

Measurement of lipoprotein lipase activity in post heparin plasma: description of technique*

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SUMMARY

A method has been described for the quantitative assay of lipoprotein lipase activity in post heparin plasma. The conditions of the assay were subjected to critical study. The technique described permits the reaction to proceed in the test tube as a zero order reaction. It is believed that this technique will permit accurate quantitative study of lipoprotein lipase activity in different groups of patients. The rate of lipolysis was compared with the rate of clearing of lipemic plasma. The ratio of the rate of lipolysis to the rate of clearing was quite variable in normal subjects. This suggests that the use of the clearing technique to study lipoprotein lipase activity in different groups of patients, or under different experimental conditions, may give misleading results.

In 1943 Hahn (1) reported the rapid *in vivo* clearing of alimentary lipemia following the injection of heparin. In 1950 Anderson and Fawcett (2) demonstrated that plasma obtained after heparin injection contained a factor which cleared lipemia *in vitro*. Since then there have been several hundred studies of the clearing of lipemia by heparin-induced clearing factor. In 1955 Korn (3) established that "clearing factor" is an enzyme, lipoprotein lipase, that splits the triglyceride moiety of chylomicrons and low density lipoproteins to form free fatty acids (FFA) and glycerol. The characteristics of this reaction have been studied by numerous investigators and are summarized in several recent reviews (4, 5). Lipoprotein lipase has been extracted from heart, adipose tissue, lung, and other tissues. Without heparin, the enzyme can be detected in the plasma only in negligible amounts, but the plasma level rises markedly immediately after the intravenous injection of heparin.

When post heparin plasma is incubated with suitable substrates—lipemic plasma, chylomicron suspensions, thoracic duct lymph, or an activated artificial fat emulsion—there is a striking decrease in turbidity of the incubation mixture. It has been shown that under appropriate conditions the clearing proceeds at a

constant rate and is proportional to the concentration of the enzyme. The simplicity of this phenomenon has encouraged investigators to use the measurement of optical density as an indication of lipoprotein lipase activity.

The absorbancy of a complex mixture of plasma and emulsified fat is dependent upon the scattering of a beam of light passed through it. The scattering of light by such a mixture is determined principally by the size and number of particles. The particle size is affected by many variables, including chemical composition, electric charge of the particulate matter, and protein distribution. The change in plasma turbidity produced by a given change in plasma triglyceride varies among individuals.¹ Thus plasma turbidity is affected by many incompletely understood physicochemical phenomena, and quantification of lipolytic activity based on rates of decrease in absorbancy might be misleading. Baeder *et al.* (6) have reported *in vitro* clearing of lipemic dog plasma without the occurrence of lipolysis. Evidence is now presented that a decrease in absorbancy does not bear a consistent relationship to lipase activity, and in a related study it has been shown that misleading conclusions can indeed be reached by use of the turbidity technique.²

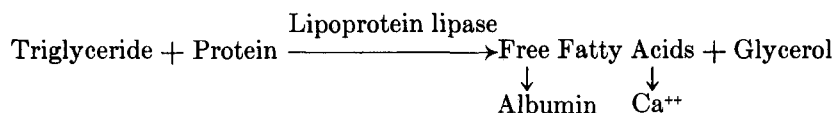
¹ F. Kern, Jr., B. Sanders, G. McDonough, and L. Steinmann. In preparation.

² F. Kern, Jr., B. Sanders, H. Singh, and L. Steinmann. In preparation.

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It is believed that a quantitative chemical technique which measures one of the products of lipolysis provides more accurate and useful data.

The reaction catalyzed by lipoprotein lipase as demonstrated by Korn (7) is shown below:



Triglyceride is not a substrate for this enzyme until it has been activated by protein. When mixed with plasma, the triglyceride is activated very rapidly.

FFA are transported bound to serum albumin and they combine with albumin *in vitro* as well (Ca^{++} also exhibits some affinity for these anions). Havel and Fredrickson (8) and Laurell (9) have shown that the half life of FFA *in vivo* is about 2 minutes. Because of this rapid turnover, albumin is normally available to bind the FFA produced *in vivo*, but this is not necessarily true *in vitro*. Since unbound FFA inhibit the lipolysis catalyzed by lipoprotein lipase, it is essential that an adequate amount of albumin be present in the *in vitro* assay. (10)

The requirements of a satisfactory *in vitro* assay of this enzyme are as follows: (a) the substrate should be a standardized artificial fat emulsion without inhibitors; (b) abundant fatty acid acceptors must be available and optimum conditions for the binding of fatty acids must be achieved; (c) the reaction should proceed with zero order kinetics during the period of measurement; (d) the chemical technique of measuring the end product must be sensitive, reproducible, and feasible technically for a large number of simultaneous determinations.

Korn's technique for measurement of tissue lipoprotein lipase activity has been modified for use with post heparin plasma so that these criteria are met.

METHODS

Nineteen studies were done on 14 normal medical students and hospitalized patients not acutely ill. All experiments were done after an overnight fast. After drawing a sample of blood, 50 mg of heparin (heparin sodium, 100 mg/ml, Upjohn) was administered intravenously. Exactly 20 minutes later the post heparin sample was collected. Nine ml of blood was mixed with 1 ml of 3.2% sodium citrate and immediately placed in an ice water bath of 0-1°. Within 2 hours the

plasma was separated by centrifuging at 0-4° at $1500 \times g$ for 10 minutes. In 8 of the 19 studies the post heparin plasma was used for simultaneous measurement of lipoprotein lipase activity by both the chemical and turbidity techniques, and in 11, only the

chemical determination was made. The 50 mg dose of heparin and the 20-minute post heparin interval were chosen arbitrarily.

For each 1.0 ml of plasma the incubation mixture contained 1.0 ml of 0.05 M tris buffer (pH 8.5) (Sigma 7-9 tris [hydroxymethyl] amino methane); 100 mg (0.4 ml of a 25% solution) bovine albumin (Fraction V powder, Armour) dissolved in distilled H_2O and pH adjusted to 8.5; and 0.1 ml of the substrate, a 15% fat emulsion. In the technique adopted for use, the substrate used was Ediol®,³ a commercial 50% coconut oil emulsion diluted with water to a 15% emulsion. In all experiments, except as otherwise indicated, the above reaction mixture was used. Ediol® was compared with Lipomul—I.V.®, a commercial 15% cottonseed oil emulsion, and a specially prepared 15% olive oil emulsion.⁴ Some experiments were done using human serum albumin (Cutter) as the fatty acid acceptor, but this proved to be unsatisfactory (see below). Duplicate 0.25 ml aliquots of the reaction mixture were incubated for 10, 20, 30, and 60 minutes and compared with the unincubated samples. In all studies incubation mixtures without added albumin were run simultaneously, but they were assayed usually only at 1 hour, and compared with the glycerol produced at 1 hour in the presence of added albumin.

The albumin palmitic acid complex used in some experiments was prepared by the technique described by Fillerup *et al.* (11). The palmitic acid-albumin ratios were calculated and were not exact since the 0.05 mole fatty acid per mole of albumin already present in bovine serum albumin (Fraction V) was ignored (12).

All plasma samples were kept cold until they were added to the incubation mixture, which was immediately placed in a water bath at 37°. At the same time,

³ Ediol® was supplied by Schenlabs Pharmaceuticals.

⁴ Lipomul I.V. and the olive oil emulsions were kindly provided by Dr. J. B. Lawson of the Upjohn Company.

the unincubated sample was deproteinized. At the various time intervals, incubation tubes were withdrawn, placed in an ice bath, and immediately deproteinized with 2 ml of 0.1 N H_2SO_4 and 0.25 ml 25% sodium tungstate and centrifuged. Glycerol was determined essentially by Korn's modification (3) of the method of Lambert and Neish (13). Deproteinized plasma (0.5 ml) was measured into a 125×16 mm Pyrex tube containing 0.5 ml 1.0 N H_2SO_4 . H_2O (0.5 ml) was used for the reagent blank and 0.5 ml of a glycerol solution (containing 8 μg glycerol) was used as standard. The tubes were transferred from ice water to a 20° water bath, and 0.1 ml .05 M NaIO_4 was added to each tube at the rate of 8 tubes per minute. Exactly 5 minutes after the addition of the periodate, 0.1 ml 0.5 M Na arsenite was added, following the same rate and sequence. After disappearance of the yellow color, 9 ml chromotropic acid solution⁵ was added; tubes were covered with marbles and placed in a covered boiling bath for 30 minutes. After cooling in ice water, samples were read against the reagent blank at 570 $m\mu$ in a Coleman Junior Spectrophotometer in 15 ml cuvettes. Results were calculated as follows:

$$\frac{\text{Absorbancy test} - \text{absorbancy unincubated sample}}{\text{absorbancy standard}} \times \mu\text{g glycerol of standard} \times$$

$$\frac{\text{dilution of plasma in incubation mixture}}{\mu\text{g glycerol/ml plasma}} \times \frac{\text{dilution of incubation mixture}}{\text{by deproteinization}} =$$

To 10×75 mm Coleman photometric cuvettes were added 0.4 ml 25% albumin, 0.3 ml 1.5% coconut oil (Ediol®), and 1.3 ml 0.05 M tris buffer. These tubes were kept at $0-4^\circ$ and 1.0 ml of the post heparin plasma was added. The tubes were then placed in a 37° water bath and a preincubation period of 4 minutes was allowed before the initial absorbancy reading, which was approximately .600. The readings were made in a Coleman Junior Spectrophotometer at 700 $m\mu$ wave length using water as a blank. Following the initial reading, the tubes were removed from the water bath and absorbancy was measured at 10-minute intervals, 30 seconds being required for each reading.

Critique of Method: The data obtained using the technique described are shown in Figure 1. Zero order

⁵ Chromotropic acid was prepared by adding 6 volumes concentrated H_2SO_4 to 3 volumes of distilled water. After cooling, 1 volume of a 1% aqueous solution of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid disodium salt, practical grade obtained from Eastman Organic Chemicals) was added. The solution keeps for two weeks stored in a brown bottle at room temperature.

kinetics were achieved for 30 minutes in the presence of added albumin.

Experiments comparing Ediol® with Lipomul and with the olive oil emulsion showed that the different substrates were hydrolyzed at different rates. It was possible for the reaction to proceed at a constant maximum rate only with Ediol®, the coconut oil emulsion. Other experiments demonstrated that smaller amounts of Ediol® did not provide sufficient substrate for zero order kinetics during the reaction catalyzed by the amount of enzyme usually present.

When post heparin plasma was diluted with pre-heparin plasma in varying concentrations, it was found that there was a linear relationship between glycerol production and the amount of enzyme present, so long as the reactions were proceeding at a maximum rate (Fig. 2). This re-emphasizes the importance of following the reaction closely so that the velocity is measured at its maximum, since a falling-off of glycerol production is noted in this system when the plasma has a very high activity.

It appeared logical to use human serum albumin as the supplementary fatty acid acceptor in the reaction

mixture. When it was used, however, the results suggested that an inhibitor was present, and when compared with bovine albumin (Fraction V powder), it was found that the latter was invariably associated with a greater glycerol production. It was suspected that this difference might be the result of inhibition by stabilizers (sodium caprylate and sodium acetyltryptophanate)⁶ present in the human albumin preparation. When these stabilizers were added to the bovine albumin preparation, significant inhibition of lipolysis was observed.

The importance of the amount of albumin in the reaction mixture is demonstrated in Figure 3. There was a progressive increase in reaction rate as the amount of added albumin was increased from none to 150 mg per ml of plasma. In this particular experiment even 150 mg of albumin was not sufficient to maintain a straight line for 1 hour. It may be important to note that the subject for this experiment was a male medi-

⁶ Supplied by Dr. E. B. McQuarrie, Cutter Laboratories.

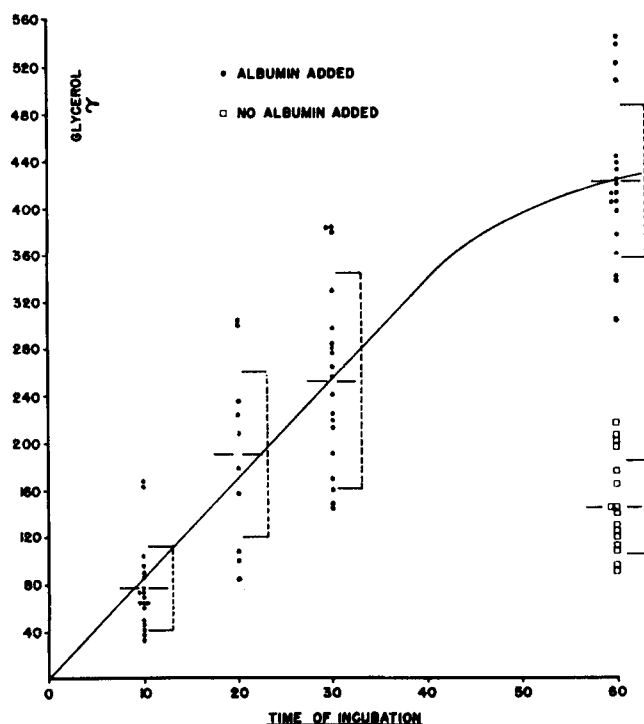


FIG. 1. Lipoprotein lipase activity. Glycerol production as a function of time in 19 normal subjects.

cal student on a voluntarily imposed low calorie, low fat diet.

It was found that 100 mg albumin per ml plasma provided adequate fatty acid acceptor in subjects whose serum albumin was 4.0 gm%. In all of these studies the patient's total serum protein was measured by the CuSO_4 technique and the albumin was determined by paper electrophoresis. Sufficient bovine albumin was added to the reaction mixture to bring the total albumin concentration to 140 mg per ml plasma.

Robinson and French (14) reported that when FFA were present in excess of a 6:1 molar ratio to albumin, lipolysis was inhibited. Figure 4 shows an experiment in which enzyme activity was compared using no added albumin, 100 mg of albumin as a 3:1 palmitate-albumin complex, 100 mg of albumin as a 6:1 palmitate-albumin complex, and 100 of unaltered bovine albumin. It is clear that the reaction rate using the 6:1 fatty acid-albumin complex was the same as the reaction rate without any added albumin, and that the 3:1 fatty acid-albumin complex accelerated the rate only about one-half as much as the albumin alone. It therefore appears that the mechanism of action of albumin is solely that of binding the fatty acids released from the triglycerides, thereby preventing their inhibitory effect upon the reaction.

The effect of pH on the kinetics of the reaction is shown in Figure 5. In the absence of added albumin, the reaction proceeds at a more rapid rate at pH 8.5 than at pH 7.5, as shown by Korn (3). The most important point to note, however, is that when additional albumin is made available, there is a very marked increase in reaction rate as the pH is increased. These observations suggest that pH 8.5 is optimum for lipoprotein lipase activity, and that at pH 8.5 the ability of albumin to combine with FFA is greatly enhanced. It was not possible to raise the pH higher than 9.0 with tris buffer. A higher pH might have denatured the proteins and inhibited the reaction. Phosphate buffers were tried but could not be used because the phosphate interfered with the glycerol assay. Using 0.05 M tris buffer (pH 8.5) and the standard reaction mixture as described, it was shown by means of a con-

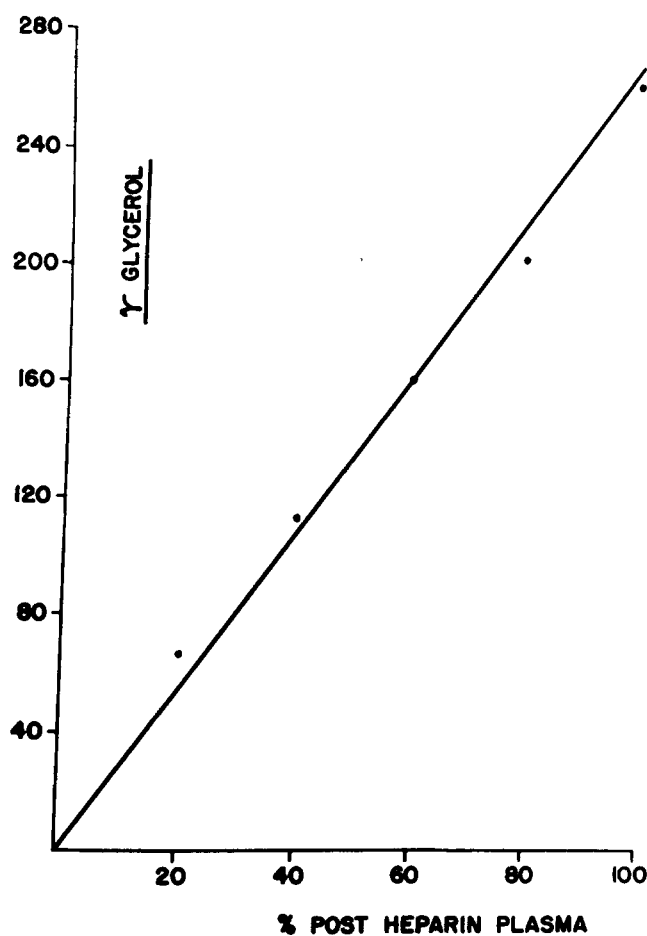


FIG. 2. Lipoprotein lipase: variation in enzyme concentration. The abscissa represents the relative amounts of post heparin plasma mixed with pre-heparin plasma from the same subject. Glycerol production was measured after a 20-minute incubation.

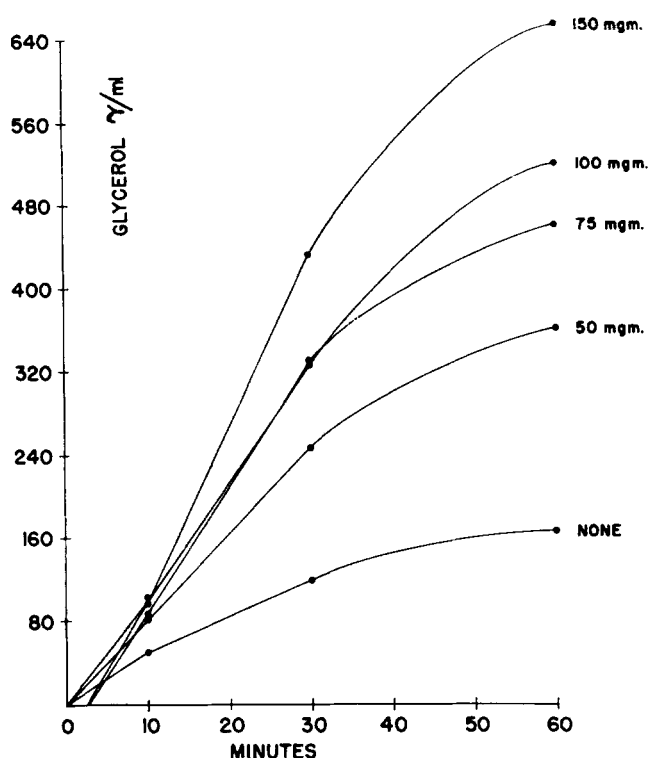


FIG. 3. Lipoprotein lipase activity: effect of added albumin.

tinuous recording of pH that the pH fall during 1 hour of incubation did not exceed 0.6 pH units.

In most experiments the fall in absorbancy was linear for 20 to 30 minutes. A straight line was drawn through this period of most rapid fall and the slope (k) was calculated in terms of Δ absorbancy per ml plasma per hour, as described by Baker (15).

RESULTS

The results of these studies are shown in Table 1. The mean glycerol production was 422 gamma glycerol per ml plasma per hour with added albumin, in contrast to 143 without added albumin. The variation in speed of the reaction in this group of patients is apparent. In almost all experiments the reaction rate was constant during the first 30 minutes of incubation, and in those patients with less enzyme, it continued at a constant rate for 60 minutes. It is to be noted, however, that when the initial reaction rate was very rapid, glycerol production slowed sometime between 20 and 30 minutes. This is easily seen by comparing the measured glycerol produced at 60 minutes with the rate of glycerol production calculated from the

straight-line portion of the slope (Table 1). Figure 1 illustrates some of these findings.

Table 1 also shows the comparison between the rate of the reaction as determined by the quantitative chemical technique and by the turbidity technique. In every experiment the rate of glycerol production was proportional to the rate of turbidity decrease (Fig. 6).

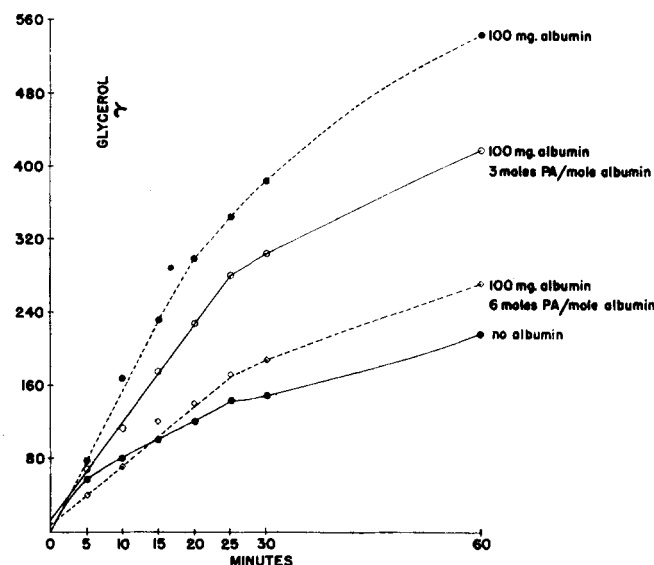


FIG. 4. Lipoprotein lipase activity: effect of albumin-fatty acid complex. This shows the inhibitory effect of prior complexing of palmitic acid with the albumin.

It is apparent, however, that although these two types of measurement bear a definite relationship to each other in the individual patient, there was marked variation from patient to patient. This variability is expressed mathematically in Table 1 as the ratio of the k for glycerol production to the k for Δ absorbancy. The ratio varied from 0.75 to 1.57.

DISCUSSION

A method for measuring the *in vitro* lipolytic activity of lipoprotein lipase has been described. With the amount of enzyme produced by 50 mg of heparin, zero order kinetics are maintained *in vitro* for at least 30 minutes in most subjects. The need for a large amount of albumin to bind the FFA produced has again been demonstrated and the importance of maintaining the optimum pH for maximum enzyme activity and for maximum albumin binding of FFA has been shown. The measurement of an end product of the lipoprotein lipase activity under conditions permitting zero order

kinetics permits quantitative interpretation of the lipolytic potential of the enzyme *in vivo*. For example, rough calculation of the amount of triglyceride split in a 70 kilogram man with average *in vitro* activity during one hour following 50 mg of heparin (assuming the same level of activity for 1 hour) shows that 14.4 g of triglyceride could be hydrolyzed in the plasma.⁷

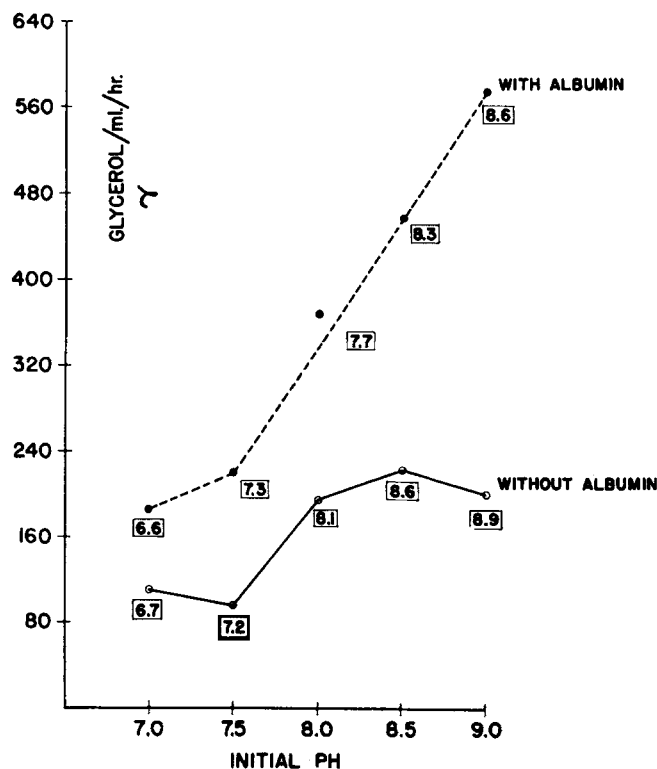


FIG. 5. Effects of pH on lipoprotein lipase activity. The boxed numbers indicate the final pH after incubation for one hour.

The rate of lipolysis observed in these studies is rapid because the reaction was allowed to achieve its maximum rate. If large amounts of heparin are used, it is possible that more enzyme would be mobilized, necessitating more substrate and more albumin in proportion to the amount of plasma in the reaction mixture.

⁷ An *in vitro* assay of 550 gamma of glycerol per ml plasma per hour is equivalent to 6 μ M glycerol per ml plasma per hour. Assuming that each mole of glycerol represents a mole of triglyceride (since a small part of the glycerol measured is monoglyceride, this assumption leads to the slight overestimation of triglyceride). Then in 3000 ml of plasma 18,000 μ M of triglyceride could be split. Using an average molecular weight of triglyceride with long-chain fatty acids, 800, 18,000 μ M is equivalent to 14.4 g of triglyceride.

TABLE 1

No.	Subject	Glycerol/ml Plasma/hour			Clearance k	Glycerol k Clearance k
	Diagnosis, Age, Sex	Without Albumin	With Added Albumin			
			Measured	k Calculated		
1.	Normal-35-F	175	305	305		
2.	Pleural effu- sion-35-F	110	338	338		
3.	Rheumatoid arthritis-43-F	144	342	342		
4.	Arsenic poi- soning-73-M	108	359	359		
5.	Cerebral vas. accident-73-M	94	377	560	0.56	1.0
6.	Convalescent pneumonia- 53-M	123	395	448	0.60	0.75
7.	Gastric ulcer-50-M	127	405	405	0.29	1.39
8.	Normal-23-M		405	560		
			413	700		
			432	598	0.39	1.53
			438	572		
		165	522	688		
		217	543	906		
9.	Gastric ulcer-47-M	206	413	413		
10.	GI bleeding cause undet. 40-M	119	420	512	0.35	1.46
11.	Duodenal ulcer-25-M	200	425	425		
12.	Pyelonephritis- 56-M	92	444	560	0.53	1.06
13.	Recovered hepatitis-31-M	129	508	658	1.16	0.56
14.	Gastric ulcer-53-M	144	535	912	0.58	1.57
Mean		143.5	422	540	0.56	1.16

The rate of decrease in absorbancy (clearance k) was calculated from the straight-line portion of the curve extrapolated to 1 hour. The glycerol k was calculated in the same manner.

The relationship between lipolysis, as measured chemically, and the difference in absorbancy of the plasma is of considerable interest and of great practical importance. In the individual patient these measures are proportional to each other; that is, as lipolysis proceeds, the absorbancy decreases. This observation has been made by many others; of equal importance, however, is the finding that the ratio between lipolysis and turbidity decrease in different patients is highly variable. It can be readily seen from Figure 6 that it is impossible to predict the rate of lipolysis from the rate of decrease in absorbancy. This finding raises considerable doubt about the meaning of studies of post heparin lipoprotein lipase activity in different age

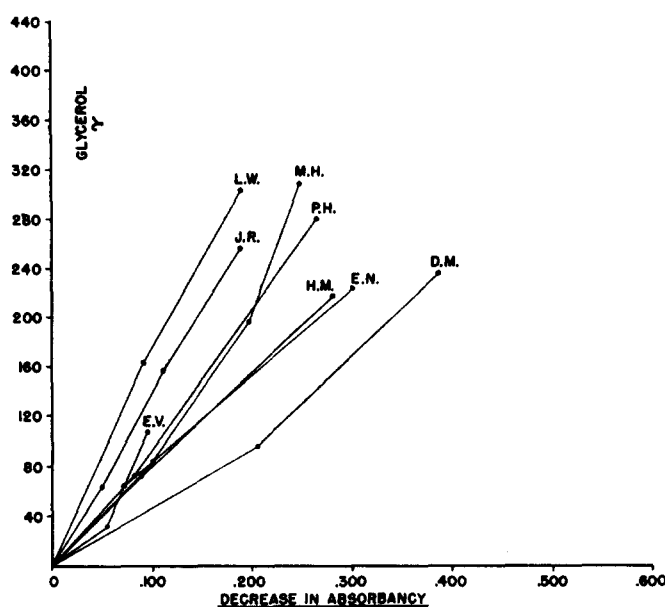


FIG. 6. Lipoprotein lipase: comparison of glycerol production with decrease in absorbancy. Glycerol production plotted against clearing of turbidity in 8 normal subjects.

groups and disease states when the turbidity technique was employed as the means of measuring the enzyme activity.

Studies of the role of certain organs of the body in the production and destruction of this enzyme have also been based upon the lipemic clearing action rather than chemical measures of lipolysis. For example, the liver is generally believed to inhibit lipoprotein lipase activity, but most studies leading to this conclusion have measured decrease in absorbancy as the major index of activity (16 to 19). Evidence collected in our laboratory concerning the role of the liver, using both the chemical measure of lipolysis and the turbidity technique, strongly suggests that rapid clearing *in vitro* of post heparin plasma of cirrhotic subjects is not adequately accounted for in terms of lipolysis.⁸

⁸ See footnote 2.

The results of the studies reported here indicate that measuring the decline in absorbancy can be useful to detect the presence or absence of post heparin clearing factor, but it should not be used to quantify or compare the rates of lipoprotein lipase activity under different experimental or clinical conditions.

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