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The Activity of Hepatic Lipase and Lipoprotein Lipase in Glycogen Storage Disease: Evidence for a Circulating Inhibitor of Postheparin Lipolytic Activity

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Summary

This study was designed to investigate the greatly reduced activities of hepatic lipase and lipoprotein lipase in postheparin plasma of patients with glycogen storage disease (GSD). Evidence for the presence of a circulating inhibitor in the plasma of GSD patients prior to the establishment of metabolic control was provided by the following observations.

The hepatic lipase activity from patients with GSD gave a nonlinear relationship with time, maximum activity being obtained after 5 min whereas normal postheparin plasma showed a linear relationship for at least 20 min. The addition of GSD plasma to a normal postheparin plasma resulted in inhibition of the normal postheparin lipolytic activity. The inhibition showed a dose response, with 10 and 30 μl of the GSD plasma giving 22 and 65% inhibition after 20 min, respectively. Serial dilution of GSD postheparin plasma gave increasing activity of hepatic lipase when expressed per ml of plasma. Thus, a 1:100 dilution gave a normal activity of 15 μmol/ml/h whereas a 1:10 dilution gave less than 10% of this activity. After affinity chromatography of the GSD postheparin plasma on heparin/Sepharose, the inhibition was removed and a normal lipolytic activity was obtained.

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Abbreviations

GSD, glycogen storage disease
FH, familial hypercholesterolemia
HLip, type I hyperlipoproteinemia

Type I glycogen storage disease (glucose-6-phosphatase deficiency) is characterized by hepatomegaly, growth retardation, fasting hypoglycemia, metabolic acidosis, and hyperlipidemia. The hyperlipidemia which is predominantly a hypertriglyceridemia appears to result from both an increase in synthesis (16) and a decrease in clearance (9) of circulating triglyceride.

Triglycerides are transported in the circulation by triglyceride-rich lipoproteins (chylomicrons and very low density lipoprotein). They are cleared from the circulation by the action of lipoprotein lipase which is located on the capillary endothelium of extrahepatic tissues but can be released into the circulation by low doses of intravenous heparin. Postheparin plasma, however, also contains a lipase of hepatic origin which has properties different from lipoprotein lipase. We (12, 13) and others (8) have reported that activities of both the lipoprotein lipase and hepatic lipase are reduced in postheparin plasma from patients with type I GSD. Treatment of this disorder by frequent feeds or nocturnal gastric drip results in marked improvement of the hyperlipidemia (15) and an increase in the activity of lipoprotein and hepatic lipase has been reported (8). These changes suggested to us that treatment might have resulted in the removal of a circulating

inhibitor of lipase activity and this hypothesis was investigated by studying postheparin plasma from patients with GSD before and after metabolic control was established. In general, hepatic lipase activity was estimated because of the comparative ease and speed of the assay but where appropriate, results were confirmed by estimating lipoprotein lipase activity using chylomicrons as a natural substrate.

SUBJECTS AND METHODS

Subjects. The subjects studied were selected from a group of patients described in detail previously (12, 13). They comprised a normal adult and children with FH, HLip, and type I GSD. Details are shown in Table 1 and unless otherwise stated the studies were carried out on postheparin plasma. Informed consent was obtained in all cases.

Methods. Blood collection. Blood was collected by venipuncture [into glass tubes containing dipotassium EDTA (1.5 mg/ml)], immediately before and 10 min after an intravenous injection of heparin. The plasma was stored at −20°C prior to assay.

Hepatic lipase activity. Hepatic lipase activity was specifically and selectively estimated using an artificial radiolabeled substrate as described and fully validated previously (12). In this procedure, use was made of the differing requirements of hepatic lipase and lipoprotein lipase for apoprotein CII and their different sensitivities to high concentrations of sodium chloride. It was normally necessary to dilute postheparin plasma 10- to 20-fold in 0.2 mol/liter Tris-HCl buffer (pH 9.0, containing 1.0 mol/liter sodium chloride) prior to assay for hepatic lipase activity.

Lipoprotein lipase activity. In some experiments, lipoprotein lipase activity was estimated using rat chylomicrons as a natural substrate. The chylomicrons were collected from the mesenteric lymphatics of male Wistar rats as described by Bollman *et al.* (4) and were diluted 2-fold in 0.2 mol/liter Tris-HCl buffer, pH 8.0 containing 0.15 mol/liter sodium chloride. This gave a typical final triglyceride concentration of 32 mmol/liter (mean of five separate estimations). Postheparin plasma (0.1 ml) was incubated with 0.1 ml diluted chylomicrons at 37°C and the reaction was stopped at fixed times by the addition of 0.2 ml chloroform. The mixture was then mixed and spun, and the liberated free glycerol in the aqueous phase was estimated enzymatically using a commercial kit (Calbiochem-Behring Corporation). The assay was linear with time for at least 2 h and with increasing volumes of plasma up to the production of at least 130 nmol of glycerol which corresponded to the hydrolysis of approximately 4% of the chylomicron triglyceride. Substrate concentration was not limiting, as a 4-fold increase in the chylomicron triglyceride concentration had no effect on activity. A quality control specimen was assayed on 12 occasions over a 2-month period using 12 different chylomicron substrate preparations and gave a coefficient of variation of 22%.

Heparin/Sepharose chromatography. Affinity chromatography using heparin/Sepharose 4B was used to partially purify the postheparin lipases. Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals Limited and the heparin/Sepharose (1 ml heparin/ml swollen gel) was prepared according to the instructions provided with the gel. The chromatography was carried out essentially as described by Boberg *et al.* (3). The lipases were coupled to the gel by rotating 0.5 ml postheparin plasma, 2.5 ml gel, and 1.4 ml buffer (0.2 mol/liter Tris-HCl, pH 9.0) for 30 min and the supernatant was removed following centrifugation. The gel was then transferred to a small glass column (6.0-mm diameter), washed with the Tris-HCl buffer, and the lipase activity eluted with 10 ml of the buffer containing heparin (1.5 mg/ml). The hepatic lipase activity in the eluate was assayed after dilution with an equal volume of saline buffer. Greater than 85% of the initial activity was recovered in the eluate, the majority appearing in the third 1-ml fraction.

Miscellaneous lipid assays. Serum and chylomicron triglyceride concentrations and serum cholesterol concentrations were estimated enzymatically using commercial kits. Plasma free fatty acids were estimated by gas-liquid chromatography (10).

RESULTS

Lipase activity. The increase of hepatic lipase activity with time using representative postheparin plasma samples is shown in Figure 1*A*. The activity obtained from normal adults, and patients with FH and HLip, showed a linear relationship with time with the line going through the origin. However, plasma from three patients with type 1 GSD and poor metabolic control, all showed a nonlinear relationship with time with a relatively normal initial velocity which reached a plateau by 5 min. Similarly shaped curves were also obtained when lipoprotein lipase activity was assayed using chylomicrons as substrate (Fig. 1*B*).

Addition experiments. To further investigate the nonlinearity observed with postheparin plasma from patients with GSD, the effect on lipase activity of adding different postheparin plasma samples to a normal postheparin plasma was investigated. Equal volumes (50 μl) of the two plasmas were combined and diluted 1:10 with saline buffer, and the combined hepatic lipase activity was estimated. The results of various additions are shown in Figure 2 *A* and *B*. The activities obtained after adding samples from a patient with FH or HLip were linear with time and equal to the sum of the two activities assayed separately (Fig. 2*A*). Results obtained with various GSD plasmas are shown in Figure 2*B*. When postheparin plasma, collected at a time when metabolic control was poor, was added, the reaction was not linear, and after 10 min the normal hepatic lipase activity was inhibited. A similarly shaped curve was obtained following the addition of a preheparin plasma sample collected at the same time. This

Table 1. *Details of patients studied*

Patient No.*	Diagnosis	Treatment	Serum triglyceride (mmol/liter)	Serum free fatty acids (mmol/liter)	Lipoprotein lipase (μmol/h/ml)	Hepatic lipase (μmol/h/ml)
4	Normal		1.0	0.8	3.8	10.8
8	Familial hypercholesterolemia	Cholestyramine	1.3	0.4	4.3	9.9
16a	Glycogen storage disease	None	100.0	8.0	0.8	1.6
16b		Frequent meals night & day	6.0	1.4		
24	Type I hyperlipoproteinemia	Low fat diet	7.0	1.2	0.3	1.6
Reference range			0.5–1.8	0.5–1.15	1.1–3.8	10.2–18.0

* Patient numbers are as in a previous report (13).

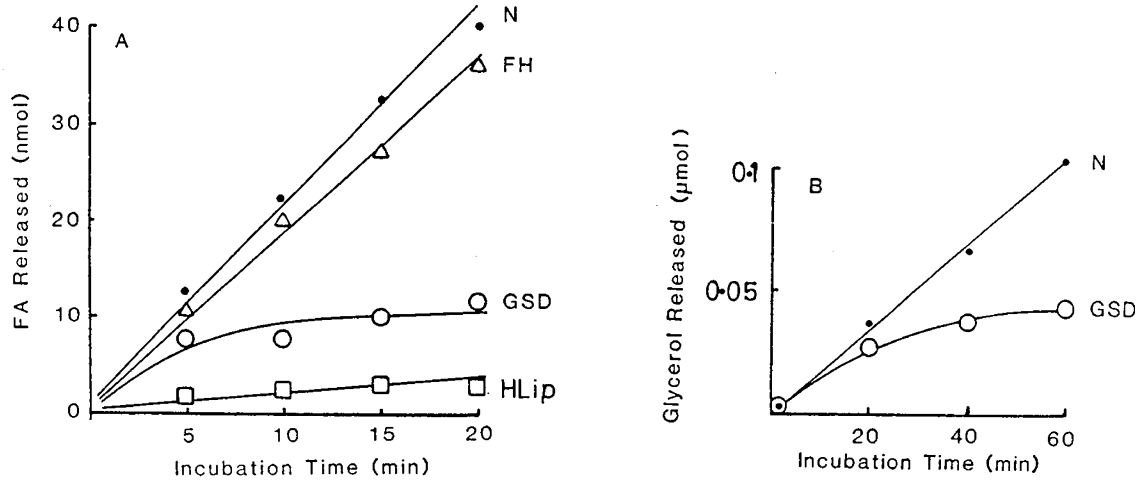

Fig. 1. *A*, the activity of hepatic lipase in postheparin plasma from a normal control (N) and patients with FH, type I GSD and poor metabolic control, and HLip. *FA*, fatty acids. *B*, the activity of lipoprotein lipase in post-heparin plasma from a normal control (*N*) and a patient with type I GSD and poor metabolic control.

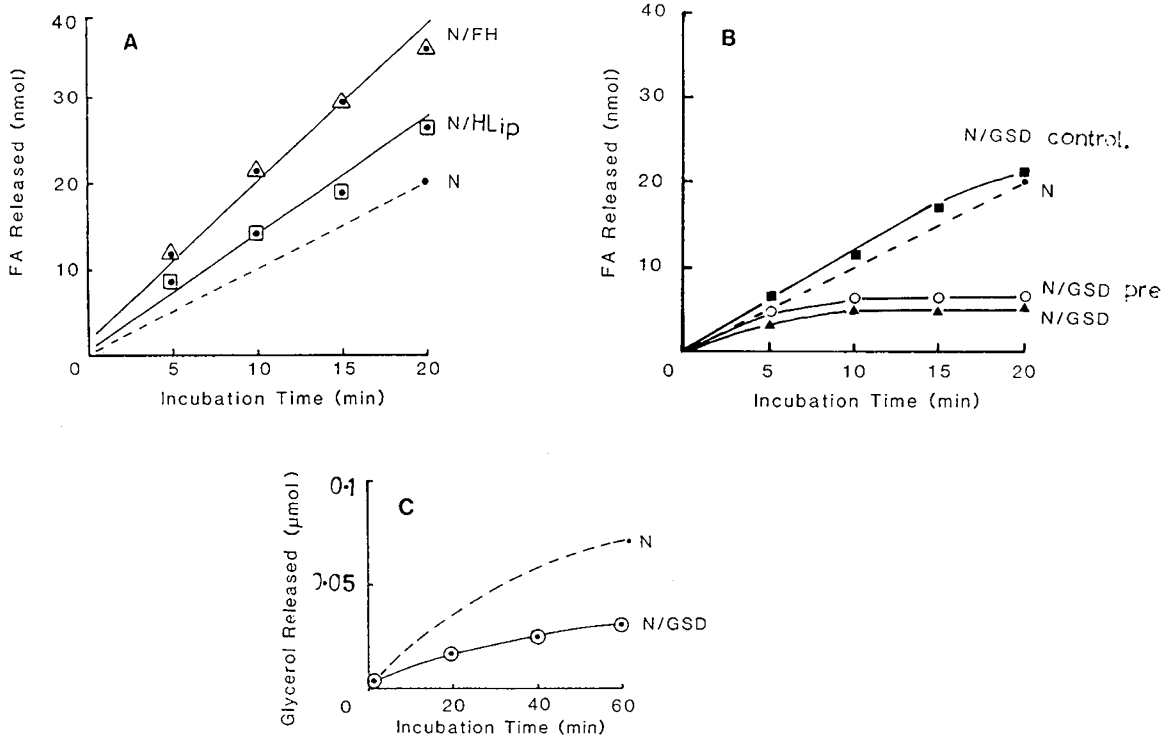

Fig. 2. *A*, the effect on hepatic lipase activity of adding various postheparin plasma samples to that of a normal control. Normal control alone (*N*), plus familial hypercholesterolaemia (*N/FH*), plus type I hyperlipoproteinemia (*N/HLip*). *FA*, fatty acids. *B*, the effect on hepatic lipase activity of adding various post-heparin plasma samples from the patient with type I GSD to that of a normal control. Normal control alone (*N*), plus a postheparin plasma sample when metabolic control was good (*N/GSD control*), plus a preheparin plasma sample when metabolic control was poor (*N/GSD pre*), plus a postheparin plasma sample when metabolic control was poor (*N/GSD*). *C*, the effect on lipoprotein lipase activity of adding postheparin plasma from the patient with type I glycogen storage disease and poor metabolic control. Normal control alone (*N*), plus glycogen storage disease (*N/GSD*).

inhibition and plateau of activity were not observed, however, after the addition of a postheparin sample from the same GSD patient taken at a time when metabolic control was improved.

Inhibition of normal lipoprotein lipase activity was also seen with postheparin plasma collected from the GSD patient when metabolic control was poor (Fig. 2*C*).

Dose-response experiments. The addition experiments suggested that both pre- and postheparin plasma specimens collected from GSD patients when metabolic control was poor contained a circulating inhibitor of lipase activity. This was corroborated by dose-response and serial dilution studies. The addition of 10 μl postheparin plasma from the patient with GSD to a normal postheparin plasma gave activity of hepatic lipase which was linear with time and showed no inhibition, whereas the addition of 30 and 50 μl of GSD plasma gave nonlinear curves with 22 and 65% inhibition of the normal activity after 20 min, respectively. A similar study with postheparin plasma from a patient with FH gave linear curves and a dose-related increase in total hepatic lipase activity.

The effect on hepatic lipase activity of serial dilutions of the GSD postheparin plasma is shown in Figure 3. When activities are expressed as μmol fatty acid released/ml plasma, there was increasing activity and improved linearity with increasing dilution of the sample. The 1:100 dilution gave an activity of 15

μmol/ml/h which falls within the normal range (mean ± 2SD) for adults of 12.1 ± 6.2 μmol/h (12). Serial dilutions of normal and FH postheparin plasma gave consistent activities when expressed per ml of plasma.

Affinity chromatography. Affinity chromatography on heparin/Sepharose was carried out in an attempt to separate the inhibitor from the lipase activity. The activities of a normal and GSD postheparin plasma before and after chromatography are shown in Figure 4. Prior to chromatography, the GSD plasma had less activity than the normal plasma, whereas after chromatography the reverse was the case. This suggested that a circulating inhibitor had been removed by the procedure and this was confirmed by various recombination experiments (Fig. 5). Normal postheparin plasma (50 μl) was mixed with the fraction under investigation, and saline buffer added to give a final volume of 1.0 ml and a sodium chloride concentration of 1.0 mol/liter. 500 μl of the mixture was assayed for hepatic lipase activity. The unchromatographed GSD postheparin plasma inhibited normal activity as shown previously and a similar inhibition was observed with the supernatant obtained after binding the lipase activity on the heparin/Sepharose. The "purified" GSD plasma did not inhibit the normal activity and gave a combined activity similar to that obtained after the addition of the chromatographed and unchromatographed normal postheparin plasma. Essentially similar results were obtained when lipoprotein lipase activity was assayed in a similar series of experiments.

DISCUSSION

The characteristic lipid abnormalities in patients with poorly controlled GSD are a marked hypertriglyceridemia (7, 17, 20) associated with increased levels of the triglyceride-rich lipoproteins (20, 25). These features could result from increased synthesis (17, 19) and/or decreased catabolism (9) of the circulating triglyceride-rich lipoproteins. Significant decreases in the triglyceride elimination rate and postheparin lipolytic activity in patients with type I GSD have been documented (9) and reduced activities of both lipoprotein lipase and hepatic lipase have also been reported (8, 12, 13).

The synthesis and activity of lipoprotein lipase activity are thought to be under hormonal control and to vary with glucose concentrations (23, 24). This led Forget *et al.* (9) to suggest that the reduced serum insulin and glucose concentrations characteristic of type I GSD (18) might account for the reduced lipase activities. Alternatively, the reduced enzyme activities could be explained by the presence of a circulating inhibitor. A number of observations in the present study strongly support this latter possibility. (a) Postheparin plasma from patients with untreated GSD gave nonlinear activity with time, although the initial velocity appeared to be normal. (b) There was inhibition of normal postheparin lipolytic activity when combined with GSD plasma (pre- or postheparin) collected at a time when metabolic control was poor. (c) When increasing dilutions or decreasing amounts of GSD postheparin plasma were assayed, there was an

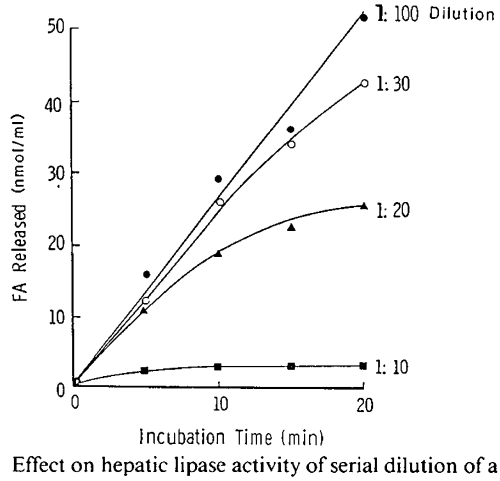

Fig. 3. Effect on hepatic lipase activity of serial dilution of a postheparin plasma sample of the patient with type I glycogen storage disease when metabolic control was poor. *FA*, fatty acids.

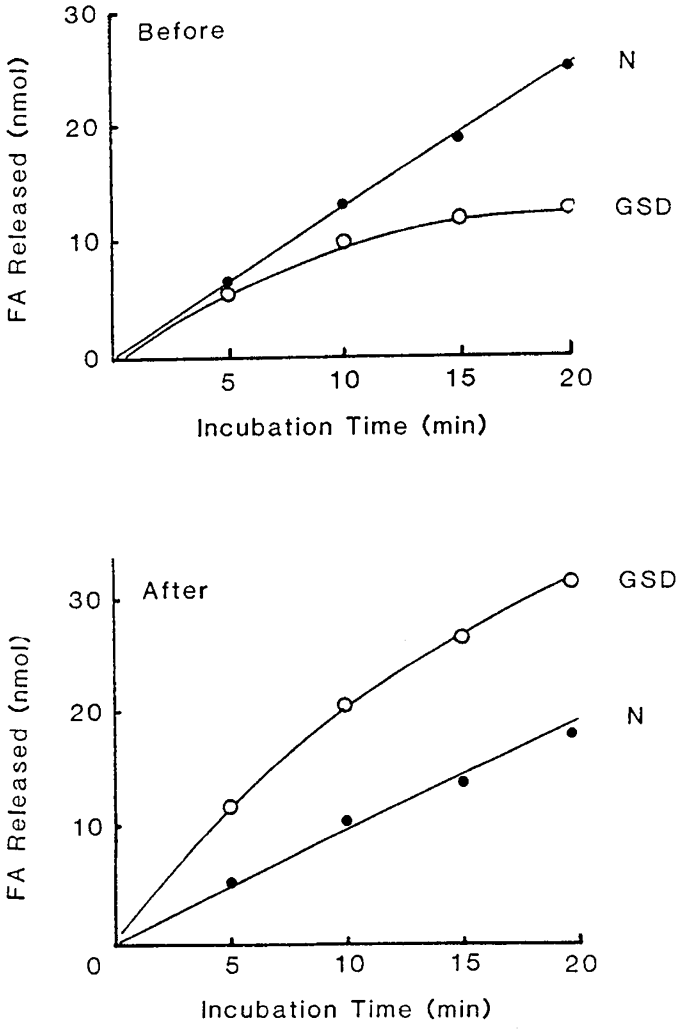

Fig. 4. The activity of hepatic lipase of the normal control (*N*) and the patient with GSD before and after affinity chromatography on heparin/Sepharose. *FA*, fatty acids.

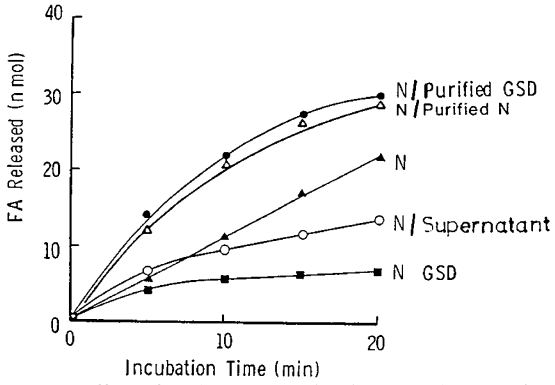

Fig. 5. The effect of various recombination experiments after heparin/Sepharose chromatography of the postheparin plasma sample from the patient with GSD when metabolic control was poor. Normal control alone (*N*), plus the GSD sample after chromatography (*N/purified GSD*), plus the supernatant of the GSD sample after chromatography (*N/supernatant*), plus the unchromatographed GSD sample (*N/GSD*), plus the control sample after chromatography (*N/purified N*). *FA*, fatty acids.

increase in linearity with time and an increase in apparent activity. (d) The inhibitor could be removed following affinity chromatography on heparin/Sepharose and its presence confirmed by appropriate recombination experiments.

The presence of a circulating inhibitor in GSD plasma will result in very different results being obtained for postheparin lipolytic activity depending on the methods used for the assay. The analysis of unfractionated sera will give the "available" activity, whereas following chromatography the "potential" activity is determined. As the *in vitro* assays for hepatic lipase were routinely carried out using a 10–20 fold dilution of the plasma, the inhibition *in vivo* might be expected to be even more pronounced. Differences in methodology could also explain why the postheparin lipolytic activities and activities of the individual enzymes reported in GSD by Forget *et al.* (9) and Fernandes *et al.* (8), respectively, were only moderately reduced compared to the very low activities we have reported previously (12, 13) and in the present study.

In a recent study, Wang *et al.* (28) estimated the total postheparin lipolytic activity in unfractionated serum and the hepatic and lipoprotein lipase activities following heparin/Sepharose chromatography, in patients with a type V hyperlipoproteinemic pattern on electrophoresis. They found that, in contrast to normal postheparin plasma, the activity of the patients' plasma was inversely related to the volume of the plasma used in the assay and that the activity of the lipolytic enzymes increased following chromatography. Their results are therefore similar to those reported in the present study.

The results with GSD plasma suggested the possibility of product inhibition by nonesterified fatty acids which are known to be increased in GSD. Circulating nonesterified fatty acids are normally bound to serum albumin (14) but when concentrations are raised the available high affinity binding sites on albumin are likely to become saturated. Preliminary *in vitro* studies have shown that increasing oleic acid concentrations result in increasing inhibition of both hepatic and lipoprotein lipase activities in a dose-related manner which could be reversed by the addition of excess albumin (11). The *in vitro* inhibition of lipoprotein lipase activity by fatty acids has been reported before (1, 2, 26) and it has been suggested that fatty acids may have an important physiological role in the feedback control of lipase activity *in vivo*. It is unlikely that the increased serum triglyceride concentrations in GSD were inhibitory as postheparin plasma from the patient with type 1 hyperlipoproteinemia did not inhibit normal postherapin plasma activity.

The findings of a circulating inhibitor of lipase activities in GSD may have implications for the understanding of other secondary hypertriglyceridemic conditions such as those found in renal disease and poorly controlled diabetes mellitus. In both conditions, decreased catabolism of the triglyceride-rich lipoproteins may be involved. Reduced activities of lipoprotein lipase (5) and hepatic lipase (22) have been reported in renal disease and Staprans *et al.* (27) and Crawford *et al.* (6) have suggested the presence of a circulating inhibitor of lipase activity. The concentrations of unbound nonesterified fatty acids could be increased and therefore inhibitory in renal disease as a result of reduced serum concentrations of albumin. In this regard, it is interesting to note that infusion of albumin is known to reverse the hypertriglyceridaemia associated with the nephrotic syndrome (21). The hypertriglyceridemia of uncontrolled diabetes mellitus could, like GSD, be explained on the basis of increased serum concentration of nonesterified fatty acids.

In situations where a deficiency of lipoprotein or hepatic lipase is suspected, we strongly recommend that the activities of unfractionated serum should be assayed as a time course rather than at a single fixed end point and that various volumes or dilutions of the enzyme source should be assayed. A reaction which has a normal initial velocity but which is not linear with time or enzyme concentration is suggestive that a circulating inhibitor may be present. This can be confirmed by estimating lipase activities after a fractionation technique such as affinity chromatography.

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