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Nature of postheparin plasma clearing factor

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NATURE OF POSTHEPARIN PLASMA CLEARING FACTOR

by

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INTRODUCTION

The lipids of biological systems are combined with proteins. A group of lipid-protein complexes containing varying amounts of cholesterol, phospholipids and neutral fat in combination with proteins, are referred to as lipoproteins. Depending on the lipid- protein ratio the lipoproteins vary in specific density.

When neutral fat is absorbed from the intestinal tract it combines with small amounts of phospholipids, cholesterol and protein to form chylomicra which may be considered as a particular class of low-density lipoproteins. Chylomicra are present in the lymph and blood as separate particles approximately 0.5 - 1.0 micron in diameter and contain from 90 - 98 per cent or more of neutral fat.

Because of their large size, chylomicra scatter light and therefore when they are present in high concentration the plasma appears milky. After a fatty meal the concentration of chylomicra in the plasma begins to rise, reaches its maximum after a few hours, and then slowly falls to normal levels. As the chylomicron concentration decreases, the plasma becomes more transparent. The phenomenon of chylomicron disappearance has been called lipemia clearing. As the concentration of chylomicra decreases during lipemia clearing, a concomitant increase in the concentration of lipoprotein molecules of higher specific densities takes place. These observations suggest a lipid transport mechanism in which lipids first appear in the lymph and blood as large particles, are gradually transferred into smaller and denser lipoprotein molecules

and then leave the blood stream to be metabolized or stored. It is not known to which extent the intravascular transformation of low-density lipoproteins to high-density lipoproteins is a prerequisite for the transfer of fats from the blood to the tissues or for their movement in the reverse direction.

Hahn (46) in 1943, observed that intravenous administration of heparin to lipemic dogs increases the rate of lipemia clearing. Since addition of heparin to lipemic plasma in vitro had no effect on the concentration of chylomicra, it was concluded that after heparin administration a particular factor (later named, "clearing factor") is produced. There are reports suggesting that clearing factor is present in normal human blood and is activated when, after a fatty meal, the neutral fat reaches the blood stream.

It has been shown that clearing factor acts on the triglycerides of chylomicra mainly by lipolysis, i.e., by hydrolyzing the triglycerides into fatty acids and glycerol. Very little is known as yet about the nature of the lipolytic activity of clearing factor. Some of the views as to the nature of the lipolytic activity of clearing factor are listed below:

(a) Clearing factor is a distinctive lipase containing heparin or another negatively charged mucopolysaccharide in its structure (104).

(b) Clearing factor is a heparin-phospholipid complex and displays its lipolytic activity by activating blood esterases and these in turn hydrolyze chylomicra present in the blood (1, 79).

(c) Clearing factor is identical with the lipoprotein lipase (63, 65). Lipoprotein lipase is an enzyme found in mammalian tissues which hydrolyzes the neutral fat of lipoproteins to free fatty acids

and glycerides or glycerol.

In this work it has been attempted to isolate the clearing factor and to study its nature and kinetics. Since until recently clearing factor could be obtained only in very crude preparations, the main objective of this work has been to develop a method for purifying clearing factor from the blood of humans to whom heparin had been administered.* The kinetic properties and lipolytic action of purified clearing factor have also been investigated and compared with those of pancreatic lipase.

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* hereafter called "post-heparin blood."

REVIEW OF THE LITERATURE

Physico-chemical properties of lipoproteins: At the beginning of this century, Nerking (93) and later Haslam (47) suggested that the lipids present in body fluids are associated with proteins and not floating in a free state. In 1929, Macheboeuf (85) first isolated a lipoprotein moiety from horse plasma, which in addition to the protein contained 18 per cent cholesterol and 23 per cent phospholipids. In 1946, Cohn and collaborators (17) obtained more complete plasma lipoprotein complexes by introducing a low-temperature ethanol-fractionation method. By this method in which the hydrogen ion concentration, ethanol concentration, ionic strength, protein concentration and temperature are controlled, the components of blood plasma are precipitated into several distinctive fractions. Studying the physico-chemical properties of lipoproteins thus obtained Cohn et al. (17), Oncley, Gurd, and Melin (101) and Oncley, Scatchard, and Brown (102) found that the lipoproteins were almost exclusively present in plasma fractions IV-1 and III-0. Since these fractions migrated electrophoretically with the speed of alpha-1-globulin and beta-1-globulin, respectively, they were named alpha and beta lipoproteins. Beta lipoproteins (molecular weight, 1,300,000) were found to consist of: unesterified cholesterol, 8.4 per cent; cholesterol ester, 22.5 per cent; phospholipid, 29.3 per cent; and protein 23.0 per cent. The chemical composition of alpha lipoproteins (molecular weight, 200,000) was found to be: protein, 65 per cent; phospholipids, 24 per cent; and cholesterol, 12 per cent. These figures

are in good agreement with those given by other workers for the composition of lipoprotein complexes (107, 71, 112, 48). The literature on the lipoproteins in human plasma has been more completely summarized by Oncley (99,98) and Oncley and Gurd (100).

Ultracentrifugal analysis of the lipoproteins of the blood: The introduction of the ultracentrifuge by Svedberg (119) opened a new approach to the study of the lipoproteins present in blood plasma. Besides its successful application in the investigation of the molecular dimensions of proteins (89, 106) the ultracentrifuge has proved itself very useful for isolation of lipoproteins.

Gofman, Lindgren and Elliott (36), increasing the density of serum by the addition of NaCl and applying high-speed centrifugation, floated serum beta lipoproteins to the top of the mixture. This method (82, 33) permits the isolation of blood plasma lipoproteins of the specific density desired simply by varying the type and amount of salt added to the plasma under investigation.

Using the Svedberg unit (S_f 1 x 10^{-13} cm/sec/dyne/gm) to measure the rate of flotation of lipoprotein molecules in a centrifugal field, Gofman et al. (34) and others (58) found beta lipoproteins to consist of many classes of molecules ranging from large, less dense molecules of S_f 40,000 to those of high density with S_f values of 4. Analyzing serum lipoproteins by chemical and ultracentrifugal procedures, Gofman (32) and Gofman and collaborators (35, 36) found that the lipid portion of serum beta lipoproteins above S_f 17 consists of cholesterol, phospholipids and neutral fat, while the lipid portion of high density beta lipoproteins (S_f 4-17) contains only cholesterol and phospholipids.

These authors also observed that with decreasing density the neutral fat content of lipoproteins increases. Jones and collaborators (58), studying lipoproteins of human sera by ultracentrifugal and tracer techniques, concluded that the lifetime of low-density protein is in a range of several hours. On the basis of this finding and the quantitative studies of lipoprotein S_f classes in the ultracentrifuge, they suggested that the lipoprotein components with high S_f values are progressively transformed into those of the lower S_f classes.

Chylomicrons: - The milky appearance of blood plasma after fatty meals has been recognized for many years. In 1924, Gage and Fish (28), using dark field microscopy, observed the presence in the blood stream of fatty particles 0.5 - 1.0 u in diameter. These they named chylomicrons. They also observed that the concentration of chylomicrons in the serum increased after ingestion of fat and became maximal approximately 2 to 3 hours after a fatty meal.

Fraser and Stewart (25) and Cunningham and Peters (19) found the chylomicrons to consist almost entirely of neutral fat. Fraser (23), studying fat absorption and metabolism, concluded that neutral fat, after being absorbed into the lymph and the blood, is present as finely dispersed particles which carry a thin film of protein on their surface. Later, Fraser (24) reported that the physico-chemical properties of the chylomicrons found in lymph differ from those of the chylomicrons found in blood, an observation which suggests redistribution of neutral fat on the way from the lymphatic system to the blood stream. Havel and Fredrickson (49) found that half

life of palmitic acid-1-C^{14} - labeled chylomicra in dog to be 15 to 20 minutes.

Although chylomicrons consist (90 to 98%) of neutral fat and contain only small amounts of cholesterol, phospholipid and protein (22,23,24) nevertheless, they are lipid-protein complexes, and as such are to be considered low density lipoproteins. The ultracentrifugal studies reveal that the S_f classes of lipoproteins which have fletation rates higher than S_f 400 actually represent chylomicrons. Studying N-terminal groups and electrophoretic migration of proteins associated with dog and human chylomicrons, Rodbell and Fredrickson (111) concluded that at least three types of proteins are associated with these chylomicrons.

Heparin-induced clearing factor: - In 1943, Hahn (46) using heparin as an anticoagulant in his studies on the circulating mass of red cells in dogs, first observed the lipemia-clearing action of heparin. He noticed that the lipemia of those dogs which received intravenous heparin, disappears at a much faster rate than the lipemia of dogs which had not received heparin. If heparin was added *in vitro* to lipemic plasma or serum, lipemia clearing did not take place.

Wald (24) in 1944 reported that the intravenous administration of heparin causes clustering and disappearance of chylomicrons. Anderson and Fawcett (1) found that lipemia clearing continued *in vitro* when plasma or serum was collected a few minutes after intravenous administration of heparin to lipemic dogs. In addition to the clearing of lipemic serum, they found that administration of heparin lowered the surface tension of the plasma. On the basis of these findings, they

postulated that *in vivo* heparin may combine with a phospholipid and thus produce a lipemia-clearing "antichylomicronemic factor." In 1952, Aufinsen Boyle and Brown (2), employing a combination of Cohn's methods VI and IX of low-temperature ethanol fractionation of post-heparin plasma, achieved four-fold purification of the lipemia-clearing component which they named "clearing factor." They also reported that the supernatant of tissue minced with heparin and human plasma produces clearing of an added fat emulsion. Nikkila and Pesola (96) in 1956 obtained forty-fold purification of post-heparin clearing factor by increasing the hydrogen ion concentration of post-heparin plasma to pH 5.6, adsorbing the resultant precipitate on calcium phosphate gel and eluting it again with sodium citrate. Recently, Hollett and Meng (52) reported up to 1,480 times purification of clearing factor by fractionating post-heparin plasma with ammonium sulfate. The present author, however, following their procedures, has been unable to purify clearing factor by this method.

Hood *et al.* (55), Meng, Hollett and Cole (91) and Grossman (41) studied conditions under which the lipemia clearing is optimal. They all agreed that the greatest concentration of clearing factor is found in post-heparin plasma 30 - 60 minutes after the intravenous administration of heparin. Meng and collaborators reported that the optimal rate of clearing factor activity occurs at pH 6.4 - 7.0 and within the temperature range of 35 - 40° C. Grossman (41) on the other hand, found the optimal temperature to be 25° C. He found the clearing factor to be very thermolabile, so that after incubation for one hour at 40° C. it was completely inactivated .

Brown, Boyle and Anfinsen (12) attacked the problem of the mechanism of clearing factor activity. They postulated the occurrence of a two-stage reaction mechanism:

First: plasma precursor protein + heparin + tissue factor $\longrightarrow$ clearing factor

Second: low density beta lipoproteins + plasma co-proteins $\xrightarrow{\text{clearing factor}}$ smaller high density beta lipoproteins + alpha lipoproteins.

Kunkel and Bearn (66) administered P_{32} labeled phospholipids to human subjects and found the radioactivity distributed among alpha and beta lipoproteins. They also demonstrated exchanges of phosphatides taking place between alpha and beta lipoproteins. Employing C_{14} and S_{35} labeling, Gould (39) found that cholesterol can also interchange between alpha and beta lipoproteins. Supported by the findings of their electrophoretic studies on plasma lipoproteins after the administration of heparin, Herbst, Lever, Lyons and Hurley (51) criticized the reports on the conversion of beta lipoproteins into alpha lipoproteins during lipemia clearing. Gitlin et al (31) studied the interconversion and turnover of peptides connected with lipoproteins of varying densities. Peptide moieties of lipoprotein were labeled with I_{131} and tracer doses were injected to normal individuals and to children with nephrotic syndrome. On isolation of alpha and beta lipoprotein molecules of various dimensions, no interchange of peptide moieties between alpha and beta lipoproteins was found. The findings of Avigan, Redfield and Steinberg (3) that alpha and beta serum lipoproteins have different N-terminal residues supports the view that there is no interconversion between alpha and beta lipoproteins.

Shore, Nichols and Freeman (117) and Nichols, Rubin and Lindgren (94), after incubating post-heparin plasma or "heparin active factor" with lipoproteins obtained from egg yolk, found partial hydrolysis of the glyceride component of the lipoproteins, suggesting a lipolytic action of the clearing factor.

Levy and Swank (79) found increased activity of blood esterases after the administration of heparin. They suggested that increased esterase activity may be responsible for the lipolytic activity observed after administration of heparin.

Overbeck and Van der Vies (104), studying the effects of various substances known to inhibit and accelerate the action of pancreatic lipase, alli-esterases (108) and clearing factor, concluded that clearing factor is not identical with alli-esterases or pancreatic lipase. The lipolytic activity of clearing factor now is well established and has been confirmed by many investigators (12, 43, 63, 109).

French, Robinson and Florey (27) have reported that the addition of normal plasma to a mixture of post-heparin plasma and chyle increases the rate of chyle clearing. They suggested that a plasma factor other than heparin may determine the rate of the clearing reaction. Gordon et al. (38), using post-heparin plasma active fraction and coconut oil emulsion as substrate, found that the addition of albumin (bovine or human) increases the rate of clearing of the lipemic mixture. They also found that the addition of 0.005 ml of normal serum to the mixture of their clearing factor preparation and coconut oil increases the rate of the clearing reaction by 50 per cent. On the basis of these findings the authors postulated that a plasma co-factor is necessary for the

lipemia clearing reaction. The importance of albumin in lipemia clearing has been stressed by Nikkile (95), Grossman et al. (44) and others (115, 26). Korn (63) has reported that the role of albumin in the clearing reaction is to accept free fatty acids and that albumin can be replaced by other free fatty-acid acceptors as, for example, calcium ion. Selden and Westphal (116), supporting the idea that serum albumin acts as a free fatty-acid acceptor, have suggested that the term "nonesterified fatty acids (NEFA)" be used instead of "free fatty acids" since serum fatty acids are bound to the albumin and therefore not free.

Levy and Swank (78) and later Worley and Lequire (125) found lipemia clearing to be accelerated in fat-fed dogs when the dogs were put in peptone or anaphylactic shock. These authors suspected that the release of heparin was responsible for the observed lipemia clearing. Injecting 20 -50 ml of cottonseed oil emulsion or merely the emulsifying agent[1] without fat to fasting dogs, Lever and Baskys (70) observed that clotting time and lipemia clearing activity were prolonged in the collected plasma of these dogs. Since these changes were reversed by the subsequent administration of protamine, they concluded that the administration of phosphatide preparation to the dogs stimulated the release of heparin or a heparin-like material. Brown (11), Gilman, Hathorn and Penn (30) and others (18) have found that dextran sulfate and some other sulfated polysaccharides have a heparin-like lipemia-clearing effect when administered intravenously.

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1. Emulsifying agent was 1.2 per cent soybean phosphatide; 4.2 per cent dextrose.

Lipoprotein lipase versus clearing factor: - Incubating mouse pylorus extract with heparin Brown and Kaufman (13) found that production of clearing factor is also taking place in vitro.

Korn (63, 62) extracted from acetone-dried heart and adipose tissue powder a lipolytic agent which he named lipoprotein lipase and identified it as behaving like postheparin clearing factor. He suggested that lipoprotein lipase is a native lipolytic enzyme of blood and tissues which hydrolyzes the triglyceride moieties of alpha as well as beta lipoprotein molecules to free fatty acid and glycerol. The following overall reaction was proposed by Korn as taking place:

1. Alpha lipoproteins + triglycerides $\xrightarrow{\text{non enzymatically}}$ chylomicrons
2. Chylomicrons $\xrightarrow{\text{lipoprotein lipase}}$ fatty acids + glycerol + alpha lipoprotein

Overbeck (103) in summarizing the fat hydrolyzing enzymes of the blood suggests that clearing factor is a simple lipase containing heparin or another negatively charged mucopolysaccharide in its structure. When heparin of postheparin plasma was removed on an ion exchange resin, Robinson (109) found that the effluent still had lipolytic activity but was less stable. Addition of small amounts of heparin restored the stability to the level of the starting material. Korn (64) found 60 per cent inactivation of its lipoprotein lipase preparation when incubated with heparin-metabolizing bacteria (105). He interpreted this as evidence that heparin is an essential component of lipoprotein lipase.

Action of protamine and other inhibitors on clearing factor: - From the work of Chargaff and Olson (15) and others (123) it has been known that protamine has an antagonistic effect on heparin in respect to blood

coagulation. Brown (11) in 1952, studying the mutual effects of heparin and protamine on alimentary hyperlipemia, found that protamine antagonized heparin also in regard to lipemia clearing. Under the action of heparin, the disappearance of lipemia is reversed within a few minutes after administration of protamine. Spitzer (118) reported that lipemic dogs became more lipemic on administration of protamine. Bragdon and Hovel (10), studying substances which form insoluble compounds with heparin, found that only protamine reverses the effect of heparin on alimentary lipemia in non-toxic doses. The inhibitory and reversing action of protamine on lipemia clearing has been well substantiated by many other investigators (45, 73, 54, 55).

Studying alimentary lipemia clearing after heparin administration to 100 subjects with coronary diseases and 100 control subjects, Oliver and Boyd (97) found that alimentary lipemia clearing rate increased only in 50 of the individuals with coronary disease, whereas it increased in all the control subjects. Hollett and Meng (53), Lever and Klein(74) and Klein, Lever and Fekete (60) have reported the presence of specific clearing factor inhibitors in human and animal blood and tissues.

Effect of clearing factor on lipemia clearing and on the lipoproteins of the blood: - In 1951, Graham and collaborators (40) observed the redistributing action of heparin on the lipoproteins. They reported:

> "Heparin administered to humans and rabbits causes profound reorientation of the distribution of low density lipoproteins characterized by a shift of lipoproteins of high S_f rates to those of successively lower S_f rates. The association of these two changes suggests a progressive conversion of the higher S_f lipoproteins into those of lower S_f rates."

Loewe et al. (84) studying the effect of heparin on the serum lipoproteins of normal subjects, and later Lever, Herbst and Lyons (72) studying the effect of heparin on the serum lipoproteins of patients with idiopathic hyperlipemia and primary hypercholesteremic xanthomatosis, confirmed the previous reports that the administration of heparin causes a shift of lipoproteins from the higher to the lower S_f classes. Lindgren, Freeman and Graham (81) reported that lipoprotein transformation also takes place in vitro. These authors incubated the "active fraction" of post-heparin plasma (obtained in the bottom of the centrifuge tube after high speed centrifugation) with lipoproteins of the S_f 20 - 200 class and obtained lipoproteins of the S_f 4 - 20 class after 60 minutes of incubation. Boyle, Bragdon and Brown (9) reported that the conversion of alpha lipoproteins of low density to alpha lipoproteins of higher density takes place in vitro in post-heparin human plasma. Carlson and Wadstrom (14) suggested that when the triglyceride fraction of the lipoprotein molecule is hydrolyzed to fatty acids and glycerol, the lipoprotein is automatically transformed into denser molecule. The findings of Levine, Kaufman and Brown (77) that the protein portions of low-density lipoproteins are antigenically similar seem to support such thinking. Employing free-boundary and paper electrophoresis methods, Lever, Smith and Hurley (75) and Herbst, Lever and Hurley (50) observed that administration of heparin to patients with idiopathic hyperlipemia and to subjects with alimentary hyperlipemia increases the speed of electrophoretic migration of serum lipoproteins. They found that in all cases after intravenous administration of heparin the concentration of chylomicrons decreases and that the beta and alpha lipoproteins

migrate at an increased rate. These authors suggested that the lipolytic action of clearing factor is responsible for the increased electrophoretic mobilities of serum alpha and beta lipoproteins. Following is a direct quotation from Herbst, Lever and Hurley (50):

> "It seems more likely that products released by the action of the heparin-induced clearing factor cause the electrophoretic changes. If we assume that the increase in electrophoretic mobility of the alpha and beta lipoproteins after an injection of heparin is due to the binding of fatty acids by these lipoproteins several findings in our study would find an explanation."

These suggestions were later substantiated by the observation (7) that incubation of pancreatic lipase with triglycerides in the presence of normal plasma or even in vitro addition of small amounts of oleic acid to normal plasma increases the electrophoretic mobility of serum lipoproteins. The findings of McDaniel and Grossman (88) also support the view that the lipolytic action of clearing factor causes the changes in electrophoretic migration of serum lipoproteins.

Physiologic action of clearing factor: - Engelberg (20) found endogenous clearing factor present in the untreated plasma of 25 out of 39 normal individuals tested, but he was unable to detect clearing factor activity in the serum of these same individuals. Gates and Gordon (29) found lipolytic activity in the serum of 7 human donors. They concluded that the weak lipolytic activity is due to the presence of lipoprotein lipase in the serum of these donors. Robinson et al. (110) reported that lipemic human plasma after a fatty meal, when incubated at 37° C., shows slight spontaneous clearing activity. These findings have been confirmed by other investigators (7, 76). The above reports

suggest that clearing factor is a physiological substance which comes into play particularly after absorption of fat. Lindgren and collaborators (80), studying blood lipoprotein transformation, concluded that

> "the introduction of a fat load into the blood stream in the form of chylomicra or lipomicra probably is the initiating factor in lipoprotein transformation. In some way this fat load must stimulate the clearing factor mechanism, possibly through heparin release. As the result of an increase in serum lipoprotein lipase activity the lipoprotein transformation reaction is driven through the entire S_f 20 - 10^5 range of lipoprotein complexes. The actual transformation of high S_f lipoprotein complexes to lower S_f lipoprotein complexes is achieved by glyceride hydrolysis (at the surface of the glyceride core) with fatty acid release."

Sexl (113, 114), studying the lipid metabolism of aging arterial tissues, reported a mechanism for clearing factor production. She found that incubation of elastase (4, 5, 6) with fresh ligamentum nuchea or human aorta for 20 hours, releases fat globules into the buffer. Addition of $alpha_1$ and $alpha_2$ fractions of serum globulin to that mixture resulted in rapid clearing of the solution. The disappearance of chylomicra during that clearing reaction was demonstrated by the electron microscope. The author concluded that elastase (in particular the mucolytic portion of elastase) and a mucopolysaccharide complex react with $alpha_1$ and $alpha_2$ fractions of serum globulin to form clearing factor.

This brief survey of the literature shows the importance of the role of clearing factor in the metabolism and transport of lipids. The present investigation has been undertaken in the hope that it may contribute to better understanding of the nature and mode of action of this lipolytic agent.

EXPERIMENTAL

I. SEPARATION AND CONCENTRATION OF CLEARING FACTOR

A. MATERIALS

Postheparin human plasma was used as starting material. One milligram of sodium heparin was administered intravenously per kilogram of body weight to normal human subjects, and 30 minutes later blood was collected into acid citrate dextrose solution. Plasma was separated by centrifugation for 30 minutes at 2,000 g and stored at 5° C. Sterile precautions were maintained throughout these procedures.

B. METHODS OF DETERMINING LIPEMIA CLEARING ACTIVITY OF POSTHEPARIN PLASMA AND ITS FRACTIONS

1. Grossman's method of measuring optical density (41).

This method is employed for the purpose of screening and comparing various plasmas and plasma fractions with clearing factor activity after addition of a fat emulsion.

(a) Principle

Optical density of a solution, in the limits of Beer's law, is directly proportional to the concentration of light adsorbing or light scattering material present in that solution. When in these tests fat emulsion is cleared by the action of clearing factor (probably due to the disappearance of chylomicrons) the optical density of the emulsion decreases and is measured in a spectrophotometer.

(b) Procedure

To 1.2 ml of postheparin plasma or to the same volume of the other system, 0.3 ml of standard fat emulsion,[2] diluted 1:30 with water, is added and the optical density of the mixture is measured at various time intervals in a Coleman Junior Spectrophotometer at 700 using distilled water as a blank. Between readings the samples are kept in the 37° C. water bath.

The lipolytic action of clearing factor is measured by determining the products of triglyceride hydrolysis, namely unesterified fatty acids and glycerol.

2. Gordon's method for the determination of fatty acids (37).

(a) Principle

In this method unesterified fatty acids are extracted and titrated with dilute alkali of known normality.

(b) Procedure

From 1 ml to 2.5 ml portions of the reaction mixture are quantitatively placed into 50 ml screw-topped tubes and immediately frozen in an ethanol-dry ice bath. During freezing the tube is revolved on slant in the bath so that the frozen sample covers 1½" to 2" up the side of the tube. The water from these samples is removed by vacuum-drying in a frozen state for 4 or more hours. To the dry

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2. Standard fat emulsion was generously supplied by Dr. E.A.Hawk, Medical Division, Dept. of Clinical Investigation, The Upjohn Co., Kalamazoo, Michigan. It consisted of: 15% coconut oil dispersed as one micron particles in water containing 0.5% Pluronic (a non-ionic detergent) and 1.0% polyglyceroloieate.

sample 30 ml of a 1:1 mixture of acetic acid glacial and iso-octane (2,2,4-trimethylpentane) solution is added, the mixture is gently shaken for a few seconds and left standing for 30 minutes. At this time the blank is prepared consisting of 30 ml of the acetic acid-iso-octane solution and also the standard consisting of 1 ml of 0.002 N palmitic acid in iso-octane, 14 ml of iso-octane and 15 ml of glacial acetic acid. The tubes are centrifuged for 2 minutes at 5,000 g and from each tube 25 ml of the solution is quantitatively transferred into another 50 ml screw-topped tube. To the transferred solution 25 ml of 1:2,000 dilute sulfuric acid are added and shaken by inverting the tube 10 times. (The screw top of the tube must be provided with teplon lining in order to prevent leaking). The mixture in the tubes is centrifuged for 1 minute at 3,000 g and the intranatant (acetic and sulfuric acid solution) is removed by means of a long needle and syringe. The washing with dilute sulfuric acid of the remaining iso-octane and fatty acid solution is repeated twice, leaving the mixture after the last washing to stand overnight at 5° C.

Again the phases are separated by centrifugation and the aqueous phase is removed by means of a syringe. 5 ml of triple-washed supernatant and 1 ml of Nile Blue indicator[3] are quantitatively placed into 15 ml conical centrifuge tubes and 0.02 N NaOH solution is obtained. The contents in the titration tube are mixed by bubbling of

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3. 1:9 stock aqueous solution of commercial Nile Blue A (0.02 per cent in water washed with iso-octane until free of extractable colored impurities) and absolute ethanol.

nitrogen gas. For the calculation of titratable acidity present in the sample under determination, the following equation is used:

$$\frac{\text{microequivalents of standard} \times (\text{ml 0.2 N NaOH for unknown titration} - \text{ml 0.02 N NaOH for blank titration})}{(\text{ml 0.02 N NaOH for standard titration} - \text{Ml 0.02 N NaOH for blank titration})} = \text{microequivalents of acid present in the sample}$$

3. Glycerol determination by the method of Lambert and Neish (68) (slightly modified)

(a) Principle

Oxidation of glycerol by periodic acid to formaldehyde and coupling the latter with chromotropic acid reagent[4] to give a colored solution.

(b) Procedure

From 1 to 2.5 ml of reaction mixtures are quantitatively placed into 50 ml test tubes and 9 ml of 2.5 per cent solution of trichloracetic acid are added and the mixture is centrifuged for 15 minutes at 5,000 g. 10 ml of the supernatant is quantitatively placed into 100 ml volumetric flask and diluted to 20 ml with distilled water. The solution is acidified by the addition of 1.0 ml of 1 ON H_2SO_4 and then is reacted with 5 ml of 0.1 M sodium periodate ($Na_3H_2IO_6$) for exactly 5 minutes. After 5 minutes the oxidizing action of sodium periodate is stopped by the addition of 5 ml of 1.0 M solution of sodium arsenite

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4. 1.0 g of chromotropic acid (1.8 dihydroxynaphtalene - 3.6-disulphonic acid) is dissolved in 100 ml of distilled water and 400 ml of 12 N sulphuric are added.

($NaASO_2$). After standing for 10 minutes, the contents in the volumetric flask are diluted to 100 ml mark with distilled water. Then 1.0 ml of the solution is placed into a 25 ml pyrex test tube and 10 ml of chromotropic acid reagent (86) added. The contents in the test tubes are mixed and placed in boiling water bath for 30 minutes. To prevent direct light from falling on the tubes, the water bath is kept in a dark place. After heating, the tubes are cooled to room temperature by running cold water and the solution is transferred into 19 x 105 mm. Coleman cuvettes. The optical density readings are taken at 570 millimicrons using distilled water as a blank.

The standard glycerol curve is prepared (by determining optical densities of known concentration of pure glycerol in fasting plasma) and the obtained optical density values are converted into micrograms of glycerol.

C. PROCEDURES EMPLOYED FOR CLEARING FACTOR SEPARATION

1. Fractionation of postheparin plasma employing Cohn's low temperature ethanol method X (16).

Reports in the literature on clearing factor activity found in the postheparin plasma fractions after low temperature ethanol fractionation suggested a promising method for obtaining crude clearing factor preparation for possible further purification.

(a) Reagents

Reagent A

250 ml 95 per cent ethanol

2.4 ml acetate buffer

Diluted to 1 liter by distilled water

Acetate Buffer

20 ml 4 M sodium acetate

40 ml 10 N acetic acid

Diluted to 1 liter with distilled water

Reagent B

175 ml 95 per cent ethanol

45 gm glycine

1.0 ml 1 M sodium acetate

1.0 ml 1 N acetic acid

Diluted to 1 liter with distilled water

Reagent C

160 ml 95 per cent ethanol

45 gm glycine

2.5 ml sodium glycinate buffer

3.2 ml 0.5 M Na_2HPO_4 solution

2.4 ml 0.5 M Na_2HPO_4 solution

Diluted to 1 liter with distilled water

Sodium Glycinate Buffer

75 gm glycine

20 gm NaOH

Dissolved in 1 liter of distilled water

(b) Procedure

100 ml of postheparin plasma and 400 ml of reagent A are precooled to 0° C. and mixed in a 1,000 ml Erlenmeyer flask. The mixture is kept at -5° C. for 15 minutes in an alcohol-water bath

and is continuously mixed by means of a mechanical stirrer. The formed precipitate (Fraction I + II + III) is separated by centrifugation for 30 minutes at 8,000 g and -5° C. Samples of Fractions I + II + III and of the supernatant containing Fractions IV + V + VI are taken for the determination of clearing factor activity.

To the precipitate (containing Fractions I + II + III) 160 ml of reagent B are added and the mixture is stirred for one hour maintaining the temperature at -5° C. The resulted precipitate containing Fractions I and III is separated by centrifugation at 3,000 g and -5° C. for 30 minutes. The samples of the precipitate and of the supernatant containing Fraction II, are used for the determination of clearing factor activity. To the rest of the precipitate containing Fractions I and III, 200 ml of reagent C are added and the mixture is kept in an alcohol-water bath at -5° C. while stirring mechanically for one hour. The mixture is centrifuged at 3,000 g and -5° C. for 45 minutes and the presence of clearing factor activity in the supernatant and precipitated fractions is determined. After removal of ethanol by evaporation under vacuum, the following systems of the collected samples were prepared in duplicate and the lipemia clearing action tested.

To each of the following tubes containing:

(a) 100 mg. of wet weight Fractions I + II + III dissolved in 0.6 ml phosphate buffer at pH 7.4

(b) 0.6 ml of supernatant containing Fractions IV + V + VI

(c) 0.6 ml of supernatant containing Fractions IV + V + VI plus 50 mg of wet weight Fractions I + II + III

(d) 100 mg. of wet weight Fraction I + III dissolved in 0.6 ml phosphate buffer at pH 7.4

(e) 0.6 ml supernatant containing Fraction II

(f) 0.6 ml supernatant containing Fraction II plus 100 mg. of wet weight Fraction I + III

(g) 100 mg. of wet weight Fraction I + III - 1, 2, 3

(h) 0.6 ml of supernatant containing Fraction III - 0

(i) 0.6 ml of phosphate buffer at pH 7.4

(j) 0.6 ml postheparin plasma

0.6 ml of normal preheparin plasma and 0.3 ml of standard fat emulsion are added and the optical density and glycerol value are determined before and after incubation for 60 minutes at 37° C. The results of these determinations are reported in Table I.

TABLE I.

Lipolytic activity of postheparin plasma fractions obtained by ethanol fractionation.

* Exp. No.	Fractions Tested	Optical Density Value		Glycerol Value in Micrograms		Substances in the Fraction
		Time of Incubation		Time of Incubation		
		0 min.	60 min.	0 min.	60 min.	
1.	Fractions I+II+III in 0.6 ml phosphate buffer pH 7.4 + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	65	53	360	440	
2.	Fractions IV+V+VI in 0.6 ml supernate + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	64	65	370	380	
3.	Fraction II in 0.6 ml supernate + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	60	58	360	370	γ-globulins
4.	Fractions I+III in 0.6 ml phosphate buffer + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	64	49	365	420	
5.	Fractions III-0 in 0.6 ml supernate + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	65	65	355	360	Lipid poor β-globulin B_1 lipoproteins Ceruloplasmin
6.	Fractions I+III-1,2,3 in 0.6 ml phosphate buffer + 0.6 ml preheparin plasma + 0.3 ml standard fat emulsion	64	47	360	420	Plasminogen, fibrinogen, prothrombin, iso-glutinins
7.	0.6 ml of postheparin plasma + 0.6 ml phosphate buffer pH 7.4 + 0.3 ml standard fat emulsion	63	28	370	465	
8.	0.6 ml of normal preheparin plasma + 0.6 ml phosphate buffer pH 7.4 + 0.3 ml standard fat emulsion	64	62	365	370	

* Each experiment represents an average of two determinations.

2. Isolation of clearing factor by extracting postheparin plasma with ethyl ether.

(a) Reagents

Anhydrous ethyl ether

Ethanol reagent:

250 ml absolute ethanol

2.5 ml 4 M sodium acetate

Diluted to 1 liter with distilled water

Glycine Buffer at pH 8.6

250 ml of 0.2 M solution of glycine

20 ml of 0.2 M solution of NaOH

Diluted to a total of 1 liter with distilled water

(b) Development of the preliminary procedure.

In order to determine the effect of fat solvents on the lipemia clearing activity present in the postheparin plasma Fraction I + III - 1, 2, 3, this fraction was extracted with solvents of differing polarity. Small volumes of original postheparin plasma were also extracted as controls. The following extractants were used: benzene, cyclohexane, carbon tetrachloride, petroleum ether (B.P. 30 to 60° C.), chloroform, ethyl ether, 3 parts ethanol plus 1 part ether, acetone, ethanol, and ethylene glycol.

It was found that with the exception of ethyl ether all solvents used decreased or destroyed the lipemia clearing activity of postheparin plasma and that of Fraction I + III - 1, 2, 3. When postheparin

plasma and ethyl ether, or Fraction I + III - 1, 2, 3 plus some water and ethyl ether, were mixed at -5° C. a precipitate was formed, which on centrifugation appeared as a semi-solid interphase between the aqueous and ether layers. That interphase comprising approximately 2 per cent of the original plasma volume, contained approximately 90 per cent of the clearing activity of the starting material when tested by the optical density method. On comparing the lipemia clearing activity extracted with ether from postheparin plasma Fraction I + III - 1, 2, 3, with the lipemia clearing activity extractable from the amount of postheparin plasma used in obtaining that fraction, it was found that direct extraction of postheparin plasma with ether gives approximately the same purity and a much better yield than prolonging the procedure by the low temperature ethanol plasma fractionation steps.

The following preliminary procedure for the separation of crude clearing factor from postheparin plasma was developed. Equal volumes (5 ml) of postheparin plasma and ethyl ether are precooled separately to 0° C. and then mixed in a test tube at -5° C. for a few minutes until a jelly-like state of the mixture is attained. The mixture is kept for 24 hours in a water-alcohol bath at -5° C. By that time the mixture has separated into an emulsified upper layer and an aqueous lower layer. The mixture is shaken to resume the jelly-like state and again is kept in the alcohol-water bath at -5° C. for 3 to 5 days and shaken thoroughly once a day. Then the mixture, containing numerous floating particulates, is centrifuged at room

temperature for 30 minutes at 12,000 g. As a result of centrifugation the emulsion separates into an upper layer and a bottom aqueous layer separated by an interphase ("pellicle") containing the particulates previously in suspension. The particulates are densely packed and thus forms a semi-solid pellicle which is removed from the tube by means of a wooden applicator.

The above procedure is readily applicable only if small amounts of starting material are used. By centrifuging larger volumes of the plasma-ether mixture it is more difficult to separate the emulsion and to obtain the formation of a semi-solid layer. In adapting the method for the processing of larger amounts of postheparin plasma, it becomes necessary to introduce some changes in the above procedure.

(c) Development of the final procedure.

Therefore, the following final procedure for obtaining crude clearing factor from postheparin human plasma was adopted. 200 ml of postheparin plasma and 200 ml of ethyl ether are precooled separately to 0° C. and mixed in a 1,000 ml volumetric flask by shaking for 30 seconds. Then 400 ml of ethanol reagent, chilled to -5° C. are added and the contents are mixed by gently shaking, while keeping the container immersed in an alcohol-water bath at -5° C. After 10 minutes of mixing the plasma-ether-ethanol mixture is placed in four 250 ml centrifuge bottles and centrifuged for 20 minutes at 3,000 g and 3° C. (see page 86 for illustration). After centrifugation the aqueous and ether layers are removed by decanting and the

remaining semi-solid substance ("pellicle") is washed with a 25 ml portion of ethyl-ether at -5° C. while mixing the contents for 3 minutes with aglass rod. Then the ether is decanted and the washing is repeated two more times. The remaining ether is removed first by blowing N_2 gas into the bottles and finally placing the centrifuge bottles with the substance into a desiccator and applying suction by means of a motor pump. From each bottle the semi-solid material is extracted with 20 ml of glycine buffer at pH 8.0 in a Potter-Elvehjem homogenizer and the supernatant is collected by centrifugation for 20 minutes at 40,000 g. and 5° C. The collected fraction is dried under vacuum in a frozen state. 5 mg. of this preparation, representing crude clearing factor, as well as the remaining plasma fractions are dissolved in 1.2 ml of postheparin, normal plasma and on addition of 0.3 ml of standard fat emulsion are incubated at 37° C. in a water bath. The optical density and glycerol concentration are determined at 0 time and after 60 minutes of incubation.

The results of these determinations are reported in Table II.

TABLE II.

Lipolytic Activity of Postheparin Plasma Fractions Obtained by Ethyl Ether Extraction Method

* Exp. No.	System Determined	Change in Optical Density Time of Incubation 0 min.	Change in Optical Density Time of Incubation 60 min.	Change in Glycerol Value in Micrograms Time of Incubation 0 min.	Change in Glycerol Value in Micrograms Time of Incubation 60 min.	△ Glycerol Millimoles/ Liter
1.	1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	.65	.65	350	360	-
2.	1.2 ml preheparin plasma + residue of ether layer + 0.3 ml standard fat emulsion	1.16	1.16	360	350	-
3.	1.2 ml preheparin plasma + pellicle from 1.5 ml postheparin plasma + 0.3 ml standard fat emulsion	.64	.29	355	525	1.23
4.	1.2 ml infranatant layer + 0.3 ml standard fat emulsion	.62	.56	365	400	0.25
5.	1.2 ml postheparin plasma (starting material) + 0.3 ml standard fat emulsion	.63	.31	380	520	1.11
6.	1.2 ml distilled water + pellicle from 1.5 ml postheparin plasma + 0.3 ml standard fat emulsion	.60	.57	20	20	-
7.	5 mg. (dry weight) of crude enzyme from pellicle extract + 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	.64	.34	370	490	0.94

* Each experiment represents an average of two determinations.

D. PROCEDURES FOR THE ATTEMPTED PURIFICATION OF CLEARING FACTOR

In an effort to increase the purity of clearing factor preparation the following procedures were employed:

1. Fractionation of clearing factor containing preparation by ammonium sulfate.

To 40 ml of the clearing factor solution obtained by extraction of postheparin plasma with ether varying quantities of ammonium sulfate are added and the solution is brought to 10; 20; 30; 40; 50; 60; 70; 80; and 100 per cent saturation with respect to the ammonium salt. The appearing precipitate, at different levels of saturation, is removed by centrifugation at 40,000 g and at 5° C. for 15 minutes. Part of the obtained fractions are dissolved in 0.3 M NaCl solution and dialyzed against 5 gallons of distilled water. 100 mg. (wet weight) of respective dialyzed and nondialyzed fractions are dissolved in 1.2 ml preheparin plasma and on addition of 0.3 ml of standard fat emulsion are incubated at 37° C. in a water bath. After 60 minutes of incubation the clearing factor activity is tested in duplicate by optical density and glycerol determination methods.

The results of these determinations are reported in Table III.

TABLE III.

Lipolytic Activity of Fractions Obtained by Ammonium Sulfate Precipitation of Crude Clearing Factor

Exp. No.	Systems Determined	Change in Optical Density		Change in Glycerol Value in Micrograms		Glycerol Millimoles/ Liter
		Time of Incub.		Time of Incub.		
		0 min.	60 min.	0 min.	60 min.	
1.	1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.64	.62	360	360	-
2.	5 mg. dry crude enzyme in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.64	.34	370	490	0.96
3.	50 mg. 10% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.60	.35	365	390	0.19
4.	Same system as above only the fraction first dialyzed	.64	.63	350	370	0.15
5.	50 mg. 20% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.62	.37	355	390	0.25
6.	Same system as above only the fraction first dialyzed	.65	.63	360	370	-
7.	50 mg. 30% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.64	.38	370	440	0.51
8.	Same system as above only the fraction first dialyzed	.66	.63	355	370	0.10
9.	50 mg. 40% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.63	.32	370	425	0.40
10.	Same system as above only the fraction first dialyzed	.65	.61	355	370	-
11.	50 mg. 50% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.63	.36	360	400	0.29
12.	Same system as above only the fraction first dialyzed	.64	.69	365	370	-
13.	50 mg. 60% saturation fraction in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.66	.38	360	390	0.20
14.	Same system as above only the fraction first dialyzed	.65	.64	360	355	-
15.	50 mg. 100% saturation fraction in 1.2 ml prehseparin plasma + 0.3 standard fat emulsion	.62	.41	350	380	0.20
16.	Same system as above only the fraction first dialyzed	.63	.61	360	370	-
17.	5 mg. crude enzyme first dialyzed in 1.2 ml prehseparin plasma + 0.3 ml standard fat emulsion	.65	.55	355	390	0.25
18.	1.2 ml of 0.1 M ammonium sulfate solution + 0.3 ml standard fat emulsion	.60	.48	25	20	-

* Each experiment represents an average of two determinations.

2. Attempted purification of crude clearing factor preparation by adsorption on calcium phosphate gel.

(a) Preparation of calcium phosphate gel.

Calcium phosphate gel was prepared by the method of Keilin and Hartree (59).

150 ml of calcium chloride solution (132 g $CaCl_2.6H_2O$ per liter) is diluted to about 1,600 ml with tap water and shaken with 150 ml trisodium phosphate solution (152 g $Na_3PO_4.12H_2O$ per liter). The mixture is brought to pH 7.4 with dilute acetic acid and the precipitate washed three or four times by decantation with large volumes of water (15 - 20 liters). The precipitate is finally washed with distilled water in a centrifuge. 9.1 g of calcium phosphate are thus obtained.

Other Reagents:

Physiological saline (870 mg. NaCl in 100 ml water)

0.1 M citric acid (9.6 g citric acid in 500 ml of water)

(b) Procedure

10 ml of clearing factor solution are mixed with 5 g of freshly made calcium phosphate gel and the mixture is centrifuged at 3,000 g and 5° C. for 10 minutes. The collected precipitate is twice extracted with 40 ml portions of physiological saline followed by extraction with 40 ml of 0.1 M citric acid. After each extraction the mixture is separated by centrifugation at 3,000 g and 5° C. for 10 minutes. The obtained extracts are lyophilized and the 15 mg. of solids from each extraction respectively, are dissolved in 1.2 ml of normal preheparin plasma and on addition of 0.3 ml standard fat emulsion,

the mixture is incubated at 37° C. in a water bath. After 60 minutes of incubation the clearing factor activity of these fractions is tested in duplicate by optical density and glycerol determination methods.

The results of these determinations are reported in Table IV.

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TABLE IV.

Lipolytic Activity Present in the Eluates Obtained after Washing of Calcium Phosphate Gel with Adsorbed Clearing Factor

* Exp. No.	System Determined	Change in Optical Density		Change in Glycerol Value in Micrograms		Δ Glycerol Millimoles/ Liter
		Time of Incubation		Time of Incubation		
		0 min.	60 min.	0 min.	60 min.	
1.	15 mg. of starting material (crude clearing factor) in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	68	27	370	530	1.16
2.	15 mg. of solids from 1st washing with physiological saline in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	65	63	360	365	-
3.	15 mg. of solids from 2nd washing with physiological saline in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	63	60	355	380	0.18
4.	15 mg. of solids from 1st washing with 0.1 M citric acid in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	64	26	360	510	1.09
5.	15 mg. of solids from 2nd washing with 0.1 M citric acid in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	64	58	365	375	-
6.	15 mg. of citric acid in 1.2 ml preheparin plasma + 0.3 ml standard fat emulsion	61	60	370	370	-

* Each experiment represents an average of two determinations.

3. Fractionation of clearing factor containing preparation by increasing hydrogen ion concentration.

The semi-solid layer (obtained by extracting 50 ml of post-heparin plasma with ether) is minced with 20 ml of 0.03 M sodium chloride solution in a Potter-Elvehjem homogenizer and the supernate is collected by centrifugation at 4,000 g and 5° C. for 20 minutes. The pH of the starting material is 7.0. By the addition of small amounts of 0.2 N HCl the hydrogen ion concentration of the solution is brought up to pH 6.8; 6.5; 6.0; 5.5 and 5.0. The appearing precipitate after each pH adjustment is removed by centrifugation at 20,000 g and 5° C. for 10 minutes. Duplicate samples of 50 mg. (wet weight) of each precipitate are extracted with 0.6 ml of glycine buffer at pH 8.6. The mixture is centrifuged for 5 minutes at 1,000 g and the respective supernatants are transferred to Coleman microcuvettes. To each microcuvette 0.6 ml preheparin plasma and 0.3 ml of standard fat emulsion are added and the samples are incubated in a water bath at 37° C. At 0 time and after 5 min.; 10 min.; 20 min.; 40 min. and 60 min. of incubation the lipemia clearing activity is tested by the optical density method.

The results of these determinations are reported in Table V.

TABLE V.

Lipemia Clearing Activity of the Fractions Obtained by Increasing Hydrogen Ion Concentration In a Crude Clearing Factor Preparation.

Exp. No. *	System Determined	Change in Optical Density Time of Incubation 0 min.	5 min.	10 min.	20 min.	40 min.	60 min.
1.	Starting material 0.6 ml (crude enzyme prep.) +0.6 ml preheparin plasma at pH 7.0 +0.3 ml standard fat emul.	70	61	48	36	31	24
2.	Precipitate in 0.6 ml glycine buffer at pH 6.8 + 0.6 ml preheparin plasma + 0.3 ml standard fat emul.	61	41	38	28	24	22
3.	Precipitate in 0.6 ml glycine buffer at pH 6.5 + 0.6 ml preheparin plasma + 0.3 ml standard fat emul.	61	44	35	28	25	24
4.	Precipitate in 0.6 ml glycine buffer at pH 6.0 + 0.6 ml preheparin plasma + 0.3 ml standard fat emul.	61	48	44	39	31	27
5.	Precipitate in 0.6 ml glycine buffer at pH 5.5 + 0.6 ml preheparin plasma + 0.3 ml standard fat emul.	61	54	49	41	34	30
6.	Precipitate in 0.6 ml glycine buffer at pH 5.0 + 0.6 ml preheparin plasma + 0.3 ml standard fat emul.	57	53	52	49	45	41
7.	0.6 ml of remaining solution pH adjusted to 7.0 0.6 ml preheparin plasma 0.3 ml standard fat emulsion	68	67	67	67	67	67
8.	0.6 ml glycine buffer pH 8.6 0.6 ml preheparin plasma 0.3 ml standard fat emulsion	65	64	64	64	63	63

* Each experiment represents an average of two determinations.

E. ISOLATION AND PURIFICATION OF CLEARING FACTOR BY REPEATED EXTRACTIONS WITH ETHYL ETHER

100 ml of postheparin plasma and 100 ml of ethyl ether are precooled to 0° C. and mixed in a 1,000 ml volumetric flask by shaking for 30 seconds. Then 200 ml of ethanol reagent, chilled to -5° C. are added and the contents are mixed for 10 minutes at -5° C. The mixture is placed in four 250 ml centrifuge bottles and centrifuged at 3,000 g and -3° C. for 20 minutes. The ether and aqueous layers are removed and the semi-solid layer ("pellicle") remaining in the bottle is extracted with three 25 ml portions of ethyl ether. Ether is decanted and completely removed by evaporation under vacuum. The precipitate is extracted with 40 ml of glycine buffer at pH 8.6 and the supernatant is collected by centrifugation at 3,000 g and 5° C. for 20 minutes. The collected supernatant is referred to as Fraction I. One and one-half ml of the supernatant was lyophilized for determination of lipolytic action, and 0.5 ml was taken for protein concentration tests. The remaining 38 ml of the supernatant and 38 ml of ethyl ether are precooled to 0° C. and mixed in a 250 ml Erlenmeyer flask. The contents are continually mixed for 5 minutes while the flask is kept in an alcohol-water bath at -5° C. On centrifugation at 3,000 g and -3° C. for 20 minutes, four layers are obtained from the above mixture: precipitate on the bottom of the tube, aqueous layer, ether layer, and semi-solid layerat the interphase of aqueous and ether layers. The liquid phases are decanted, the precipitate and the semi-solid layers are combined and extracted with two 25 ml portions of ethyl ether at -5° C. The

ether is decanted and completely evaporated under vacuum. The obtained precipitate is extracted with 40 ml of glycine buffer at pH 8.6 and the supernatant is collected after centrifugation for 15 minutes at 20,000 g and 5° C. The collected supernatant is referred to as Fraction II. One and one-half ml of the supernatant was lyophilized for lipolytic activity determinations and 0.5 ml was taken for protein concentration tests. The remaining 38 ml of supernatant are extracted with 38 ml of ethyl ether by repeating the above described procedure. The obtained 40 ml of supernatant (by extracting semi-solid and solid layers with glycine buffer) represents Fraction III. One and one-half ml of the Fraction III containing solution is lyophilized for lipolytic action determination and 0.5 ml is taken out for protein concentration tests. The remaining 38 ml of supernatant and 38 ml of ethyl ether are precooled to 0° C. and again mixed together, following the previously described procedures. After the centrifugation four layers are obtained from the mixture. At this point only the semi-solid layer is removed (by means of a wooden rod). It is washed with 25 ml of ethyl ether at -5° C. and extracted with 30 ml of glycine buffer at pH 8.1. The collected supernatant, after centrifugation for 20 minutes at 20,000 g and 5° C., is referred to as Fraction IV and represents the final fraction containing purified clearing factor. One and one-half ml of the Fraction IV containing solution was taken for the protein concentration tests and the remaining material is lyophilized and stored in the dry state at -5° C. The summary of the steps taken for isolation and purification of clearing factor by the ether extraction method is given in the attached flow sheet.

100 ml Postheparin Plasma + 100 ml Ethyl Ether + 200 ml Ethanol Reagent

Mixture kept at -5° C. for 10 minutes
Centrifuged at 3,000 g and -5° C. for 20 minutes

- Intermediate semi-solid layer (pellicle) 92-95% activity
- Ether layer No activity
- Infranatant aqueous layer 5-8% activity

Intermediate semi-solid layer (pellicle) 92-95% activity

Washed 3 times with 25 ml portions of ethyl ether at -5° C.

- Intermediate semi-solid layer (pellicle) 90-95% activity
- Ether layer No activity

Intermediate semi-solid layer (pellicle) 90-95% activity

Extracted with 40 ml glycine buffer of pH 8.6 and centrifuged at 20,000 g and +5° C. for 20 minutes

- Supernatant 80-82% activity
- FRACTION I
- Precipitate No activity

Supernatant 80-82% activity

+ 40 ml ethyl ether, mixed for 5 min. at -5° C., centrifuged at 3,000 g and -5° C. for 20 minutes

- Intermediate semi-solid layer 45-50% activity
- Precipitate 25-30% activity
- Ether layer No activity
- Aqueous layer 1-5% activity

Precipitates combined and twice extracted with 25 ml ethyl ether at -5° C.

- Precipitate 75-80% activity
- Supernatant No activity

Extracted with 40 ml glycine buffer Centrifuged at 20,000 g, + 5° C. for 20 minutes

- Supernatant 70-74% activity
- FRACTION II
- Precipitate No activity

Supernatant 70-74% activity

+ 40 ml ethyl ether, mixed for 5 min. at -5° C., centrifuged at 3,000 g and -5° C. for 20 minutes

- Intermediate semi-solid 40-45% activity
- Precipitate 18-20% activity
- Ether layer No activity
- Aqueous layer No activity

Precipitates combined and twice extracted with 25 ml ethyl ether at -5° C.

- Precipitate 65-70% activity
- Ether layer No activity

Extracted with 40 ml glycine buffer centrifuged at 20,000 g and +5° C. for 20 minutes

- Supernatant 65-68% activity
- FRACTION III
- Precipitate No activity

40 ml ethyl ether mixed for 5 min. at -5° C., centrifuged at 3,000 g and -5° C. for 20 minutes

- Intermediate semi-solid layer 55-60% activity.
- Precipitate 2-5% activity
- Ether layer No activity
- Aqueous layer No activity

Washed with 25 ml ethyl ether at -5° C.

Extracted with 30 ml glycine buffer Centrifuged at 20,000 g and 5° C. for 20 minutes

- Supernatant 50-52% activity
- FRACTION IV
- Precipitate No activity

The lipolytic activity of the isolated fractions is tested in duplicate by optical density and free fatty acid determination methods. 5 mg. of a respective dry fraction are dissolved in 2.0 ml of normal preheparin plasma. On incubation for 60 minutes with 0.5 ml standard fat emulsion the changes in optical density and unesterified fatty acids are determined.

The protein content of the isolated fractions was determined by the following Biuret method (90).

(a) Preparation of Biuret Reagent

To a solution of 15 gm. of copper sulfate with five waters of hydration (10.25 gm. of copper chloride with two waters of hydration) in 250 ml of water, 75 ml of ethylene glycol are added, followed by 150 gm. of sodium hydroxide in aqueous solution and the whole mixture made up to 500 ml. The solution is transferred to a large Erlenmeyer flask, covered with a watch glass, and heated 4 hours on a steam bath. The solution is filtered after cooling.

(b) Procedure

One-half ml aliquots of the respective fractions in solution are placed in 25 ml Erlenmeyer flasks and brought to 10 ml by addition of 0.15 M sodium chloride. A blank is set up using 10 ml of the 0.15 M sodium chloride. In each series of readings is also included a bovine serum albumin standard containing 1 ml of 1.0 per cent albumin (the standard is checked by micro Kjeldahl analysis, and the factor 6.30 used to convert N into protein content) and 9 ml of 0.15 M sodium chloride.

To the contents of each flask is added by swirling 1 ml of Biuret reagent. After standing 20 minutes at room temperature, the solutions are read against the blank in a Klett colorimeter, using a filter transmitting maximally near 550 millimicrons.

Calculation:

$$\frac{\text{volume of solution}}{\text{volume of aliquot}} \times \frac{\text{unknown reading}}{\text{standard reading}} \times 10 = \text{mg. protein in the sample}$$

The results of these determinations are reported in Table VI. The additional data on clearing factor purification is presented in Table VII and is partially derived from the results reported in Table VI.

In order to assure that the same concentration of clearing factor is used in a given set of tests, an arbitrary unit of clearing factor, at this point, is introduced:

One unit is the amount of clearing factor, which on incubation for 60 minutes at 37° C. with 0.2 ml of normal preheparin plasma and 2.5 mg. of coconut oil (present in the form of 0.5 ml of standard emulsion*), increases titratable acidity by one millimicroequivalent or 1×10^{-9} of an equivalent.

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* For description see page 18.

TABLE VI.

Lipolytic activity present in the postheparin plasma fractions after ether extractions.

Exp. No.	System Determined	Protein in Fraction Used for Activity Test (mg.)	Change in Optical Density Test of Incubation 0 min.	60 min.	Change of NEFA Value in Microequivalents Test of Incubation 0 min.	60 min.	Increase of Titratable Acidity per Sample in Microequivalents	Total weight of Fraction (mg.)	Total Protein in Fraction (mg.)	Total Units of Clearing Factor in Fraction
1.	2.0 ml postheparin plasma (starting material) + 0.5 ml standard fat emulsion	141	.66	.23	1.8	4.3	2.5	100,720	7,050	125,000
2.	5 mg. of fraction I in 2.0 ml postheparin plasma + 0.5 ml standard fat emulsion	3.5	.65	.51	1.6	3.2	1.6	365	224	102,000
3.	5 mg. of fraction II in 2.0 ml postheparin plasma + 0.5 ml standard fat emulsion	3.2	.64	.37	1.6	3.7	2.1	280	141	92,400
4.	5 mg. of fraction III in 2.0 ml postheparin plasma + 0.5 ml standard fat emulsion	3.1	.65	.30	1.6	3.9	2.3	185	115	85,100
5.	5 mg. of fraction IV in 2.0 ml postheparin plasma + 0.5 ml standard fat emulsion	2.1	.65	.23	1.6	4.4	2.8	115	55	44,400
6.	2.0 ml postheparin plasma + 0.5 ml standard fat emulsion	-	.64	.62	1.6	1.68	-	-	-	-

* Each experiment represents an average of two determinations. ** NEFA = Non-esterified fatty acids.

For definition of a unit of clearing factor see page

TABLE VII.

Isolation and Purification of Clearing Factor by Extraction From Postheparin Human Plasma with Ethyl Ether

100 ml of starting material.

Fraction	Total Protein mg.	Total Units*	Units per mg. of protein	Purification	Yield %
Postheparin plasma	7,050	125,000	17.7	1	100
Fraction I	224	102,000	456	26	81.6
Fraction II	141	92,400	654	37	73.9
Fraction III	115	85,100	740	41.8	68
Fraction IV	55	64,400	1,170	66	51.9

* One unit is the amount of clearing factor which on incubation for 60 minutes at 37° C. with 2 ml of normal preheparin plasma and 2.5 mg. of coconut oil (present in the form of 0.5 ml of standard emulsion) increases titratable acidity by one millimicroequivalent or 1×10^{-9} of an equivalent.

II. PROCEDURES FOR THE DESCRIPTION OF PHYSIOCHEMICAL PROPERTIES OF ISOLATED CLEARING FACTOR

A. PURITY OF THE ENZYME PREPARATION

The purity of the fractions with clearing factor activity was tested by electrophoretic and ultracentrifugal procedures.

1. Free boundary electrophoresis

110 mg. of Fraction IV, obtained through successive ether extractions of postheparin plasma, are dissolved in 2.4 ml of water and are set overnight for dialysis against 2 liters of barbital-sodium citrate buffer of pH 8.6 and ionic strength of 0.1. The electrophoretic analysis is carried out in a Perkin-Elmer Tiselius electrophoresis apparatus, model 58, using barbital-sodium citrate buffer of pH 8.6 and ionic strength 0.1.

2. Ultracentrifugal analysis

30 mg. of Fraction IV, obtained after successive ether extractions of postheparin plasma, are dissolved in 1.2 ml of 0.1 M sodium chloride and subjected to centrifugation in an E type Spinco analytical centrifuge at 56,100 RPM and 22° C. for 80 minutes. Pictures of the sedimentation patterns are taken at 0, (time at which the full speed is achieved) 4, 8, 16, 24, 32, 38, 44, 64 and 80 minutes.

The patterns obtained are presented in Figs. 1 and 2.

FIGURE 1.

Electrophoretic Pattern of Purified Clearing Factor Preparation

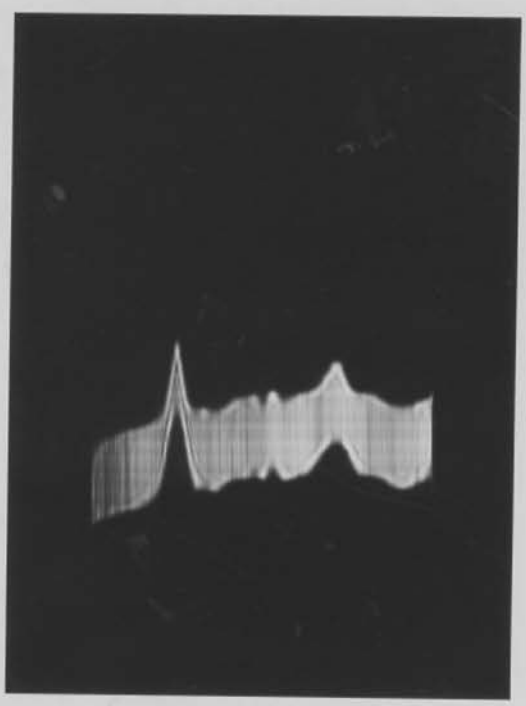
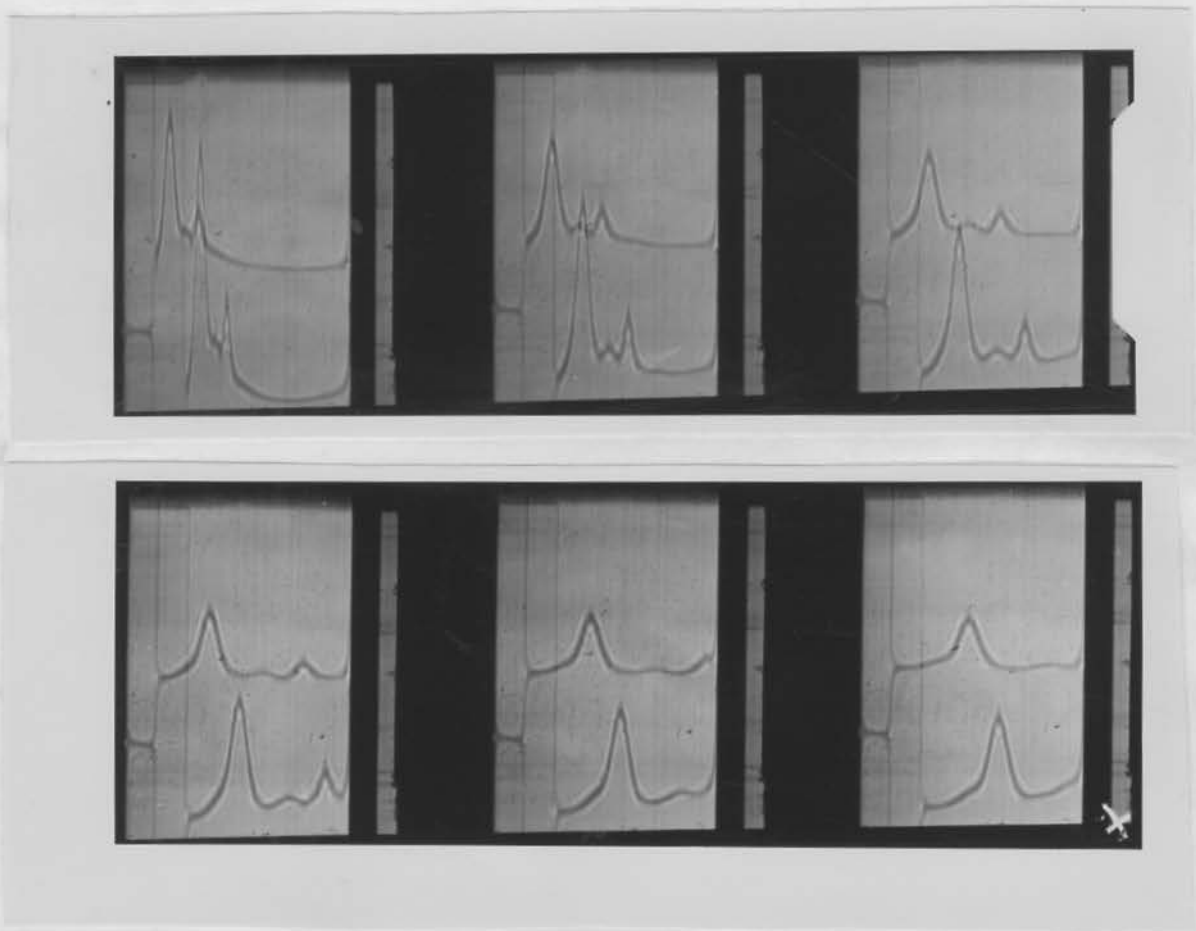


FIGURE 2.

Ultracentrifugal Patterns of Purified Clearing Factor Preparation



B. STABILITY OF THE PURIFIED CLEARING FACTOR

The stability of the clearing factor was evaluated in regard to its physical state, temperature and requirement of protective protein.

Samples containing 2,000 units of clearing factor are prepared in triplicate in the following manner:

(a) in the form of a dry powder

(b) dissolved in 0.8 ml of veronal buffer of pH 8.6

(c) dissolved in a mixture of 0.8 ml of veronal buffer and 0.4 ml of 10 per cent bovine albumin solution (the resulting pH is 8.1)

The above prepared samples are kept at -20° C., 0° C. and 25° C. for 7 days. Then the physical state of the samples are adjusted so that all samples are dissolved in a mixture of 0.8 ml veronal buffer and 0.4 ml 10 per cent bovine albumin solution of pH 8.1. On addition of 0.3 ml of standard fat emulsion to each tube the samples are incubated at 37° C. for 30 minutes and the clearing factor activity is determined by optical density method. As a control the activity of 2,000 units of freshly prepared enzyme (dissolved in the same veronal-albumin mixture) is determined.

The results of these determinations are presented in Table VIII.

TABLE VIII.

Effects of Temperature and Protein on the Stability of Clearing Factor Solutions

2,000 Units of Clearing Factor Kept for 7 Days	Change in Optical Density after Incubation with SFE* at 37° C. for 60 min.					
	Kept at -20° C.		Kept at 0° C.		Kept at 25° C.	
	Incubation Time		Incubation Time		Incubation Time	
	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.
Dry form	.65	.32	.64	.32	.66	.36
Dissolved in Veronal Buffer	.63	.41	.64	.49	.65	.57
Dissolved in Veronal Buffer + Albumin	.66	.40	.65	.44	.66	.48

* SFE = Standard fat emulsion.

For control purposes 2,000 units of freshly prepared enzyme were dissolved in a Veronal-Albumin mixture and the optical density was determined. The reading was .64 at 0 time and .31 at 60 min. of incubation.

Each figure represents an average of three determinations.

C. TESTS ON THE CHEMICAL COMPOSITION OF THE PURIFIED CLEARING FACTOR

1. Determination of total protein.

The protein content of clearing factor preparations obtained by extracting postheparin plasma with ether was determined by the biuret method (page 42) and the results are reported in Table VII.

2. Determination of phospholipids and cholesterol.

The cholesterol and phospholipid content of the clearing factor containing Fraction IV were determined; employing the revised method of Fiske, C.H. and Y. Subbarow (21) for phospholipids determination and Bloor, W.R. and A. Knudson (8) for cholesterol. No cholesterol and only traces of phosphorus representing phosphatides were found.

3. Determination of the presence of lipoprotein and glycoprotein in the clearing factor preparation.

The presence of lipoprotein and glycoprotein in clearing factor containing Fraction IV was determined by the staining methods of Swahn (120, 121) and of Koiw and Gronwall (61).

A few drops of a staurated solution of clearing factor are placed on 3 strips of Whatman #1 filter paper and dried. Then the strips are fixed in an ethanol-formalin solution and stained with Sudan black B for lipoproteins and with Toluidine blue for glycoproteins. For control purposes dilute solutions of beta lipoprotein and heparin are stained by the above methods and the colors developed are compared with the test strips. By these methods no lipoproteins or glycoproteins were detected in the clearing factor preparation.

III. KINETIC STUDIES OF THE ISOLATED CLEARING FACTOR

A. DETERMINATION OF THE EFFECT OF TEMPERATURE ON THE REACTION VELOCITY OF CLEARING FACTOR

To the test tubes containing 1.0 ml of veronal buffer of pH 7.8, 0.2 ml of 10 per cent albumin solution and 6,000 units of clearing factor; 0.3 ml of standard fat emulsion is added and the contents are incubated at various temperatures for 15 minutes. At At the beginning and the end of incubation the lipolytic activity of the duplicate samples are tested by determining the changes in optical density and the concentration of unesterified fatty acids.

The results of these determinations are presented in Table IX and in Fig. 3.

B. DETERMINATION OF THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE REACTION VELOCITY OF CLEARING FACTOR

To the test tubes containing 4,000 units of clearing factor dissolved in 1.0 ml citrate or veronal buffers of varying pH., 0.2 ml of 10 per cent albumin solution and 0.3 ml of standard fat emulsion are added and the mixture is incubated for 15 minutes at 37° C. At the beginning and the end of incubation the non-esterified fatty acid content is determined in duplicate.

The results of these determinations are reported in Table X and in Fig. 4.

C. DETERMINATION OF THE EFFECT OF CONCENTRATION OF CLEARING FACTOR ON THE VELOCITY OF THE REACTION

Duplicate samples containing 3.6; 7.2; 14.4; 28.8; 43.2; and 57.6 mg. of clearing factor protein (Fraction IV from ether extractions

of postheparin plasma) are dissolved in 6.0 ml of preheparin normal plasma respectively, and on addition of 1.5 ml of standard fat emulsion are incubated at 37° C. in the water bath. At 0 time and 4 and 8 minutes of incubation 1.0 ml aliquots are taken and optical density and non-esterified fatty acids are determined.

The results of these determinations are reported in Table XI and in Fig. 5.

D. DETERMINATION OF THE EFFECT OF SUBSTRATE CONCENTRATION ON THE REACTION VELOCITY OF CLEARING FACTOR

To the duplicate samples containing 2,000 units of clearing factor dissolved in 1.0 ml of preheparin normal plasma; 0.03; 0.06; 0.09; 0.12; 0.15 and 0.18 ml of standard fat emulsion are added and the mixtures are incubated at 37° C. for 4 minutes. After 4 minutes of incubation and at 0 time the concentration of non-esterified fatty acid in the samples is determined.

The results of these determinations are reported in Tables XII and XIII and in Fig. 6.

TABLE IX.

Effect of Temperature on Reaction Velocity of Clearing Factor

* Exp. No.	Temp. in Centrigrade	Temp. in Absolute Degrees	$\frac{1}{T} \times 10^4$	ml. .02 N NaOH Used	Microequivalents of Titratable Acidity per Sample	Log of Microequivalents of Titratable Acidity
1.	0	273	36.6	.0015	.075	$\bar{2}.87$
2.	5	278	35.9	.003	.15	$\bar{1}.18$
3.	10	283	35.3	.0045	.25	$\bar{1}.35$
4.	20	293	34.1	.0085	.425	$\bar{1}.63$
5.	30	303	33.0	.0016	.800	$\bar{1}.90$
6.	37	310	32.2	.024	1.200	0.08
7.	40	313	31.9	.0233	1.17	0.07
8.	45	318	31.4	.0131	0.65	$\bar{1}.81$
9.	50	323	30.9	.006	0.24	$\bar{1}.38$
10.	55	328	30.5	.003	0.15	$\bar{1}.18$

* Each experiment represents an average of two determinations.

Reaction time = 15 minutes.

FIGURE 3.

ARRHENIUS PLOTS FOR THE ACTIVATION TEMPERATURE OF CLEARING FACTOR

$$\text{LN } K = \frac{-E}{R}\left(\frac{1}{T}\right) + \text{CONSTANT}$$

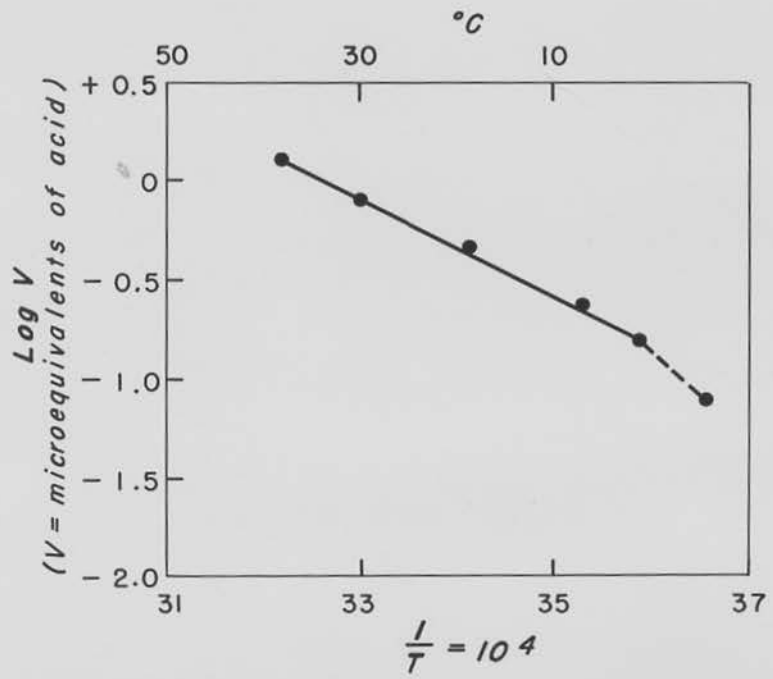


TABLE I.

The Effect of Hydrogen Ion Concentration on the Reaction Velocity of Clearing Factor

pH of the System	Buffer	ml 0.02 N NaOH at 0 Time of Enzyme Addition	ml 0.02 N NaOH after 60 min. Incubation	ml 0.02 N NaOH Increase after 60 min. Incubation	Microequivalents of Titratable Acidity per Sample
5.6	Citrate	0.0256 0.0265	0.0271 0.0289	0.002	0.05
6.0	"	0.0224 0.0241	0.0275 0.0291	0.005	0.200
6.3	"	0.0254 0.0271	0.0330 0.0328	0.007	0.300
6.7	"	0.0255 0.0248	0.0348 0.0356	0.010	0.450
7.0	Veronal	0.0252 0.0241	0.0372 0.0383	0.013	0.600
7.4	"	0.0254 0.0271	0.0436 0.0450	0.018 0.018	0.850
7.7	"	0.0264 0.0280	0.0487 0.0500	0.022	1.050
7.8	"	0.0220 0.0248	0.0468 0.0480	0.024	1.150
7.9	"	0.0258 0.0291	0.0511 0.0542	0.025	1.200
8.0	"	0.0225 0.0223	0.0480 0.0492	0.026	1.250
8.1	"	0.0224 0.0250	0.0479 0.0505	0.025	1.200
8.2	"	0.0261 0.0248	0.0510 0.0493	0.024	1.150
8.3	"	0.0255 0.0230	0.0465 0.0460	0.022	1.050
8.4	"	0.0242 0.0235	0.0428 0.0425	0.019	0.900
8.5	"	0.0250 0.0241	0.0408 0.0414	0.016	0.750
8.7	"	0.0240 0.0240	0.0352 0.0355	0.011	0.500
8.9	"	0.0221 0.0218	0.0310 0.0291	0.008	0.350
9.2	"	0.0220	0.0274	0.005	0.200
9.5	"	0.0208 0.212	0.0211 0.0226	0.002	0.05
8.0	"	0.0210 0.0218	0.0221 0.0230	0.001	No enzyme present
6.3	Citrate	0.0234 0.0246	0.0251 0.0250	0.001	No enzyme present
Standard		0.0408 0.0405			

FIGURE 4.

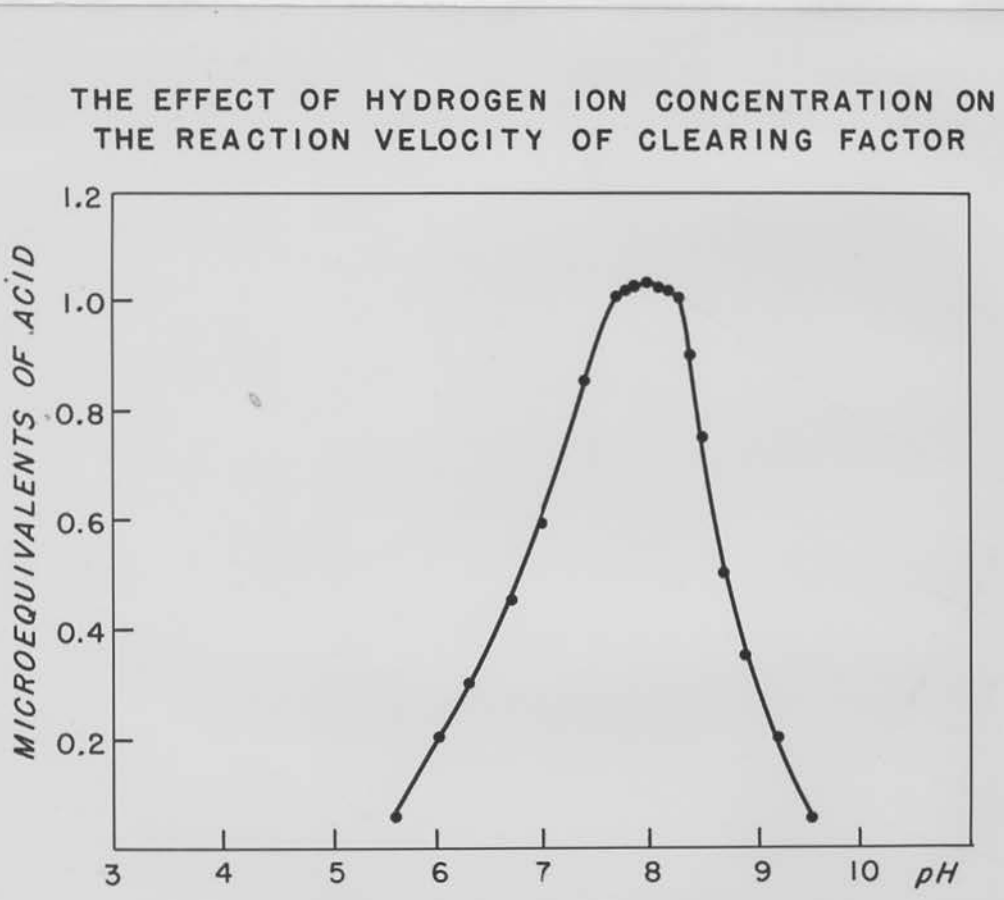

THE EFFECT OF HYDROGEN ION CONCENTRATION ON
THE REACTION VELOCITY OF CLEARING FACTOR
MICROEQUIVALENTS OF ACID
1.2
1.0
0.8
0.6
0.4
0.2
3
4
5
6
7
8
9
10
pH

TABLE XI.

Effect of Clearing Factor Concentration on the Velocity of Lipolytic Reaction

Exp. No.*	Clearing Factor as mg./ml Protein	Change in Optical Density			Change in Microequivalents of Titratable Acidity		
		Time of Incubation			Time of Incubation		
		0 min.	4 min.	8 min.	0 min.	4 min.	8 min.
1.	0.48	.63	.61	.60	1.614	1.625	1.638
2.	0.96	.64	.59	.58	1.602	1.639	1.662
3.	1.92	.64	.59	.58	1.615	1.655	1.689
4.	3.84	.63	.57	.51	1.610	1.670	1.720
5.	5.73	.63	.53	.48	1.612	1.672	1.746
6.	7.68	.62	.51	.43	1.610	1.680	1.758

* Each experiment represents an average of two determinations.

FIGURE 5.

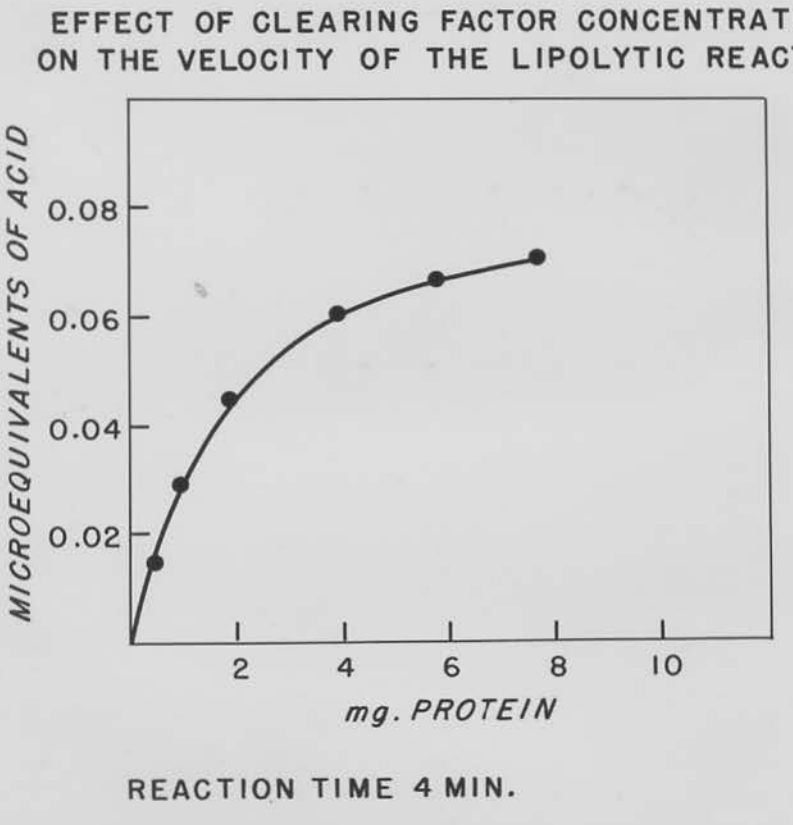

EFFECT OF CLEARING FACTOR CONCENTRATION
ON THE VELOCITY OF THE LIPOLYTIC REACTION
MICROEQUIVALENTS OF ACID
0.08
0.06
0.04
0.02
2
4
6
8
10
mg. PROTEIN
REACTION TIME 4 MIN.

TABLE XII.

Effect of Substrate Concentration on Clearing Factor Activity

* Exp. No.	Coconut Oil Present Micrograms/ml	Time of Incubation 0 min. 0.02 N NaOH	Time of Incubation 4 min. 0.02 N NaOH	Difference in Microequivalents per Sample Titrated	Change in Microequivalents of titratable acidity
1.	25	.0309 .0311	.0317 .0316	.013	.017
2.	50	.0313 .0310	.0320 .0318	.018	.030
3.	75	.0311 .0313	.0322 .0321	.023	.042
4.	100	.0308 .0312	.0323 .0323	.026	.050
5.	125	.0307 .0303	.0324 .0325	.029	.057
6.	150	.0314 .0307	.0326 .0325	.031	.062
7.	Control No substrate	.0311 .0314	.0314 .0317	.006	-
8.	Standard	.0404 .0406	-	-	-

* Each experiment represents an average of two determinations.

TABLE XIII.

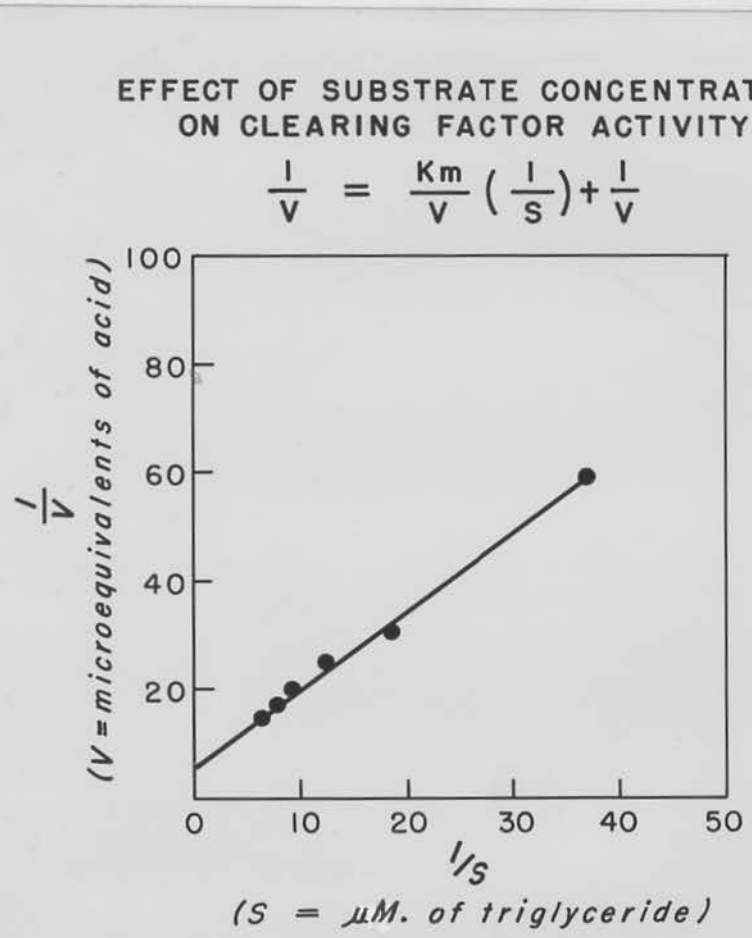
Effect of Substrate Concentration on Clearing Factor Activity

Exp. No.*	Coconut Oil Micrograms/ ml	Coconut Oil Micromoles/** ml	$\frac{1}{\text{Substrate}}$	Relative Velocity Microequivalents Acid	$\frac{1}{\text{Relative Velocity}}$
1.	25	.027	37	.017	59
2.	50	.054	18.5	.03	33
3.	75	.081	12.3	.042	24
4.	100	.108	9.3	.05	20
5.	125	.135	7.4	.057	17
6.	150	.162	6.2	.062	15

* Each experiment represents an average of two determinations.

** Molecular weight of coconut fat is considered 923 and that of fatty acids 277.

FIGURE 6.


EFFECT OF SUBSTRATE CONCENTRATION
ON CLEARING FACTOR ACTIVITY
$\frac{1}{V} = \frac{Km}{V}\left(\frac{1}{S}\right) + \frac{1}{V}$
100
80
60
40
20
0
10
20
30
40
50
$\frac{1}{V}$
(V = microequivalents of acid)
$1/S$
(S = μM. of triglyceride)

E. SUBSTRATE SPECIFICITY FOR CLEARING FACTOR CATALYZED REACTIONS

Employing the experimental conditions as uniform as possible the action of the clearing factor on the following substrates were tested:

1. Standard fat emulsion

(a) Preparation of substrate

One part of standard fat emulsion and 19 parts of water.

Kept at -5° C.

(b) Procedure

To a mixture containing 8.0 ml veronal buffer of pH 8.6, 4.0 ml of 10 per cent albumin solution and 30,000 units of dissolved clearing factor; 3.0 ml of 1:20 diluted standard fat emulsion are added and the mixture is incubated in a water bath at 37° C. After 0, 5, 10, 20, 40 and 60 minutes of incubation, 1.0 ml aliquot of the mixture is taken in duplicate and the concentration of non-esterified fatty acids is determined.

2. Triacetin

(a) Preparation of substrate

12.0 mg. of triacetin in 1.5 ml of distilled water.

(b) Procedure

To a mixture containing 8.0 ml veronal buffer of pH 8.6, 4.0 ml of 10 per cent albumin solution and 30,000 units of dissolved clearing factor; 3.0 ml of triacetin solution are added and the mixture is incubated in a water bath at 37° C. At 0, 5, 10, 20, 40 and 60 minute time intervals 1.0 ml aliquot of the mixture is taken in duplicate and the concentration of non-esterified fatty acids is determined.

3. Chylomicrons

(a) Preparation of substrate

A normal male blood donor, after a fatty breakfast at 7 A.M., was given a pint of heavy cream at 10 A.M. and 3 hours later blood is collected in citrated bottles. After 30 minutes centrifugation at 2,000 g, 250 ml of lipemic plasma are collected and the chylomicrons separated by repeated centrifugation at 20,000 g for 30 minutes. The obtained top creamy layer is twice washed in physiological saline and the collected chylomicrons are suspended in 5 ml of distilled water. From this stock solution a final dilution is prepared so that adding 0.3 ml of chylomicron preparation to 1.2 ml of veronal buffer would give optical density reading of .65.

(b) Procedure

To a mixture containing 8.0 ml veronal buffer of pH 8.6, 4.0 ml of 10 per cent albumin solution and 30,000 units of dissolved clearing factor; 3.0 ml of above chylomicra preparation are added and the mixture is incubated in a water bath at 37° C. At 0, 5, 10, 20, 40, and 60 minute intervals, 1.0 ml aliquot of the mixture is taken in duplicate and the concentration of non-esterified fatty acids is determined.

4. Beta lipoproteins

(a) Preparation of substrate

To 10 lusteroid tubes each containing 5 ml of plasma obtained from normal, fasting person, 4 ml of sodium chloride solution of specific gravity 1.1343 are added and the mixture is centrifuged in a Spince

L type centrifuge at 36,000 RPM and 22° C. for 16 hours. One milliliter of the top layer is removed by means of syringe and needle. This represents beta lipoproteins of specific density 1.04 (67).

(b) Procedure

To a mixture containing 6.0 ml veronal buffer of pH 8.6, 3.0 ml of 10 per cent albumin solution and 22,500 units of dissolved clearing factor; 2.5 ml of the collected beta lipoproteins are added and the mixture is incubated in a water bath at 37° C. At 0, 10, 20, and 60 minute time intervals, 1.0 ml aliquot of the mixture is taken in duplicate and the concentration of non-esterified fatty acids is determined.

F. DETERMINATION OF ALBUMIN AND CALCIUM ION INFLUENCE ON THE LIPOLYTIC ACTION OF CLEARING FACTOR

The following mixtures are prepared in duplicate and after incubation at 0 time at 37° C. for 30 minutes, the concentration of non-esterified fatty acids is determined.

(1) 1.2 ml buffer + 0.3 ml standard fat emulsion + 2,000 units of clearing factor.

(2) 0.8 ml buffer + 0.4 ml 10 per cent albumin solution + 0.3 ml standard fat emulsion + 2,000 units clearing factor.

(3) 1.0 ml buffer + 0.2 ml 5 per cent calcium acetate solution + 2,000 units clearing factor.

(4) 0.6 ml buffer + 4 ml albumin solution + 0.3 ml standard fat emulsion + 0.2 ml calcium acetate + 2,000 units clearing factor.

(5) Control consists of 0.8 ml buffer + 0.4 ml albumin solution + 0.3 ml standard fat emulsion and no enzyme.

The results of these determinations are reported in Tables XIV and XV.

TABLE XIV.

Lipolytic Activity of Clearing Factor in the Presence of Different Substrates

Exp. No.*	Substrate	Amount of Triglyceride	Microequivalents of Titratable Acidity After Incubation with Substrate						Δ Acid After 60 min.
			0 min.	5 min.	10 min.	20 min.	40 min.	60 min.	
1.	SFE**	1.5 mg.	0.883	0.986	1.090	1.233	1.321	1.435	0.552
2.	Triacetin	1.5 mg.	0.880	0.878	0.884	0.880	0.884	0.888	-
3.	Chylomicron	To give optical density 65	0.885	1.019	1.161	1.385	1.547	1.687	0.653
4.	-lipoproteins	te give optical density 65	1.022	-	1.082	1.142	-	1.195	0.173
5.	SFE Control No Enzyme	-	0.880	0.880	0.880	0.880	0.884	0.884	-
6.	Control -lipoprotein No Enzyme	-	1.021	1.022	1.020	1.020	1.021	1.021	-
7.	Standard	-	0.840						

* Each experiment represents an average of two determinations.

** SFE = Standard fat emulsion.

TABLE XV.

Calcium Ion and Albumin Influence on the Lipolytic Action of Clearing Factor

* Exp. No.	Buffer ml	System Used: Albumin ml	Beta-lipoproteins ml	Ca^{++} ml	** SFE	Microequivalents of NEFA*** After Incub. 0 min.	30 min.	Change in Microequivalents of Titratable Acid
1.	1.2	-	-	-	0.3	0.870	0.875	-
2.	0.8	0.4	-	-	0.3	0.872	1.17	0.30
3.	1.0	-	-	0.2	0.3	0.870	1.005	0.135
4.	0.6	0.4	0.3	0.2	-	0.895	1.185	0.290
5.	0.8 No enzyme	0.4	-	-	0.3	0.868	0.870	-
6.	Standard					0.800		

* Each experiment represents an average of two determinations.

** SFE = Standard fat emulsion

*** NEFA = Non-esterified fatty acids.

G. THE RATE AT WHICH TRIGLYCERIDES ARE HYDROLYZED IN THE PRESENCE OF CLEARING FACTOR

To 38.4 ml of preheparin, normal plasma containing 70,000 units of dissolved clearing factor, 9.6 ml of standard fat emulsion are added and the mixture is incubated at 37° C. in a water bath. At 0 time and after 5, 10, 15, 30 minutes, 1, 2, 3, 4, 5, and 6 hours of incubation, 1.0 ml samples of the mixture are taken and the concentration of non-esterified fatty acids and glycerol are determined. In both glycerol and non-esterified fatty acid determinations, duplicate samples of 1.0 ml are taken. The decrease in optical density of the mixture during the above time intervals is also measured. Along with samples containing clearing factor, a control consisting of preheparin plasma and standard fat emulsion are incubated and the concentration of glycerol, non-esterified fatty acids, as well as optical density, is determined at the time intervals given above.

The results of these determinations are reported in Table XVI. and graphically presented in Figs. 7 and 8.

TABLE XVI.

The Rate of Triglyceride Hydrolysis in the Presence of Clearing Factor

Incubation Time min.	NEFA *		Glycerol		Optical Density	
	μM Acid	Increase μM	μM Glycerol	Increase μM	O.D.	Change O.D.
0	1.23	-	1.52	-	0.65	-
5	1.44	0.21	1.52	-	0.52	0.13
10	1.61	0.38	1.54	-	0.42	0.23
15	1.69	0.46	1.53	-	0.37	0.28
30	1.85	0.62	1.65	0.13	0.30	0.35
60	2.06	0.83	1.84	0.32	0.27	0.38
90	2.18	0.95	1.95	0.43	0.26	0.39
120	2.28	1.05	2.02	0.50	0.26	0.39
180	2.35	1.12	2.23	0.71	0.26	0.39
240	2.39	1.16	2.37	0.85	0.30	0.35
300	2.44	1.21	2.47	0.95	0.33	0.32
360	2.46	1.23	2.48	0.96	0.35	0.30
Control/ Plasma without Enzyme						
0	1.22	-	1.54	-	0.66	-
360	1.28	0.06	1.69	0.15	0.64	0.02

The figures given represent an average of two determinations.

* NEFA = Non-esterified fatty acids.

FIGURE 7.

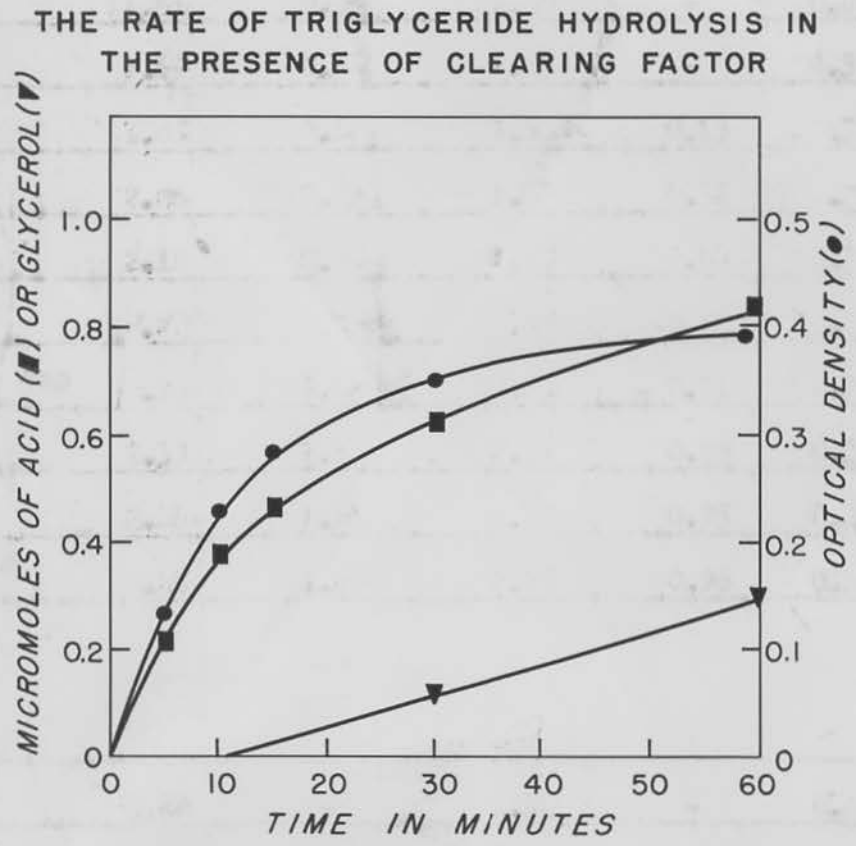
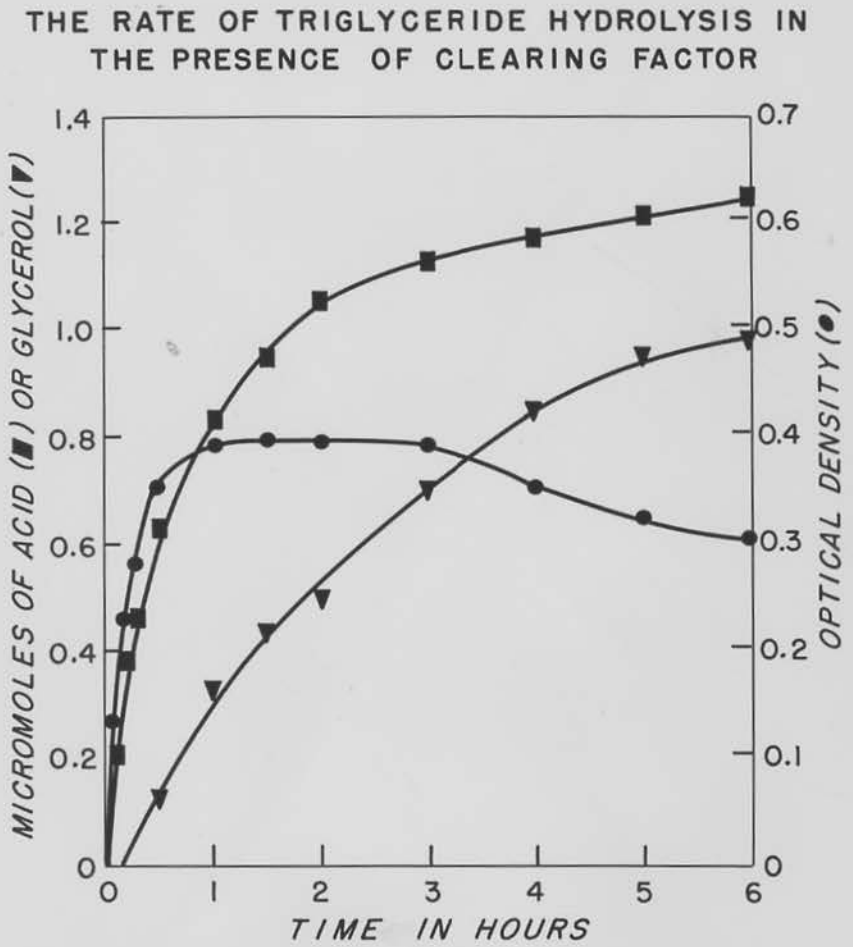

THE RATE OF TRIGLYCERIDE HYDROLYSIS IN THE PRESENCE OF CLEARING FACTOR
MICROMOLES OF ACID (■) OR GLYCEROL (▼)
OPTICAL DENSITY (●)
1.0
0.8
0.6
0.4
0.2
0
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
TIME IN MINUTES

FIGURE 8.


THE RATE OF TRIGLYCERIDE HYDROLYSIS IN THE PRESENCE OF CLEARING FACTOR
MICROMOLES OF ACID (■) OR GLYCEROL (▼)
OPTICAL DENSITY (●)
TIME IN HOURS
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.1
0.3
0.5
0.7
1
2
3
4
5
6

IV. COMPARISON OF THE LIPOLYTIC ACTION OF PANCREATIC LIPASE AND CLEARING FACTOR

The lipolytic action of pancreatic lipase and clearing factor on triglycerides are compared in respect with:

A. SUBSTRATE SPECIFICITY

To the duplicate samples containing 0.1 mg. pancreatic lipase[5] dissolved in 0.8 ml of veronal buffer of PH 8.6, 0.4 ml of 10 per cent albumin solution and 0.3 ml of standard fat emulsion are added and the mixture is incubated at 37° C. for 30 minutes in a water bath. The above experiment is repeated three times, but instead of standard fat emulsion, 0.3 ml of 0.8 per cent triacetin, chylomicra or beta lipoproteins are used respectively. After 30 minutes of incubation and at 0 time, 1.0 ml portion from each sample is taken and the concentration of non-esterified fatty acids is determined. (The preparation of the substrates used in this experiment is described on pages 63 to 65).

The above experiment is repeated and this time instead of pancreatic lipase 3,000 units of clearing factor in each sample is used. (0.1 mg of pancreatic lipase on incubation with standard fat emulsion present in preheparin, normal plasma gives approximately the same decrease in optical density as 3,000 units of clearing factor.)

The results of the above determinations are reported in Table XVII.

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5. Commercial grade, obtained from the Wilson Laboratories, Division of Wilson and Company, Inc., Chicago, Illinois.

TABLE XVII

Comparison of Lipolytic Action of Clearing Factor and Pancreatic Lipase on Various Substrates

Exp. No.*	Substrate	PANCREATIC LIPASE			CLEARING FACTOR		
		Microequivalents of Titratable Acidity		Change in Microequivalents of Titratable Acidity	Microequivalents of Titratable Acidity		Change in Microequivalents of Titratable Acidity
		0 min.	30 min.		0 min.	30 min.	
1	Standard fat emulsion	1.60	2.305	0.705	0.883	1.277	0.394
2	Triacetin	1.63	1.635	0.005	0.880	0.882	0.002
3	Chylomicrons	1.612	2.08	0.468	0.885	1.466	0.581
4	Beta-lipo-proteins	1.83	1.98	0.15	1.022	1.168	0.146

* Each experiment represents an average of two determinations.

B. EFFECT OF PROTAMINE ON THE ACTION OF PANCREATIC LIPASE AND CLEARING FACTOR

The following systems are prepared in duplicate and incubated in a water bath at 37° C. for 30 minutes.

(1) 1.2 ml veronal buffer containing 0.1 mg./ml of pancreatic lipase + 0.3 ml standard fat emulsion. The pH of the mixture is 8.6.

(2) Same as above only 0.1 ml of normal plasma added.

(3) Same as system (1) only 0.1 ml plasma and 40 mg. of albumin are added.

(4) 1.2 ml normal plasma containing 0.1 mg./ml of pancreatic lipase + 0.3 ml standard fat emulsion.

(5) Same as system (4) only 4.5 mg. protamine are added.

(6) 1.2 ml normal plasma containing 3,000 units clearing factor + 0.3 ml standard fat emulsion.

(7) Same as system (6) only 4.5 mg. protamine are added.

(8) 1.2 ml buffer containing 3,000 units clearing factor + 0.3 ml standard fat emulsion.

(9) 1.2 ml normal plasma (no enzyme) + 0.3 ml standard fat emulsion.

At 0 minutes and after 30 minutes of incubation 1.0 ml aliquot from each sample is taken and optical density and non-esterified fatty acid concentrations are determined. The results of this determination are reported in Table XVIII.

TABLE XVIII.

The Effect of Protamine and Serum Proteins on the Lipolytic Action of Pancreatic Lipase and Clearing Factor

Exp. No.*	Lipolytic Agent	System	Additions	Time of Incubation 0 min. OD**	0 min. Acid µM	30 min. OD	30 min. Acid µM	△ Microequivalents Acid
1.	Pancreatic Lipase	Buffer + SFE*** pH 8.6	-	.80	0.83	.76	.85	-
2.	Pancreatic Lipase	Buffer + SFE pH 8.6	0.1 ml Plasma	.62	0.911	.22	1.88	0.869
3.	Pancreatic Lipase	Buffer + SFE pH 8.6	0.1 ml Plasma 40 mg Albumin	.71	0.916	.7	2.164	1.248
4.	Pancreatic Lipase	Plasma + SFE	-	.65	1.60	.19	2.305	0.705
5.	Pancreatic Lipase	Plasma + SFE	Protamine 4.5 mg	.65	1.61	.38	2.23	0.620
6.	Clearing Factor	Buffer + SFE pH 8.1	-	.68	.868	.67	0.870	-
7.	Clearing Factor	Plasma + SFE	-	.68	1.65	.36	2.215	0.565
8.	Clearing Factor	Plasma + SFE	Protamine 4.5 mg	.65	1.63	.52	1.81	0.180
9.	Control No enzyme	Plasma + SFE	-	.64	1.54	.63	1.57	-

* Each experiment represents an average of two determinations. **OD = Optical Density. ***SFE = Standard fat emulsion.

RESULTS AND DISCUSSION

Purification of Clearing Factor. - The results of attempted purification of heparin-induced clearing factor by low temperature ethanol fractionation of postheparin plasma are summarized in Table I. Considerable lipemia clearing activity is found in Fraction I + III - 1, 2, 3 of postheparin plasma. On further fractionation, however, the lipolytic activity is completely lost. It has not been established which steps are responsible for the disappearance of the lipemia clearing activity.

The clearing factor was more successfully concentrated by the ether extraction method developed in the course of this study. By this method, over 90 per cent of the original clearing activity is found in a semi-solid layer comprising approximately 2 per cent of the original plasma volume. From this layer, the clearing factor is then extracted into a glycine buffer (" crude clearing factor preparation"). It has not been possible to demonstrate with this method the presence of clearing factor in plasma or serum of normal untreated individuals.

The residue of the layer after extraction of the clearing factor is a white fibrous material. This material is insoluble in dilute acids or alkali. Since the addition of human fibrinolysin[6] resulted in partial solubilization of the residue, it is presumed that at least part of the layer (containing the clearing factor) consists of fibrin.

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6. Courtesy of Dr. Eugene C. Loomis, Parke, Davis and Co., Detroit, Mich.

The crude clearing factor preparation still contains many impurities, among them fibrinogen and prothrombin. When the crude clearing factor preparation is incubated with simplastin[7] clot formation is taking place.

The association of clearing factor with prothrombin and fibrinogen i is not limited to the ether extraction method. All methods used for the separation of clearing factor (i.e., low temperature ethanol fractionation (16), precipitation at pH 5.6 (95), adsorption on barium sulfate or calcium phosphate columns (96) result in preparations containing fibrinogen and prothrombin.

The results of attempted purification of the crude clearing factor preparation by precipitation with ammonium sulfate, by fractionation at various pH levels or by adsorption on calcium phosphate gel, are presented in Tables III, IV and V.

On precipitation of the crude clearing factor preparation with ammonium sulfate, the highest lipolytic activity, as measured by glycerol evolution, is found in the fractions obtained at 30 and 40 per cent saturation. Considerable activity, however, is scattered through the other fractions. After dialysis, the lipolytic activity of the fractions is lost even when the preparation is dissolved in preheparin plasma. Optical density measurements could not be evaluated since it has been noted that 0,1 M $(NH_4)_2SO_4$ solution alone causes optical clearing of a fat emulsion. This "spontaneous" clearing phenomenon is not accompanied by evolution

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* 7. Obtained from Warner-Chilcott Laboratories, Morris Plains, N.Y.

of glycerol and is due to decreasing the stability of the emulsion.

Fractionation of the crude clearing factor preparation by changes in hydrogen ion concentration produced only negligible purification. Clearing activity was scattered among the various fractions (pH 5.0; 5.5; 6.0; 6.5; 6.8) and contained approximately the same impurities as the starting material.

Adsorption on clearing factor preparation with calcium phosphate gel did not result in further purification. By adsorption methods, Nikkila (96) obtained up to 40-fold purification of clearing factor using postheparin plasma as starting material. But the crude clearing factor preparation used in this study, already, is at least as pure as the material which Nikkila obtained by his purification method. It is quantitatively adsorbed on calcium phosphate gel and also quantitatively eluted with sodium citrate. It therefore appears that the impurities which Nikkila removed by adsorption on calcium phosphate gel have already been removed by the early steps in our ether extraction method.

More than 50 per cent purification of the crude clearing factor preparation was achieved by repeated extractions with ether (Tables VI and VII). The final preparation of clearing factor ("purified clearing factor preparation") contained less than a one-hundred-and-thirtieth (1/130) part of the protein present in the starting material and retained more than 50 per cent of the total original lipolytic activity.

Physico-chemical Characteristics of Purified Clearing Factor Preparation. - Heating at 60° C. for 15 minutes or incubation with trypsin [8]

8. Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

for 6 hours at 37° C. completely destroys the lipolytic activity of the purified clearing factor preparation. These findings indicate that protein is an essential part of clearing factor. They are in agreement with the observation that clearing activity in intact postheparin plasma is similarly destroyed by heat and trypsin.

Stability tests of clearing factor show (Table VIII) that the purified clearing factor preparation, when kept in a dry form at 25° C. for 7 days, suffers no loss of activity. In veronal buffer solution, however, at 0° C. after 7 days, only 50 per cent of the original activity is retained. Addition of albumin to the clearing factor solution increases its stability only slightly.

Analtical ultracentrifugation and free-boundary electrophoresis of the purified clearing factor preparation show one major and two minor components (Figs. 1 and 2). Attempts to determine which of the ultracentrifugally separated components is responsible for lipolytic activity of clearing factor have not been successful.

Since the ultracentrifugal and electrophoretic studies indicate that the purified clearing factor preparation still contains considerable impurities, chemical analytical studies of this preparation have been limited to the determination of protein, lipids and polysaccharides. The purified clearing factor preparation consists mainly of protein and is free of lipids. On testing for the presence of glycoproteins by staining, negative results were also obtained. However, the staining method for the determination of glycoproteins employed here is not sensitive enough to disclose dilute concentrations of glycoprotein.

Kinetic Studies on Purified Clearing Factor. - In studying the effects of temperature on the initial rate of the reactions (Table IX) it was found that the optimal temperature for clearing factor activity is in the range between 37° and 40° C. On substituting the values of clearing factor activity at various temperatures into the Arrhenius equation

$$\ln K = \frac{-E}{R}\left(\frac{1}{T}\right) + \text{constant}$$

and plotting it against x and y coordinates, a straight line is obtained. From the slope of the line (Fig. 3) the activation energy E for clearing factor is calculated and found to be approximately 10,000 cal.

The study of the reaction velocity of clearing factor over pH ranges from 5.0 - 9.0 is summarized in Table X and is graphically presented in Fig. 4. The hydrogen ion concentration for optimal clearing factor activity is found to be in the range of pH 7.9 - 8.1.

The velocity of the clearing reaction is found to increase as the concentration of clearing factor increases (Table XI and Fig. 5). A linear relation between the rate of reaction and the concentration of enzyme is present at lower concentrations of clearing factor.

Increase in the concentration of substrate is accompanied by increase in the velocity of the reaction catalyzed by clearing factor when other factors are kept constant and the concentration of the substrate is low (Tables XII and XIII and Fig. 6). The method of Lineweaver and Burk (83) has been used to determine Michaelis-Menten constant

of clearing factor. On substituting the values of clearing factor activity found at different substrate concentration levels into the Michaelis-Menten equation

$$\frac{1}{v} = \frac{Km}{V}\left(\frac{1}{S}\right) + \frac{1}{V}$$

and plotting it against x and y coordinates a straight line is obtained. From the slope of the line the Michaelis constant Km is calculated and is found to be 0.21 M.

Study of the rate of triglyceride hydrolysis in the presence of clearing factor discloses that during the early stage of the reaction the production of non-esterified fatty acids is linear with the increase in the reaction time. Thus the initial stage of the clearing reaction is of zero order. The rate of the clearing reaction was determined by measuring the end products of triglyceride hydrolysis and change in optical density. Table XVI and Figs. 7 and 8 indicate that the non-esterified fatty acid concentration sharply rises during the first 60 minutes of the reaction and continues to rise slightly during the next five hours. The rate of change in optical density during the first 60 minutes runs nearly parallel to the rate of liberation of non-esterified fatty acids. On the other hand, the decrease in optical density stops after one hour and after 3 hours the optical density begins to increase again. In contrast to the initial increase in non-esterified fatty acids, the concentration of glycerol does not begin to rise until 30 minutes after initiation of lipolysis. The concentration of glycerol increases gradually and is found to be highest after 5 hours of incubation.

It is suggested (14) that the delayed increase in glycerol concentration during the clearing reaction is due to the selective action of clearing factor on the alpha and beta positions of the triglyceride ester bonds. Such selective action of clearing factor would produce first non-esterified fatty acids and mono and di-glycerides before free glycerol is released. Glycerol estimation is based on the formaldehyde formed by the oxidation of either glycerol or monoglyceride with periodate. There is no evidence that the diglycerides take part in this reaction. Therefore, the glycerol determination would reflect only more advanced stages of lipolysis in the reactions catalyzed by clearing factor.

It was found that clearing factor hydrolyzes the standard fat emulsion and human chylomicra at about the same rate, while it did not degrade triacetin. The fact that triacetin is not hydrolyzed by clearing factor eliminates the possibility that clearing factor is similar to or identical with ali-esterases of blood.

Incubation of purified clearing factor preparation with beta lipoproteins (density 1.04 g/cc) also results in an increase in non-esterified fatty acids (Table XIV). Since this lipoprotein preparation usually contains some chylomicra, it was not established whether the released non-esterified fatty acids originated from the chylomicra, the beta lipoproteins or from both.

When the purified clearing factor preparation is dissolved in a buffer and is mixed with standard fat emulsion, no clearing takes place until at least a small amount of plasma or albumin is added to the mixture (Table XV). Addition of calcium ions partially replaces the requirement for protein. It therefore appears likely that the function

of the protein is to accept the fatty acids formed during the clearing reaction.

Korn (63, 65) reports that lipoprotein lipase hydrolyzes triglycerides only if they are in complex with protein. On the other hand, our observation indicates that the reaction catalyzed by clearing factor can proceed without added proteins provided calcium ions are present. This seems to imply that tissue lipoprotein lipase and heparin-induced plasma clearing factor are not identical.

Pancreatic lipase and clearing factor acted alike in respect to substrate specificity or protein requirement (Tables XVII and XVIII). However, the effects of many other factors (i.e., various inhibitors) on these two lipolytic agents have not been studied in this work. Therefore, it is not warranted to conclude that pancreatic lipase and clearing factor are identical. On the other hand, since both pancreatic lipase and the heparin-induced clearing factor require protein, a distinction of these two lipolytic agents on the basis of protein requirement, is not possible.

SUMMARY AND CONCLUSIONS

In order to study the lipolytic action and the kinetics of heparin-induced lipemia clearing factor, isolation and purification of this factor from human postheparin plasma has been undertaken. By a newly developed method consisting of repeated extractions of postheparin plasma with an ether-ethanol mixture, a 130-fold purification (based on protein content) of clearing factor has been obtained. The purified clearing factor preparation contains approximately 50 per cent of the total original activity. No clearing factor is found in the plasma or serum of normal untreated individuals when tested by the above method.

Analytical ultracentrifugation and free boundary electrophoresis of the purified clearing factor preparation shows one major and two minor components. Attempts to establish which of these ultracentrifugally separated component (-s) is responsible for clearing factor activity failed because of the instability of the purified clearing factor preparation in aqueous solutions.

Purified clearing factor preparation consists mainly of protein and is free of lipids. Heating of the purified clearing factor preparation for 15 minutes at 60° C. or incubation with trypsin for 6 hours at 37° C. completely destroys its activity. On the basis of these findings and from the results of kinetic studies, it is concluded that clearing factor is an enzyme catalyzing the hydrolysis of triglycerides.

Activity of clearing factor is optimal at temperatures between 37° C. - 40° C. and at hydrogen ion concentrations between pH 7.9 and 8.1. The activation temperature of clearing factor is approximately 10,000 cal.

and the Michaelis constant Km is 0.21 M. Clearing factor catalyzes the hydrolysis of triglycerides present in coconut oil emulsion or in human chylomicra at about the same rate. It does not hydrolyze triacetin. On this basis the possibility can be excluded that clearing factor is identical with alli-esterases of blood.

The initial stage of the reaction catalyzed by clearing factor proceeds at a rate of zero order. During the first 30 minutes of the reaction, the production of non-esterified fatty acids is about six times greater than the production of glycerol. This agrees with the reports that clearing factor acts selectively on the alpha and beta bonds of triglycerides.

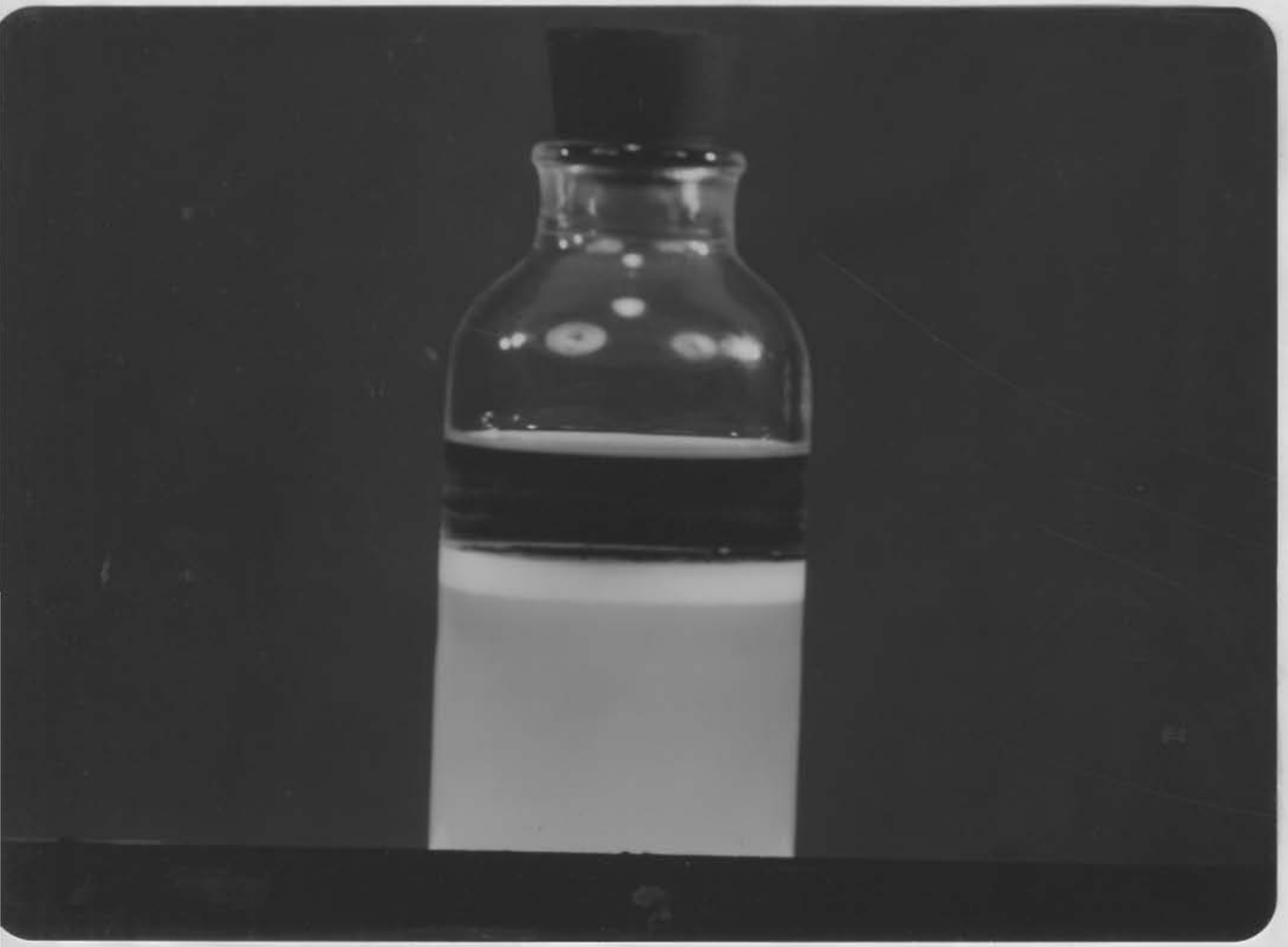
In a system containing coconut oil emulsion at least small amounts of protein are required for the lipolytic action of clearing factor to take place. Since addition of calcium ions to the system replaced the requirement of protein, it seems that the function of the added protein is acceptance of fatty acids formed during the clearing reaction.

Clearing factor and pancreatic lipase act alike in respect to substrate specificity and protein requirement. Since the effects of the other factors on clearing factor and pancreatic lipase have not been studied, no conclusion can be drawn whether or not these two lipolytic agents are identical.

These studies indicate that the heparin-induced clearing factor in plasma is closely related to the lipases present in other biological fluids and tissues. However, a full investigation of the relationships of the lipases requires purified preparations of such lipases from various sources. The purification of these lipases is beyond the scope of this study.

ILLUSTRATION

Semi-solid Layer Between Aqueous and Ether Phases after Centrifugation of Plasma-Ether Mixture.



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ABSTRACT

After the intravenous administration of heparin to animals or human subjects, an enzyme, the lipemia clearing factor, appears in the blood. As a result of the action of this heparin-induced clearing factor, neutral fat present in lipemic plasma is hydrolyzed into fatty acids and glycerol, while at the same time the turbidity of the lipemic plasma is decreased.

In order to study the kinetics of this lipolytic action of clearing factor, the separation and purification of the clearing factor from postheparin human plasma has been carried out by a method developed in this study in the following manner: Postheparin human plasma* is mixed in equal parts with a solution consisting of 2 parts ethanol and 1 part ether at -5° C. Upon incubation of this mixture at -5° C., numerous particles of low specific density appear in the mixture. On centrifugation, these particles form an interphase of semi-solid consistency between the aqueous and the ether phase.

Ninety per cent of the total activity originally present in the postheparin plasma are recovered when the semi-solid layer is suspended in plasma of normal non-heparinized subjects. Suspension of this semi-solid layer in veronal buffer of pH 8.1 and repeated extractions with ethyl ether result in further purification of the clearing factor preparation. The final purified preparation of clearing factor represents a 130-fold purification (based on protein content) and contains approximately 50 per cent

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* Postheparin plasma is plasma collected from normal fasting individuals 30 minutes after injection of 2 mg/kg body weight of heparin.

of the total original activity. Attempts to demonstrate the presence of clearing activity in the plasma or serum of normal fasting individuals by this extraction method have not been successful.

The lipolytic activity of clearing factor is measured in a system consisting of standard fat emulsion* and the purified clearing factor preparation which is dissolved either in preheparin plasma or in 3.3 per cent albumin solution. The measurements consist in determinations of the decrease in optical density and the increase in concentration of nonesterified fatty acids or glycerol.

Ultracentrifugal and electrophoretic analyses of the purified clearing factor preparation show one major and two minor components. Attempts to determine which of the three components obtained by ultracentrifugal separation might be responsible for the lipolytic action of clearing factor have not been successful because of the instability of the purified clearing factor preparation in aqueous solutions.

On chemical analysis the purified clearing factor preparation consists mainly of proteins and is free of lipids. Glycoprotein has not been demonstrated in this preparation by the staining method.

The purified clearing factor preparation exerts its optimal activity at temperatures between 37° and 40° C. and at hydrogen ion concentrations

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* Standard fat emulsion was generously supplied by Dr. E.A.Hawk, Medical Division, Dept. of Clinical Investigation, The Upjohn Company, Kalamazoo, Michigan. It consisted of: 15% coconut oil dispersed as one micron particles in water containing 0.5% Pluronic (a nonionic detergent) and 1.0% polyglycerololeate.

between pH 7.9 and 8.1. Applying the Arrhenius equation

$$\ln K = \frac{-E}{R}\left(\frac{1}{T}\right) + \text{constant}$$

to the clearing factor activity values obtained at various temperatures, the activation temperature of clearing factor is calculated to be 10,000 cal.

A linear relationship exists between concentration of clearing factor and its lipolytic activity in the range of dilute concentrations (from 0.5 to 1.0 mg. of protein). In a range of dilute concentration of substrate the velocity of the reaction catalyzed by clearing factor increases when the concentration of triglycerides increases. Applying the Michaelis equation

$$\frac{1}{v} = \frac{Km}{V}\left(\frac{1}{S}\right) + \frac{1}{V}$$

to the clearing factor activity values obtained at different substrate levels, the Michaelis constant (Km) of clearing factor is calculated to be 0.21 M.

Clearing factor hydrolyzes the triglycerides present in a coconut oil emulsion and in human chylomicra at about the same rate, while it does not hydrolyze triacetin. In the case of coconut oil emulsion, no hydrolysis takes place until a small amount of protein is added to the system. Since the protein can be replaced by the addition of calcium ions, it appears that the function of the protein is to accept the free fatty acids formed during the clearing reaction.

The rate of the reaction catalyzed by clearing factor has been studied by determining the evolution of nonesterified fatty acids and glycerol and by measuring changes in optical density. During the first 10 minutes of the reaction, the rate of increase in the concentration of nonesterified fatty acids as well as the decrease in optical density follow the curve of zero order reaction. No increase in concentration of glycerol is found until the reaction has proceeded for about 30 minutes.

This delay in the evolution of glycerol is in agreement with the reports that clearing factor primarily hydrolyzes triglyceride bonds in alpha and beta positions. The fact that the method employed for the determination of glycerol does not detect the diglycerides formed first during the reaction, but only monoglycerides and glycerol, explains the delayed evolution of glycerol.

Pancreatic lipase and clearing factor act alike in respect to substrate specificity and requirements for protein. Since no other comparisons have been carried out, no conclusion can be drawn as to the actual identity of these two lipolytic agents.

In summary, the analytical and kinetic studies on the purified clearing factor preparation show that heparin-induced clearing factor is a lipolytic enzyme which catalyzes the hydrolysis of triglycerides. Further investigations are required to determine the exact relationship between clearing factor and the lipases present in other biological fluids and tissues.

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PUBLICATIONS

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