Hepatic triacylglycerol lipase activities after induction of diabetes and administration of insulin or glucagon

Thomas E. Knauer,[1] Judith A. Woods, Robert G. Lamb, and Harold J. Fallon

Departments of Biophysics, Medicine, and Pharmacology, Medical College of Virginia, Richmond, VA 23298

Abstract Triacylglycerol lipase activities of homogenates and subcellular fractions of rat liver were measured under optimal conditions at pH 7.5 using emulsified tri[1-^{14}C]oleoylglycerol as substrate. Twenty-four hr after administration of streptozotocin, hepatic alkaline lipase activity was 39% of normal, and this lower level of activity was observed at 72 hr and 7 days. after streptozotocin injection. After 24 hr of starvation, lipase activity also was significantly lower (35%) than normal. Insulin (35 U regular/kg body weight) had no acute (90 min) effect on the hepatic lipase activity of either normal or diabetic rats. Chronic insulin administration (4 subcutaneous injections of 10 U protamine zinc insulin/kg at 16-hr intervals) to normal rats provoked a 40% increase in hepatic lipase activity. Diabetic rats given the same insulin treatment showed lipase activity that was significantly higher (155%) than normal. Lipase activity fell to 65% of normal when insulin was withheld (32 hr) from diabetic rats given chronic insulin therapy. Intracardial injection of glucagon (1 mg/kg) into normal rats had no acute (30 min) effect on hepatic alkaline lipase activity. Hepatic alkaline lipase activity varied independently from the concentrations of either glucose or triacylglycerol in the plasma. However, there was an apparent negative correlation between this lipase activity and the concentration of fatty acids in the plasma; lipase activity was highest when fatty acid concentrations were lowest, and lowest when fatty acid concentrations were elevated. From these data we conclude: *1*) changes in hepatic alkaline lipase activity are provoked by chronic, but not acute, alteration of the hormonal and metabolic status of the rat, and *2*) changes in hepatic alkaline lipase activity may be mediated through changes in the levels of circulating fatty acids presented to the liver, but the effect is not an immediate one.—**Knauer, T. E., J. A. Woods, R. G. Lamb, and H. J. Fallon.** Hepatic triacylglycerol lipase activities after induction of diabetes and administration of insulin or glucagon. *J. Lipid Res.* 1982, **23:** 631–637.

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There have been several reports of significant changes in the activity of hepatic neutral-alkaline triacylglycerol lipase (EC 3.1.1.3) accompanying alteration of the hormonal or nutritional status of experimental animals or humans (1–8). Jansen and Hülsmann (1) reported a significant increase in the post-heparin plasma activity of triacylglycerol hydrolase of rats made diabetic by streptozotocin. However, based on the identity of hepatic alkaline triacylglycerol lipase with the post-heparin plasma activity of palmitoyl coenzyme A hydrolase (EC 3.1.2.2), which was significantly reduced after induction of diabetes, these authors concluded that hepatic triacylglycerol lipase activity was significantly reduced in the diabetic state. Nakai et al. (2) measured the alkaline lipase activity of post-heparin plasma using emulsified [^{14}C]trioleoylglycerol as substrate. These authors distinguished heparin-released hepatic lipase from extrahepatic lipoprotein lipase by adding 1.0 M NaCl and deleting serum from the in vitro assays. They found that, whereas the total post-heparin lipolytic activity was significantly increased after induction of diabetes in rats by streptozotocin, hepatic triacylglycerol lipase activity in post-heparin plasma was significantly decreased from control.

Some potential problems in generating and interpreting data from experiments in which hepatic triacylglycerol lipase is measured in post-heparin plasma should be considered. First, it is unclear what proportion of the total hepatic activity a sample of post-heparin plasma lipase contains. A more fundamental problem concerns the possibility that diabetes affects the heparin-releasibility of the lipase from the liver rather than altering the actual amount or activity of enzyme in that tissue. Nakai et al. (2) acknowledged this problem with reference to the original criticism expressed by Applebaum et al. (3). Until the present report, the only information about the effect of experimental diabetes on the tissue activity of hepatic triacylglycerol lipase consisted of the brief, but conflicting, reports of Claycomb, Bynagle, and Kilsheimer (4) and Elkeles and Hambley (5). Claycomb et al. (4) reported that withholding insulin from alloxan-diabetic rats produced dramatic increases in both acidic (pH 4.0) and alkaline (pH 7.4) lipase activities. Acute (90 min) insulin treatment reduced the acidic lipase activity to an undetectable level, whereas the lipase activity at pH 7.4 fell to one-third the level of normal rats. Elk-

[1] To whom reprint requests should be addressed at: Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104.

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eles and Hambley (5) measured triacylglycerol lipase activities (pH 8.0) in acetone–ether extracts of livers from normal, starved, diabetic (streptozotocin), and diabetic rats treated with insulin. They found a significant decrease in the lipase activity when insulin was withheld from the diabetic rats.

There is insufficient information available concerning what factors might influence hepatic lipolytic activity. These may include direct effects of the hormones insulin or glucagon (4, 7, 9) or indirect effects provoked by the accompanying changes in the concentrations of metabolites such as glucose, triacylglycerol, or fatty acids (3, 6). In our studies, experimental conditions were chosen so that the circulating levels of the hormones and metabolites could be varied in a differential fashion. Our results indicate that, in the rat, hepatic alkaline triacylglycerol lipase activity is insensitive to acute changes in the levels of circulating insulin, glucagon, triacylglycerol, glucose, or fatty acids. However, in the long term, this lipase activity may be reciprocally related to the concentration of fatty acids presented to the liver by the circulation.

EXPERIMENTAL PROCEDURES

Materials

Tri[1-^{14}C]oleoylglycerol (84 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Trioleoylglycerol, dioleoylglycerol (mixture of 1,3- and 1,2-isomers), monooleoylglycerol, oleic acid, streptozotocin, and bovine serum albumin (essentially fatty acid-free) were obtained from Sigma Chemical Co., St. Louis, MO. Insulin and glucagon were products of Eli Lilly and Co., Indianapolis, IN. All chemicals and reagents were of the highest purity available. Adult (250–300 g) male rats of the Sprague-Dawley strain were purchased from Flow Laboratories, Dublin, VA. Except as noted, rats were provided with free access to food and water up to the time of killing.

Methods

Triacylglycerol lipase assay. The triacylglycerol lipase activities of homogenates and subcellular fractions of liver were measured by the method of Groener and Knauer (10). The routine assay mixture consisted of 10 mg of fatty acid-poor bovine serum albumin, 3.2 μmol of trioleoylglycerol containing 0.08 μCi of tri[1-^{14}C]oleoylglycerol, and liver protein in a final volume of 1.0 ml of 0.10 M potassium phosphate, 1 mM EDTA, pH 7.5. The trioleoylglycerol substrate was emulsified by sonification with 5% gum arabic in water. The incubations at 37°C were stopped after 10–40 min and extracted (11), and the liberation of [1-^{14}C]oleate was determined by liquid scintillation spectrometry with external standardization to determine counting efficiency.

To determine if di[1-^{14}C]oleoylglycerol or mono[1-^{14}C]oleoylglycerol accumulated during the incubations, selected reaction mixtures were extracted by the procedure of Bligh and Dyer (12). Aliquots of the extracts were taken for radioassay and for determination of the glycerolester constituents by chromatography on thin layers of silica gel G using a developing solvent of n-hexane–diethyl ether–acetic acid 50:50:1 (by vol). Authentic lipid standards were applied with the extract aliquots. After development, the chromatograms were visualized by exposure to iodine vapor and the appropriate areas of the chromatograms were transferred directly into scintillation vials for radioassay.

Homogenization of liver. The livers were removed from rats after decapitation and immediately chilled in ice-cold 0.25 M sucrose solution, pH 7.5. Homogenates (10%, w/v) were prepared using a Polytron ST10 (Brinkman Instr., Westbury, NY). In most instances the homogenates were centrifuged at 500 *g* for 5 min in a refrigerated centrifuge (2–4°C) to remove whole cells and debris, and the resulting cell-free homogenates from individual livers were assayed for triacylglycerol lipase activity. The distribution of hepatic alkaline lipase between particulate and soluble subfractions was determined by measuring lipase activities in the supernatant and resuspended sediment after centrifugation of the homogenates for 60 min at 105,000 *g*.

Induction of diabetes by streptozotocin. Rats were lightly anesthetized with diethyl ether and injected intracardially with 100 mg of streptozotocin/kg body weight. This mode of injection provides a highly reliable, reproducible, and facile method for direct introduction of the diabetogen into the blood stream. The streptozotocin was dissolved immediately before use in sufficient 0.9% NaCl, pH 4.0, to allow injection of approximately 0.2 ml.

Analytical assays. Before sacrifice, blood samples were taken from the rats by intracardial puncture using heparinized syringes. Plasma metabolic concentrations were determined as follows: glucose by an *o*-toluidine method (Diagnostest, Dow Chemical Co., Midland, MI); nonesterified fatty acids by the method of Laurell and Tibbling (13); and triacylglycerol by the method of Haux and Natelson (14). The triacylglycerol content of liver was measured as above following extraction of liver homogenates by the method of Bligh and Dyer (12). Protein was determined by the method of Lowry et al. (15) using crystalline bovine serum albumin as a standard. The statistical significance of differences was determined by a two-tailed Students *t*-test.

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RESULTS AND DISCUSSION

Triacylglycerol lipase activity of rat liver

Optimal conditions of trioleoylglycerol concentration, time, and protein concentration in the assay of lipase activity at pH 7.5 were determined from the data illustrated in **Fig. 1.** Using cell-free homogenates of liver, the liberation of oleate was found to be linear for approximately 20 min when the substrate concentration exceeded 3 mM. Both particulate and soluble lipase activities exhibited linearity with time and protein up to 1.2 mg. Under these conditions, no significant accumulation of di- or mono-[1-^{14}C]oleoylglycerol was detected by thin-layer chromatography, suggesting that the rate of degradation of triacylglycerol to glycerol and fatty acids may be limited by the rate of hydrolysis of the first glycerolester bond of triacylglycerol (10). This conclusion is supported by our observation that partial glycerides do not accumulate, and suggests that the lipolytic rate we measured reflects the rate-limiting step in the degradation of triacylglycerol.

Until recently, it was generally accepted that liver contains several triacylglycerol lipases with activity in the neutral-alkaline pH range. However, it is now clear (10) that rat liver contains one predominant alkaline lipase which is the enzyme released from hepatic plasma membranes following heparin treatment. Thus, the lipase activity at pH 7.5 measured in our studies is identical to that measured by others (2, 3, 8) in post-heparin plasma, but we have circumvented the problems alluded to by Applebaum et al. (3) and Nakai et al. (2) by measuring the lipase directly in homogenates of rat liver. Previous experiments indicated no clear compartmentation of the alkaline lipase activity in conventionally prepared subcellular fractions of liver (10, 16–19). As illustrated in Fig. 1, the total particulate subfraction was slightly enriched in alkaline lipase activity, but the 105,000 *g* supernatant always contained 30% or more of the total recovered lipase activity. Based on this information, we chose to use homogenates of liver in our studies on the effects of diabetes, hormones, and starvation on hepatic alkaline lipase activity. This choice was based on our desire to measure changes in total as well as specific activities. With regard to the relationship between the lipase activities found in homogenates and plasma membrane-enriched subfractions, there is little doubt that these activities are virtually equivalent. As we pointed out (10), nearly all (approx. 93%) of the alkaline lipase activity found in liver homogenates originates from the plasma membrane. Thus, although we did not measure changes in the lipase activities of plasma membrane-enriched subfractions of liver, we are confident that these changes would be essentially identical to the changes we observed using homogenates.

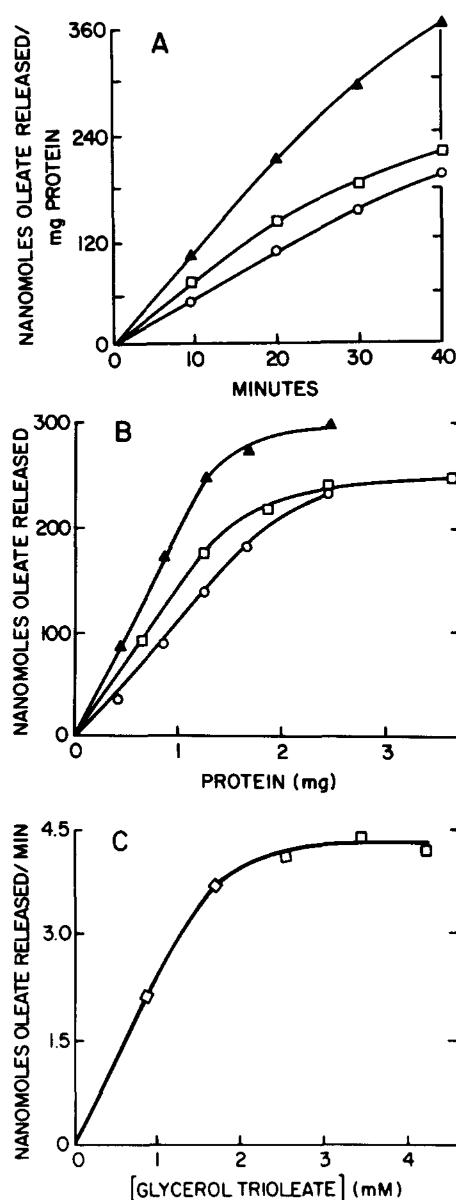

Fig. 1. Dependence of hepatic alkaline lipase activity on incubation time (A), protein concentration (B), and substrate concentration (C). Portions of cell-free homogenates (□), 105,000 *g* particulate fraction (▲), and 105,000 *g* supernatant fraction (○) were incubated in the lipase assay system described in the test. The trioleoylglycerol concentration for the experiments depicted in A and B was 3.2 mM. Dependence on substrate concentration (C) was determined using 0.71 mg of homogenate protein incubated for 20 min.

Effects of diabetes, chronic insulin treatment, and starvation

Table 1 presents the time course of changes in blood and liver metabolite concentrations and changes in hepatic alkaline lipase activity after administration of strep-

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TABLE 1. Changes in blood and liver metabolites and hepatic alkaline lipase activity after administration of streptozotocin to rats[a]

	Control	Time after Streptozotocin Administration				
		2 Hr	8 Hr	24 Hr	72 Hr	7 Days
Plasma						
Glucose (mg/100 ml)	129 ± 3	326 ± 11*	93 ± 6*	425 ± 9*	430 ± 7*	514 ± 32*
Fatty acids (mM)	0.222 ± 0.035	0.457 ± 0.016*	0.291 ± 0.088	0.460 ± 0.069*	0.893 ± 0.138*	0.626 ± 0.124*
Triglyceride (mg/100 ml)	100.5 ± 20.3	117.8 ± 35.4	109.0 ± 8.4	108.4 ± 8.3	433.6 ± 15.9*	517.0 ± 95.4*
Liver						
Triglyceride (mg/gram)	4.58 ± 0.47	5.50 ± 0.34	9.34 ± 0.58*	17.20 ± 0.59*	6.70 ± 0.43*	
Triacylglycerol lipase[b]						
Specific activity	6.36 ± 0.25	6.39 ± 0.72	6.52 ± 1.00	2.29 ± 0.23*	2.71 ± 0.18*	2.86 ± 0.08*
Total activity	789 ± 39	826 ± 85	863 ± 149	308 ± 24*	369 ± 22*	389 ± 14*

[a] Groups of five or more rats each were killed at the times indicated after intracardial injection of 100 mg of streptozotocin/kg body weight.
[b] Triacylglycerol lipase activity is expressed as nmol of oleate released/min per mg protein; total activity is expressed as nmol of oleate released/min per g equivalent of liver.
* Indicates significant difference from control ($P < 0.05$). Data are expressed as means ± S.E.

tozotocin to rats. Blood glucose and fatty acid levels exhibited the characteristic triphasic response to streptozotocin: elevated at 2 hr, decreased at 8 hr, and increased at 24 hr. A stable, permanent diabetic state was achieved by at least 72 hr. Hepatic triacylglycerol content gradually increased and abruptly dropped at 72 hr when plasma triacylglycerol concentration was greatly increased. By 24 hr the alkaline lipase activity in liver was reduced to 39% of normal and remained low thereafter.

To determine the effects of insulin, groups of normal and diabetic rats were given insulin and hepatic alkaline lipase activity was measured at various times thereafter. Chronic insulin therapy of normal and diabetic rats consisted of four subcutaneous injections of 10 U protamine zinc insulin/kg body weight at 16-hr intervals. Rats were normally killed 16–18 hr after the final injection. In an experiment in which insulin was withheld, diabetic rats were killed 32 hr after the final insulin injection. The data in **Table 2** indicate that the alkaline lipase activity in liver was approximately half normal after induction of diabetes (see also Table 1). Chronic insulin treatment of diabetic rats produced a significantly higher than normal level of this lipase activity. Normal rats given identical insulin treatment also showed a significant elevation in the total activity (per g liver) of the lipase, although a change in the specific activity in this group may have been obscured by the increase in homogenate protein observed in this group of rats.[2] Withholding insulin from diabetic rats previously maintained on insulin resulted in a decline in the alkaline lipase activity in liver, although at this time point (32 hr) the effects of insulin deprivation were not maximal. Finally, the alkaline lipase in the livers of rats deprived of food for 24 hr was significantly lower than in control livers.

[2] Chronic insulin treatment of both normal and diabetic rats always altered the amount of homogenate protein per g of liver, necessitating the dual expression of lipase activity as specific (per mg of protein) and total (per g of liver).

Acute effects of hormones on hepatic lipase activity

It is clear from the foregoing that profound alterations of the alkaline lipase activity in liver accompany chronic alterations of the hormonal status of the rat. We conducted the following series of experiments to determine the responsiveness of this lipase to acute changes in the hormonal status of the rat. Groups of five or more rats were treated with insulin or glucagon and the hepatic alkaline lipase activities were compared with those of a corresponding control group. The effect of insulin was examined 90 min after intraperitoneal injection of 35 U regular insulin/kg body weight into normal or diabetic rats. Glucagon (1 mg/kg) was injected intracardially into normal, fed rats 30 min before they were killed. The responsiveness of each group of rats to the injected hormones was monitored by measuring the concentrations of glucose and fatty acids in blood samples taken at the time of killing.

There were no significant ($P > 0.05$) changes in the alkaline lipase activity of livers from either normal or diabetic rats 90 min after injection of insulin. Furthermore, glucagon had no effect on this lipase activity. Bewsher and Ashmore (7) reported that a neutral-alkaline triacylglycerol lipase of liver (pH optimum 7.0) was stimulated more than 3-fold 30 min after injection of rats with glucagon (1 mg/kg). They also reported that

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TABLE 2. Effects of diabetes, chronic insulin treatment, and starvation on hepatic alkaline lipase activity

	Control (10)[a]	Normal + Insulin (6)[b]	Diabetic (11)[c]	Diabetic + Insulin (6)[b,c]	Diabetic, Insulin Withheld (4)[b,c,d]	Starved (5)[e]
Plasma						
Glucose (mg/100 ml)	134 ± 5	91 ± 12*	458 ± 12*	170 ± 32	471 ± 14*	102 ± 3*
Fatty acids (mM)	0.302 ± 0.020	0.186 ± 0.007*	0.731 ± 0.141*	0.141 ± 0.011*	0.757 ± 0.148*	0.514 ± 0.051*
Triglycerides (mg/100 ml)	96.7 ± 8.6	102.8 ± 4.5	391.9 ± 132.0*	103.3 ± 27.0	75.8 ± 20.0	62.4 ± 8.7*
Liver						
Triglycerides (mg/gram)	5.25 ± 0.42	4.32 ± 0.72	6.85 ± 0.33*	5.80 ± 0.32	5.30 ± 0.52	
Triacylglycerol lipase[f]						
Specific activity	5.67 ± 0.40	6.52 ± 0.34	2.78 ± 0.17*	8.79 ± 0.40*	3.69 ± 0.17*	3.68 ± 0.11*
Total activity	803 ± 47	1124 ± 63*	481 ± 36*	1252 ± 36*	616 ± 45*	670 ± 31*

[a] Numbers in parentheses indicate rats per group. Data are expressed as means ± S.E.
[b] Insulin treatment consisted of four subcutaneous injections of 10 U protamine zinc insulin/kg body weight at 106-hr intervals. Rats were killed 16–18 hr after final injection.
[c] Diabetic rats were used 72 hr after intracardial injection of 100 mg streptozotocin/kg.
[d] Rats were killed 32 hr after final insulin injection.
[e] Rats were deprived of food for 24 hr prior to being killed.
[f] Triacylglycerol specific activity is expressed as nmol oleate released/min per mg protein; total activity is expressed as nmol oleate released/min per g equivalent of liver.
* Indicates significant difference from control ($P < 0.05$).

administration of anti-insulin serum to rats increased this lipolytic activity by 52%, suggesting the possibility that a high glucagon/insulin ratio may be important in the activation of hepatic triacylglycerol lipase. These authors (7) and others (20–22) have suggested that this activation of an hepatic lipase, by increasing the supply of fatty acids for β-oxidation, may contribute significantly to the ketogenic and gluconeogenic responses to glucagon observed both in vitro and in vivo.

It is difficult to reconcile this concept with what is known about the diabetic state. Although the glucagon/insulin ratio is significantly elevated in the ketotic, diabetic state produced by streptozotocin (23), the only diabetes-induced change in hepatic lipase activity in the neutral-alkaline pH range that we observed was a significant decrease from normal. Furthermore, by direct measurement using our lipase assay, we could not detect any significant acute effect of glucagon on hepatic lipase activity at pH 7.5 under conditions similar to those employed by Bewsher and Ashmore (7). These authors based their conclusions on measurements of the disappearance of endogenous triacylglycerol during incubations of liver homogenates from normal and glucagon-treated rats. Since our data did not reveal any effect of glucagon on the neutral-alkaline lipase activity of liver, it may be that the hormone influences the availability or susceptibility of endogenous triacylglycerol to an hepatic lipase rather than altering alkaline lipase activity directly.

Relationship between hepatic lipase activity and metabolite levels

Although it is clear that hepatic alkaline lipase activity changes with the chronic insulin status of the rat, we did not observe any acute effect of either insulin or glucagon on the lipase activity. This suggested the possibility that slow alterations in hepatic lipase activity may occur in response to chronic conditions of altered metabolite levels. From our data no relationship between lipase activity and triacylglycerol levels of blood was evident. Similarly, other investigators (2, 3, 8) could find no significant correlations between diabetes or hormone-induced changes in the hepatic lipase activity of post-heparin plasma and the concentrations of circulating triacylglycerols.

To determine whether hepatic lipase activity had any relationship with either blood glucose or fatty acid concentrations, the individual data from all animals used during this study were segregated into groups of low to high glucose and fatty acid levels (**Fig. 2**). Hepatic lipase activity was significantly different from normal in those rats exhibiting a relatively severe hyperglycemia, a situation always accompanied by substantial elevation of plasma fatty acid concentrations. On the other hand, when plasma fatty acid concentrations were very low (<50% normal), lipase activity was significantly elevated. Furthermore, as fatty acid levels increased, hepatic lipase activity decreased significantly. These data suggest a correlation between lipase activity and fatty acid concen-

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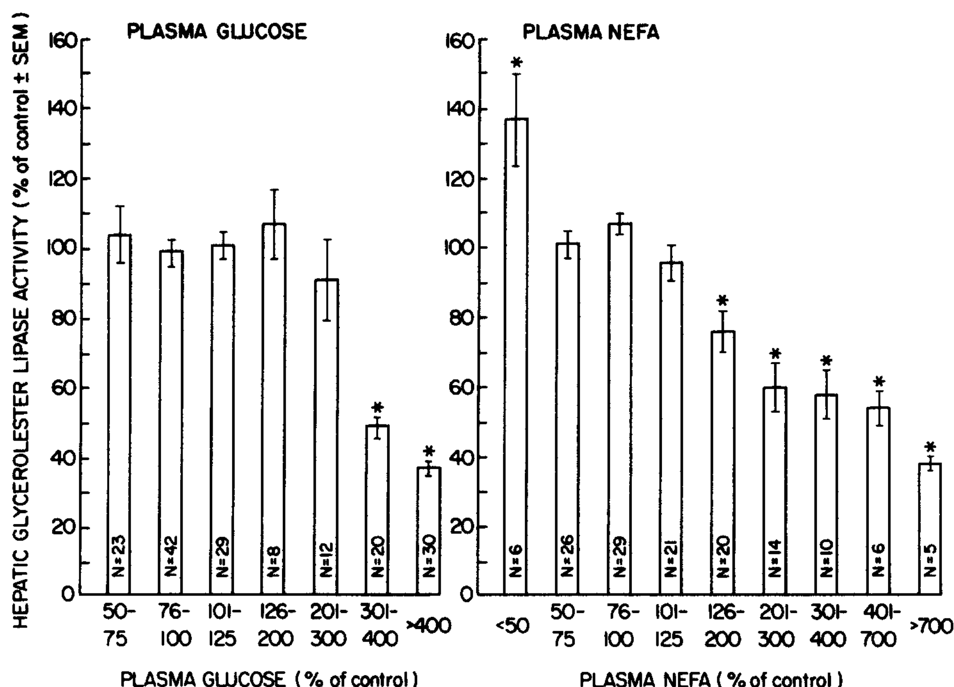

Fig. 2. Relationship between alkaline triacylglycerol lipase activities of liver and plasma concentrations of glucose and non-esterified fatty acids (NEFA). Glycerolester lipase activity is expressed as the mean percent of control specific activity ± S.E.M. for the number of rats indicated in each bar. *, Indicates significant difference from normal ($P < 0.05$).

trations. However, a direct causal relationship would seem to be ruled out by acute studies using insulin and glucagon, hormones that provoke immediate (<90 min) and significant changes in plasma fatty acid levels without immediately altering hepatic alkaline lipase activity.

The function of hepatic alkaline lipase is not fully understood. Our data show that this lipase is responsive to chronic alterations in the hormonal or nutritional status of the rat. Our data do not provide support for a major role of this lipase in the gross regulation of the concentrations of circulating triacylglycerol or fatty acids. However, with respect to fatty acid concentrations, the converse may be true. It may be that, in the long-term situation, reciprocal changes in hepatic and extrahepatic lipolytic activities (1, 2) are balanced by the concentration of circulating fatty acids. In diabetes, a likely function of a lipase in liver, to provide a ready supply of fatty acids, is obviated by the high concentrations of circulating fatty acids presented to the liver, and the activity of hepatic alkaline lipase may be decreased accordingly.

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