the injection of heparin. The values show the rise in plasma histaminase activity above the basal level.

TABLE III
Reproducibility of Response of Plasma Histaminase Activity to Heparin

Subject	Date	Plasma histaminase activity	
		Basal level	Increase after heparin*
<i>U/ml</i>			
Controls			
R. B.	2 Aug. 71	1.1	1.1
	2 Oct. 71	1.8	0.9
C. B.	31 Aug. 71	2.1	3.2
	9 Sept. 71	1.7	3.5
L. K.	29 Sept. 71	1.6	3.6
	15 Oct. 71	1.6	3.0
	20 Oct. 71	1.4	2.4
	20 Oct. 71	1.3	2.3
	1 Nov. 71	1.3	2.5
Type I hyperlipoproteinemia			
L. W.	4 Nov. 70	0.6	-0.2
	9 Dec. 70	0.8	0.0
	6 May 71	0.3	0.1
	28 July 71	0.3	0.0
D. T.	5 Sept. 71	0.7	0.2
	8 Dec. 71	0.7	0.3
G. S.	28 Sept. 71	0.6	0.7
	1 Oct. 71	0.7	0.9
Type V hyperlipoproteinemia			
R. J.	5 Aug. 71	1.7	1.1
	21 Dec. 71	1.3	1.7

* 10 min after the injection of 10 U/kg of heparin.

66 U/ml and in plasma 2.3, 1.4, 0.8, 0.8, and 62 U/ml, respectively. These data indicated that heparin in vitro did not increase histaminase activity.

TABLE IV
Increase in Plasma Histaminase Activity in Response to 10 and 75 U of Heparin/kg in Various Control Subjects

Subject	Plasma histaminase activity increase* after	
	10 U/kg	75 U/kg
<i>U/ml</i>		
R. S.	0.0	6.8
R. R.	0.3	8.2
R. N.	0.4	9.9
A. G.	0.7	25.2
D. Sp.	0.9	32.4
D. So.	1.0	26.7
G. S.	1.2	37.8
R. J.	1.4	32.2
G. J.	3.2	39.1
C. J.	3.5	49.8
W. H.	4.4	60.1

* As measured 10 min after the injection of heparin.

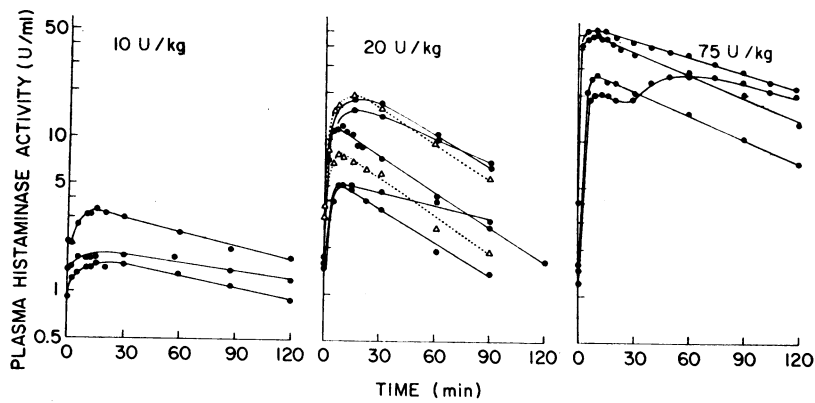


FIGURE 1 Time-course of changes in plasma histaminase activity after the i.v. administration of 10, 20, and 75 U heparin/kg to control subjects (●—●), and to two patients with medullary carcinoma of the thyroid (△··△).

After all doses of heparin, plasma histaminase activity increased rapidly, reached a maximum within 7–15 min, and then declined exponentially (Fig. 1). The half-life of this exponential decline was about 120 min after the 10/kg dose of heparin and 40–90 min after the 20 and 75 U/kg doses of heparin. This pattern of response was

also observed in the two patients with medullary carcinoma of the thyroid. In neither patient was the response greater than that of normal subjects (Fig. 1). In one control subject, an additional rise in enzyme activity was observed 30–60 min after the injection of 75 U/kg heparin (Fig. 1).

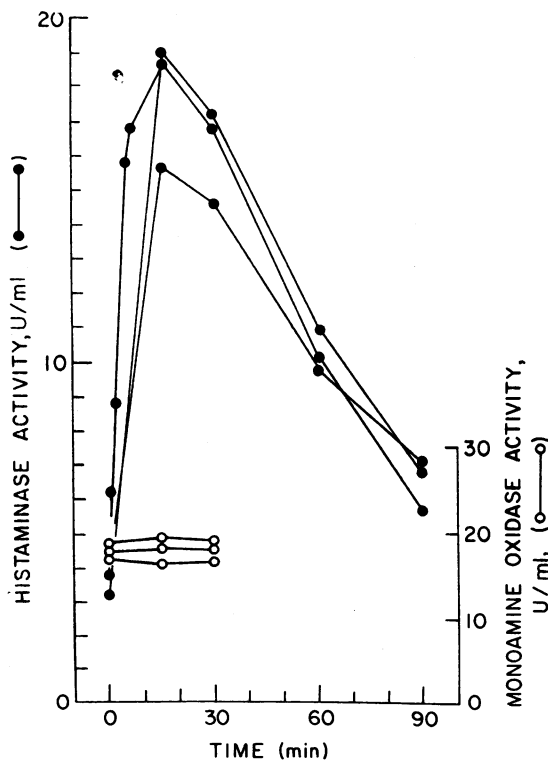


FIGURE 2 Changes in serum histaminase and monoamine oxidase activities in three normal subjects after the i.v. administration of 20 U heparin/kg. Serum histaminase activity, ●—●; serum monoamine oxidase activity, ○—○.

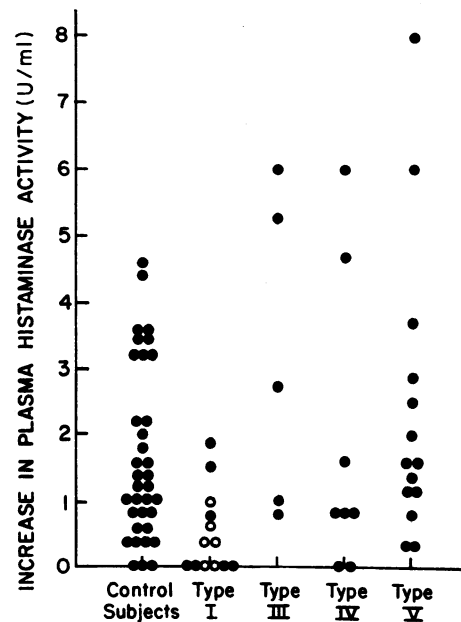


FIGURE 3 Increase in plasma histaminase activity after intravenous administration of 10 U heparin/kg to 35 control subjects and 40 patients with types I, III, IV, or V hyperlipoproteinemia. The points depict the increase in plasma enzyme activity 10 min after the injection of heparin. Six patients with type I hyperlipoproteinemia were children less than 16 yr of age and are shown by the open circles. The response in patients with type I hyperlipoproteinemia was significantly lower than that in normal subjects or patients with the type III, IV, or V disorder (see text).

Human serum contains another amine oxidase activity, a soluble monoamine oxidase, which resembles diamine oxidase in its sensitivity to inhibition by carbonyl reagents and copper-chelating agents, but differs in the range of substrates that it deaminates (19). Monoamines such as tyramine, tryptamine, and benzylamine are particularly good substrates for this enzyme. Measurement of plasma monoamine oxidase activity in three normal subjects indicated that unlike histaminase activity, monoamine oxidase activity does not increase after the injection of heparin (Fig. 2).

Increase in plasma histaminase activity in normal subjects and patients with hyperlipoproteinemia. The increase in plasma histaminase activity after the administration of 10 U heparin/kg in normal subjects and patients is shown in Fig. 3. The average increase in enzyme activity was 1.6 ± 0.2 U/ml (\pm SEM) in normal subjects, 0.5 ± 0.2 U/ml in patients with type I hyperlipoproteinemia, and 3.2 ± 1.1 , 1.8 ± 0.8 , and 2.4 ± 0.6 U/ml in patients with types III, IV, and V hyperlipoproteinemia, respectively. The mean increase in the type I patients was significantly lower than that in the normal subjects ($P < 0.001$) and in patients with other types of hyperlipoproteinemia ($P < 0.001$). The mean increase for patients with types III, IV, and V hyperlipoproteinemia (2.4 ± 0.5 U/ml) was not significantly different ($P > 0.1$) from that of normal subjects.² As seen in Fig. 3, no increase in enzyme activity was observed in 6 of our 13 patients with type I hyperlipoproteinemia, whereas an increase was observed in 32 of 35 normal subjects, and 25 of 27 patients with types III, IV, and V hyperlipoproteinemia. A response was noted in some patients with type I hyperlipoproteinemia, however, and in two patients (I. S. and J. K.) the increase was greater than 1 U/ml plasma. Low responses were observed in both children and adults with the type I disorder (Fig. 3).

In nine of the patients with type I hyperlipoproteinemia, it was possible to test the response to 75 U heparin/kg. The mean increase, 10.9 ± 5.6 U/ml, was significantly lower ($P < 0.02$) than that of controls (33 ± 4.9). Eight patients showed a low response and one patient a large response to this dose of heparin

² The differences were confirmed by analysis of covariance which was based on a common linear regression of the final level on basal level of enzyme in plasma (i.e., activity after and before heparin injection) in each group of subjects and patients. The assumption was made that the five linear regressions were parallel. The $(1 - \alpha) > 99\%$ confidence intervals for the individual groups indicated no apparent difference in response among types III, IV, and V nor between normals and types III, IV, and V combined. Differences were found between type I and normals and between type I and types III, IV, and V combined. A difference was also found between type I and normals after the 75 U/kg dose of heparin.

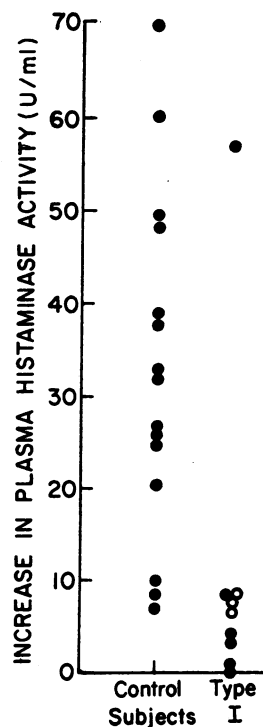


FIGURE 4 The increase in plasma histaminase activity after the i.v. administration of 75 U heparin/kg to 15 normal subjects and 9 patients with type I hyperlipoproteinemia. The points depict the increase in plasma histaminase activity 10 min after the injection of heparin. Children are depicted by open circles.

(Fig. 4). The latter patient (I. S.) was one of the two who had shown a response greater than 1 U/ml after 10 U heparin/kg. The second of these two patients was not tested with the 75 U/kg dose of heparin.

The increase in enzyme activity in normal subjects and patients with hyperlipoproteinemia appeared to be related to the individuals' basal histaminase activity. Those with low basal plasma enzyme activity had low responses and those with high basal enzyme activity had large responses to heparin. A plot of basal plasma histaminase activity against the increase in enzyme activity showed a highly significant correlation ($P < 0.001$, $r = 0.84$) (Fig. 5). Five patients with types III, IV, and V disorders had high basal enzyme levels and greater responses to heparin than normal subjects (Fig. 5).

Increase in plasma histaminase activity in a patient with type I hyperlipoproteinemia during pregnancy. During the course of this study, one woman with type I hyperlipoproteinemia (G. W.) became pregnant. This afforded the opportunity to study the appearance of histaminase activity from a second source, the maternal placenta, in this woman. The patient's plasma histaminase activity was high during pregnancy and was com-

Effect of inhibitors of lipoprotein lipase and of lipemic plasma on histaminase activity. Sodium chloride, potassium chloride, and protamine sulfate, inhibitors of lipoprotein lipase activity (20, 21), were tested for their effect on histaminase activity in plasma obtained from a control subject who had received heparin (Table VI). Plasma histaminase activity, like lipoprotein lipase activity, was completely inhibited by 1 M sodium and potassium chloride but was inhibited incompletely by high concentrations of protamine (Table VI).

Lipemia did not appear to modify plasma histaminase activity. Enzyme activity was not inhibited when lipemic plasma from two patients with type I hyperlipoproteinemia was added to plasma from the control subject above (Table VI). Treatment of one patient (L. W.) with a low fat diet resulted in a fall of plasma triglyceride levels from 4590 to 672 mg/100 ml with no change in plasma histaminase activity (6 May 71 and 28 July 71, Table III). In addition, three patients with type V hyperlipoproteinemia who had plasma triglyceride levels of 2024, 2244, and 3045 mg/100 ml, showed normal increases of 2.5, 1.5, and 2.9 U histaminase activity after 10 U/kg heparin.

DISCUSSION

The heparin-induced rise in plasma histaminase (diamine oxidase) activity has been observed in man (5, 6), guinea pig (22, 23), rabbit (23), rat (23), and other vertebrate species (24). There is evidence that this rise is due to release of enzyme from liver in guinea pig (22) and intestine in rat (25). The source of the enzyme in man is not known, but because human kidney and intestine contain appreciable histaminase activity (12), both of these organs are possible sources.

The present studies have shown that a rise in plasma histaminase activity occurs after the administration of heparin in doses as low as 10 U/kg. Although the extent of this rise varies widely among individuals, in a particular individual the response is constant and appears to be related to the individual's basal histaminase activity. One possible explanation for this relationship is that both the basal level and the rise in plasma enzyme activity after a 10 U/kg dose of heparin are determined by the level of histaminase activity in tissues. Thus, high levels of enzyme activity in tissues would lead to high basal enzyme activity in plasma and a large response to heparin. The effect of heparin on plasma histaminase is specific, in that monoamine oxidase activity is not increased in plasma after heparin administration.

The rapid rise in plasma histaminase activity after the injection of heparin suggests that the enzyme is released directly into the bloodstream. The exponential decline further suggests that the enzyme is cleared from the

blood at a single site. Other authors have noted that after large doses of heparin, an additional rise in histaminase activity may follow the initial rapid rise in enzyme activity in some subjects. This later rise was attributed to enzyme reaching the blood from lymph (5, 6). In the present studies an additional rise was observed in one of the subjects after 75 U/kg heparin.

The availability of a sensitive assay for histaminase activity has permitted studies that would not have been possible by other procedures. In the past, diamine oxidase activity in plasma has been assayed by measurement of histaminase activity or by measurement of deamination of radioactively labeled putrescine (7, 14). In our hands, the latter assay procedure was not sufficiently sensitive to measure the small changes in plasma enzyme activity that occur after the injection of 10 U heparin/kg.

The two patients with medullary carcinoma of the thyroid showed a rise and decline in histaminase activity similar to that in normal subjects. However, the wide range of responses in normal subjects and the small number of patients available for study do not permit us to conclude whether histaminase activity in medullary thyroid carcinoma contributes to the rise in plasma histaminase after heparin administration.

In the patients with type I hyperlipoproteinemia, who all had low levels of triglyceride lipase activity after heparin, the increase in plasma histaminase after 10 U heparin/kg was significantly less than that in normals or in patients with other types of hyperlipoproteinemia. This low response was even more apparent when the dose of heparin was 75 U/kg. At either dose, however, the histaminase response in some patients with the type I disorder fell within the normal range, and in one patient (I. S.) the response was particularly large. We have no explanation for the large response in this individual, although it may indicate that there is a genetic or biochemical heterogeneity in this disorder. The patient was otherwise similar in all respects to the other type I patients studied.

The high plasma histaminase activity in the type I patient who was pregnant indicated that the release of enzyme from placenta into the plasma was not impaired in this patient. In the nonpregnant state, this patient showed no increase in plasma histaminase activity after a 75 U/kg dose of heparin. The histaminase activity which appears in plasma during pregnancy has a different electrophoretic mobility and is cleared less rapidly from the circulation than the histaminase activity released by heparin (26). The possibility that isoenzymes of histaminase exist has been suggested (26).

The low plasma histaminase activity after heparin administration in patients with type I hyperlipoproteinemia did not appear to be due to lipemia or to the presence of

inhibitors. Other studies have suggested that type I patients do not show a general lack of response to heparin such as that seen in certain patients with dysproteinemia (27). Patients with the type I disorder may in fact show normal increases in plasma monoglyceride hydrolase (2, 28) and hepatic triglyceride lipase activity (16) after heparin. Heparin-induced changes in clotting parameters have not been systematically studied in this disorder, but an increase in thrombin time after heparin administration has been reported in one patient (27). Whether the deficient release of histaminase activity is due to low enzyme levels in tissues or to a defect in the release of enzyme from tissues remains to be determined.

Histaminase and lipase catalyze two different reactions. The two enzyme activities, however, have some features in common. They are inhibited by 1 M sodium and potassium chloride and by protamine, although the inhibition of histaminase activity by protamine is incomplete. Both enzymes are released rapidly into plasma by intravenously administered heparin. Our studies have shown that the time of peak plasma histaminase activity after small doses of heparin is 7–15 min, which is comparable to the time of peak activity previously described for plasma lipase activity (29, 30). Moreover, a deficiency of heparin-released plasma histaminase activity coexists with the deficiency of lipase in many of our patients with type I hyperlipoproteinemia. It is therefore possible that similar mechanisms are involved in the release of the two enzyme activities into plasma by heparin.

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