CHANGES IN BLOOD COAGULATION DURING THE CLEARING OF LIPID EMULSIONS BY LIPOPROTEIN LIPASE*

By ROBERT L. HIRSCH,‡ M.D., AARON KELLNER, M.D., AND ROSEMARY IRELAND

(*From the Department of Pathology, The New York Hospital–Cornell Medical Center, New York*)

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The enzymatic hydrolysis of triglycerides by lipoprotein lipase has been implicated in the normal metabolism of lipids in the rat, the dog, and man (1–3). The studies done thus far have dealt for the most part with details of the lipolytic process; few have explored the possibility that other phenomena may occur simultaneously. During the course of experiments dealing with lipoprotein lipase in which fresh postheparin plasma was used as the source of the enzyme, the plasma was often observed to form a solid clot despite the presence of large amounts of heparin. Investigation of this phenomenon, to be reported herein, revealed that during the clearing of lipid emulsions both *in vivo* and *in vitro* there occurred at the same time a progressive and striking reduction in the anticoagulant effect of heparin.

Materials and Methods

The clearing of lipid emulsions was carried out with freshly prepared plasma from rabbits or human beings given an intravenous injection of heparin. This postheparin plasma (PHP),[1] which has a markedly prolonged clotting time, contains clearing factor, a substance shown by Korn to be an enzyme that hydrolyzes selectively the triglycerides of lipoproteins, and hence referred to by him as lipoprotein lipase (4). When chylomicra in the form of lipoproteins or a vegetable oil emulsion are added *in vitro* to plasma containing the active enzyme, there is a progressive splitting off of the fatty acids of triglycerides, the liberated fatty acids becoming attached to the plasma albumin. This lipolysis can be measured directly by the liberation of glycerol and fatty acids, or indirectly by reduction in the optical density of the mixture.

Preparation of Clearing Factor.—Clearing factor was prepared from normal adult rabbits following the intravenous injection of heparin,[2] 7.5 mg. per kg. Fifteen minutes after the injection of heparin, the animals were bled into 3.8 per cent sodium citrate in the proportion

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‡ Present address: Columbia Research Division, Goldwater Memorial Hospital, New York.

[1] PHP, postheparin plasma.

[2] The authors are indebted to Dr. George H. Berryman of the Abbott Co., North Chicago, for a generous supply of heparin.

of nine parts of blood to one part of citrate solution. The blood was centrifuged immediately at 3000 R.P.M. for 10 minutes in a refrigerated centrifuge at 4°C. to remove the cellular elements and most of the platelets. This platelet-poor plasma was kept in an ice bath until used, usually within 2 hours after collection. In several experiments, human beings given heparin in a dose of 1 mg. per kg. were used as the source of clearing factor.

Lipid Emulsions.—Three sources of lipid were utilized for studies of the clearing reaction: (*a*) ediol,[3] a coconut oil emulsion; (*b*) lipomul,[4] a cottonseed oil emulsion; and (*c*) washed chylomicra from 500 cc. of lipemic blood obtained from a normal human being following a fat-rich meal. The stock solutions were diluted in physiological saline to yield the required concentration of lipids. Most studies were done with ediol diluted 1–100.

Clearing Reaction.—Clearing of the lipid emulsions *in vitro* was carried out using a mixture containing 4 ml. of postheparin plasma, 1 ml. of appropriately diluted lipid emulsion, and 5 ml. of 0.2 M imidazole buffer, pH 6.7. The pH of the mixture was 6.9 to 7.0. Control mixtures, containing 4 ml. of postheparin plasma and 6 ml. of buffer, but no added lipid, were included in each experiment. The mixtures were incubated in a water bath at 37°C. and optical density was measured at the outset and after 15, 30, and 60 minutes of incubation, in a Coleman, Jr. spectrophotometer at a wave length of 700 λ.

Recalcification Time.—Recalcification time was determined by a modification of the method of Biggs and Macfarlane (5). 0.25 ml. of 0.05 M $CaCl_2$ solution was blown into a 1.0 ml. aliquot of either the clearing or the control mixture and a stop-watch was started simultaneously. The tubes were kept in a water bath at 37°C. and were examined by tilting them every 15 to 30 seconds. The recalcification time was considered to be the time elapsed from the introduction of the $CaCl_2$ solution to the appearance of the first visible strands of fibrin.

Relatively large doses of heparin were administered to the rabbits to insure the production of adequate amounts of lipoprotein lipase. This resulted in considerable excess of heparin in the postheparin plasma, and test mixtures containing 4 parts of plasma to 6 parts of buffer solution often failed to clot upon recalcification. Without a finite clotting time it was impossible by this means to measure any changes in the clotting mechanism. Further dilution of the plasma as a means of reducing the effectiveness of the heparin was deemed inadvisable since it would result in reduction in the concentration of lipoprotein lipase and blood clotting factors below optimal levels. Wadsworth *et al.* have shown that the anticoagulant effects of heparin can be reduced progressively by lowering the pH of the test sample (6). Accordingly, the pH of all plasmas and plasma-lipid mixtures in these experiments was adjusted to a range of 6.8–7.0 by the use of the appropriate buffer system. This brought the baseline recalcification time of the control mixtures to a range of 25 to 35 minutes, and permitted accurate assessment of any reduction in the clotting time during the experiments. The pH range 6.8–7.0 is still within the optimal range for blood coagulation and lipolysis of lipoproteins. Numerous measurements of pH were made during the incubation period and it was found that the pH did not vary significantly during clearing. Thus it is unlikely that any change in blood coagulation was due to changes in pH.

Determination of Non-Esterified Fatty Acids.—Non-esterified fatty acids were determined by the Albrink modification (7) of the method of Dole (8).

EXPERIMENTAL

Reduction of the Prolonged Recalcification Time of Postheparin Plasma during the Clearing of Lipid Emulsions in Vitro

An experiment was first done in which rabbit postheparin plasma was incubated *in vitro* with a lipid emulsion, and the recalcification time of the mix-

[3] Ediol, manufactured by SchenLabs Pharmaceuticals, Inc., New York.

[4] Lipomul, manufactured by the Upjohn Co., Kalamazoo.

ture was measured at intervals during clearing, in order to determine what effect, if any, clearing had on the prolonged clotting time of the heparin-containing plasma.

Plasma was obtained from rabbits 15 minutes after an intravenous injection of heparin. In a typical experiment 4 ml. of plasma were mixed with 6 ml. of imidazole buffer and the recalcification time of the mixture was determined as a baseline level for the experiments to be done with the particular postheparin plasma. In a separate test tube 4 ml. of postheparin plasma were mixed with 1 ml. of ediol 1:100 and 5.0 ml. of imidazole buffer. Both the clearing and the control mixture were placed in a water bath at 37°C. The optical density of the clearing mixture was measured at the start and at regular intervals during an hour long period of incubation. At intervals, as clearing of the mixture progressed, 1 ml. aliquots of both the clearing and the control mixtures were removed and the recalcification times determined. Fig. 1

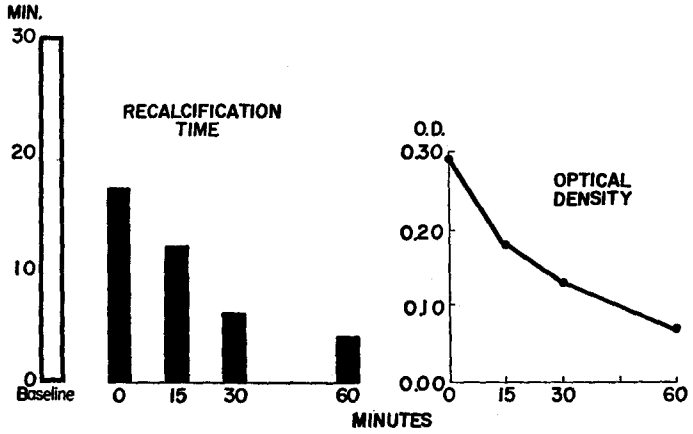

FIG. 1. Reduction of the prolonged recalcification time of rabbit postheparin plasma during the clearing of lipid emulsions *in vitro*.

summarizes one such experiment. The baseline recalcification time of the control mixture was 30 minutes and this did not change during the hour long period of incubation. The optical density of the clearing mixture was 0.300 at the start of the incubation period, 0.125 at 30 minutes, and 0.025 at 1 hour. The recalcification time of the aliquot removed at the very start of the experiment was 10 minutes, compared to the baseline time of 30 minutes. This reduction was due to the fact that considerable clearing went on during the period of time necessary for the recalcified mixture to clot. It was not due to the addition of lipid *per se*, as will be documented in a subsequent section of this paper. The recalcification times of aliquots removed as clearing progressed were progressively shorter; the sample tested after 1 hour clotted in 4 minutes.

The rate and extent of reduction in the recalcification time of the clearing mixture varied to some extent with the individual source of postheparin plasma. However, control recalcification times in the range of 25 to 35 minutes were consistently reduced by at least two-thirds after 1 hour of clearing, and usually the final recalcification time was in the range of 2 to 5 minutes. The recalcification times of five samples of normal rabbit plasma, mixed with ediol and imidazole buffer in a comparable fashion, ranged from 1 to 2 minutes; and these recalcification times were unaffected when the mixtures were incubated at 37°C. for 1 hour.

Studies with Postheparin Plasma and with Chylomicra from Human Beings.—Human post-

heparin plasma was substituted for rabbit postheparin plasma, and chylomicra from a human being in place of ediol, in a series of experiments designed to learn whether the effect on blood coagulation occurred when plasma from a species other than rabbit, or lipids from other sources, were utilized in the clearing reaction. Seven male and two female adult human beings were given heparin, 1 mg. per kg., and the postheparin plasma was prepared as previously described. The plasma samples were mixed with coconut oil emulsion and buffer as in the experiments with rabbit postheparin plasma. In addition, postheparin plasma from a rabbit and from one of the human beings were each mixed with washed chylomicra prepared from postprandial hyperlipemic human plasma. These were incubated in a water bath at 37°C. The turbidity of all mixtures decreased promptly and the recalcification times shortened pro-

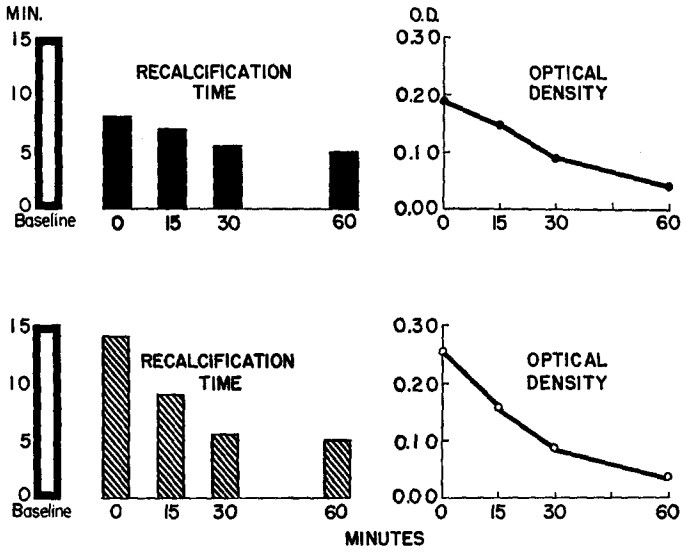

FIG. 2. Reduction of prolonged recalcification time of human postheparin plasma during clearing *in vitro*.

Substrate: Top, human chylomicra. Bottom, coconut oil emulsion.

gressively in all cases. Fig. 2 illustrates the results of one such experiment in which postheparin plasma from a human being was incubated with coconut oil emulsion, and with chylomicra from a human source. The baseline recalcification time of the human postheparin plasma before the addition of either lipid emulsion was 15 minutes. The optical density of the coconut oil–plasma mixture decreased from 0.260 to 0.040 in 1 hour, and there was a comparable reduction in the turbidity of the plasma-chylomicra mixture. The recalcification time of the mixture which contained coconut oil emulsion was 14 minutes at the start of the incubation period and fell to 5 minutes after clearing had progressed for 1 hour. In the mixture containing chylomicra of human origin, the recalcification time was 8 minutes at the outset and 5 minutes at the end of the period of incubation.

It was evident from these experiments that the recalcification time of postheparin plasma was reduced markedly during the clearing of lipid emulsions *in vitro*. Furthermore, lipoprotein lipase from at least two different species

was equally effective in this regard, and lipids from both vegetable and animal sources could be used as substrate for the reaction.

Studies Which Indicate That Clearing Is Essential for Reduction of the Recalcification Time of Postheparin Plasma

The reduction in recalcification time noted in these experiments appeared to be related directly to the process of clearing. To substantiate this, experiments were done in which postheparin plasma and lipids were incubated in a system where clearing was completely inhibited. Two such systems were studied; in one, lipoprotein lipase was inactivated by heat (9), and in the other it was inactivated by the presence of the surface active agent triton WR1339 (10).

Heat Inactivation of Lipoprotein Lipase.—Samples of normal plasma and postheparin plasma were obtained by bleeding a rabbit before, and 15 minutes after, the intravenous injection of heparin, 7.5 mg. per kg. Aliquots of these plasmas were incubated in a water bath at 49°C. for 60 minutes, conditions which have been shown previously to inactivate lipoprotein lipase (9). The heated plasmas, and in each case their unheated counterparts, were mixed with ediol and buffer in the proportions of 4.0 ml. plasma, 5.0 ml. imidazole buffer, and 1.0 ml. ediol 1–100. The pH of the mixtures was 6.9. These mixtures were placed in a water bath at 37°C. and aliquots were recalcified after 0, 15, 30, and 60 minutes of incubation. The recalcification time of normal plasma was unchanged despite the prior heat treatment, demonstrating that the temperature and duration of time chosen to inactivate the lipoprotein lipase produced no measurable effect on the blood coagulation mechanism. This treatment, however, did inactivate the clearing enzyme completely. The heated PHP failed to clear the lipid emulsion and there was no change in the prolonged recalcification time, as can be seen from the data contained in Table I. The untreated PHP, on the other hand, produced rapid clearing and a progressive fall in the recalcification time from a baseline value of 30 minutes to 7 minutes at the end of 60 minutes of incubation.

Inhibition of Clearing with Triton WR1339.—The clearing of lipid emulsions can be prevented by triton WR1339, as has been shown by Schotz (10). The mechanism of this inhibition is not known, but it appears to be due to alteration of the lipid substrate and not to inactivation of the enzyme, for Brown has shown that triton combines irreversibly with lipids and the resulting combination cannot be cleared by active lipoprotein lipase (11). A number of experiments were done in which citrated rabbit postheparin plasma was mixed with buffer and with ediol containing triton WR1339,[5] 5 mg. of the surface active agent per ml. of ediol 1–100. In a typical experiment, the optical density of the mixture at the outset was 0.300 and the recalcification time was 30 minutes. When incubated in a water bath at 37°C. for 60 minutes, there was no reduction in either the optical density or the recalcification time of the mixture. In control studies, comparable amounts of triton did not alter appreciably the recalcification time of normal rabbit plasma.

These experiments demonstrated that recalcification times were not shortened when the clearing reaction was prevented by inactivation of the enzyme, or when the reaction was blocked by modifying the lipid substrate with triton WR1339. It appeared, therefore, that the reduction in the recalcification time

[5] Triton WR1339 manufactured by Winthrop Laboratories, New York.

was dependent upon the process of clearing itself and not upon the presence of the lipid substrate. The possibility that one or more of the end products of the clearing reaction were responsible for the changes in blood coagulation was investigated next.

TABLE I

Effect of Inactivation of Lipoprotein Lipase by Heat on the Clearing of Lipids and the Recalcification Time of Rabbit Postheparin Plasma

Time of incubation	PHP-heated*		PHP-unheated	
	Optical density	Recalcification‡ time	Optical density	Recalcification‡ time
min.		*min.*		*min.*
Baseline§	0.010	29	0.010	30
0‖	0.295	33	0.280	19
15	0.280	32	0.141	15
30	0.282	33	0.101	10
60	0.289	39	0.052	7

* PHP heated in a water bath at 49°C. for 60 minutes before use.

‡ Recalcification time determined by adding 0.25 ml. $CaCl_2$ solution to 1 ml. of each test mixture.

§ Baseline mixture contained 0.4 ml. PHP (either heated or unheated), and 0.6 ml. imidazole buffer but no ediol; pH 6.9.

‖ Clearing mixture contained 4 ml. PHP, 1 ml. ediol 1–100, and 5 ml. imidazole buffer; pH 6.9.

Studies Which Indicate That the Products of Clearing Do Not Affect the Recalcification Time of Postheparin Plasma

Lipoprotein lipase catalyses the release of glycerol and fatty acids from the neutral fats of lipoproteins (4). Conceivably, the glycerol or one or more of the various fatty acids evolved during lipolysis could be responsible for the changes in the clotting time of postheparin plasma. To test this possibility, glycerol and two common fatty acids were added individually in purified form to postheparin plasma, and the recalcification time of the resultant mixtures was compared to baseline controls. In a second experiment, coconut oil was hydrolyzed by chemical means, yielding a mixture of glycerol and fatty acids which closely simulated the results of the enzymatic hydrolysis; this hydrolysate was then tested for its effect on the clotting of postheparin plasma.

Glycerol.—Previous experience in this laboratory has shown that approximately 40 μM of glycerol are produced when 0.4 ml. postheparin plasma clears 0.1 ml. ediol 1–100 in 1 hour. Appropriate dilutions of U.S.P. grade glycerol were made in imidazole buffer (pH 6.7), such that each 0.1 ml. contained 0, 10, 50, and 100 μM glycerol. One-tenth ml. of each concentration was added to test tubes containing 0.4 ml. postheparin plasma and 0.5 ml. imidazole buffer, and the mixtures were incubated in a water bath at 37°C. As shown in Table II, re-

calcification times done at the start and after 30 and 60 minutes of incubation were all in the range of 17 to 21 minutes. The glycerol failed to shorten the recalcification time significantly regardless of the amount used or the length of time of incubation.

Purified Fatty Acids.—Solutions of oleic and linoleic acid were obtained by dissolving their sodium salts in a 3 per cent bovine serum albumin solution and adjusting the pH to 6.8 with imidazole buffer. Varying amounts of these solutions were added to 0.4 ml. portions of rabbit postheparin plasma and sufficient imidazole buffer was added to bring the volume to 1.0 ml. The final concentration of the fatty acids in the various mixtures tested was 0, 1, 5, and 10 μeq./ml., respectively. The enzymatic hydrolysis of ediol under the conditions utilized throughout these experiments regularly results in a concentration of 2 to 2.5 μeq./ml. of fatty acids at the end of 1 hour of incubation, and thus the amounts added in this experiment represent a range of one-half to four times the usual amount. The recalcification times of these mixtures, summarized in Table III, revealed that these quantities of fatty acids did not reduce the clotting time of postheparin plasma; in fact, the fatty acids, particularly the larger amounts appeared to prolong it.

TABLE II

Effect of Glycerol on the Recalcification Time of Rabbit Postheparin Plasma

Time of incubation	Recalcification time, *min.*			
	Glycerol, μM/ml.			
	0	10	50	100
min.				
0	19	21	21	21
30	17	17	17	18
60	18	18	18	18

Each test mixture contained 4 ml. PHP, 1 ml. glycerol solution, 5 ml. imidazole buffer, pH 6.9.

0.25 ml. of 0.05 M $CaCl_2$ solution added to 1 ml. of test mixture for determination of recalcification time.

Products of Chemical Hydrolysis of Ediol.—Ediol was hydrolyzed by boiling for 2 hours in 1 N NaOH. The cooled solution was brought to pH 7.0 with 1 N HCl and then diluted with sufficient distilled water to bring the saline concentration of the mixture to 0.85 per cent. Four ml. of fresh postheparin plasma were added to each of three test tubes. No lipid was added to the first tube, 1 ml. of fresh ediol 1–100 was added to the second tube, and 1 ml. of the ediol which had undergone hydrolysis was added to the third tube. Sufficient imidazole buffer was added to each tube to bring the volume to 10 ml. and the pH to 6.9. All three tubes were placed in a water bath at 37°C. for 1 hour. Table IV summarizes the free fatty acid (FFA) content and recalcification time of 1 ml. aliquots removed from each tube after 0, 30, and 60 minutes of incubation. FFA concentration of the mixture containing PHP and buffer but no ediol was 0.42 μeq./ml. at the start of the experiment, due to the FFA present in the rabbit plasma. This concentration rose slowly to 0.75 μeq./ml. during the hour of incubation, presumably due to a spontaneous release of fatty acids from plasma triglycerides (8). The baseline recalcification time of this mixture was 20 minutes, and it did not change significantly during the period of incubation. By contrast, clearing did take place in the second tube; the FFA content increased fourfold from 0.49 μeq./ml. to 2.01 μeq./ml. in 1 hour. The

recalcification time of this sample was reduced sharply from the baseline value of 20 minutes (established in the first tube) to 12 minutes as clearing commenced, and to 6 minutes when clearing was completed. The mixture to which the hydrolysate of ediol was added had a FFA

TABLE III

Failure of Oleic and Linoleic Acid to Reduce the Recalcification Time of Rabbit Postheparin Plasma

Time of Incubation	Recalcification Time, *min.*						
	Fatty Acids, *μeq./ml.*						
	None	Oleic acid			Linoleic acid		
		1	5	10	1	5	10
min.							
0	19	30	30	30	26	21	30
30	17	26	24	30	24	18	30
60	18	26	24	30	25	19	30

Each test mixture contained 4 ml. PHP, 3 ml. fatty acid in 3 per cent bovine serum albumin, 3 ml. imidazole buffer.

0.25 ml. of 0.05 M $CaCl_2$ solution added to 1 ml. of each test mixture at the end of each period of incubation.

TABLE IV

Comparison of the Effects of the Products of the Chemical and Enzymatic Hydrolysis of Ediol on the Recalcification Time of Postheparin Plasma

Time of incubation	PHP, no ediol		PHP + ediol		PHP + hydrolyzed ediol	
	FFA	Recal. time	FFA	Recal. time	FFA	Recal. time
min.	*μeq./ml.*	*min.*	*μeq./ml.*	*min.*	*μeq./ml.*	*min.*
0	0.42	20	0.49	12	2.74	19
30	0.60	20	1.56	6	2.76	17
60	0.75	19	2.01	6	2.79	20

Each test mixture contained 8 ml. PHP, 10 ml. of imidazole buffer, and 2 ml. of either ediol, hydrolyzed ediol, or additional buffer.

Aliquots were removed at the start and end of each incubation period for FFA determination. Recalcification time determined by addition of 0.25 ml. of 0.05 M $CaCl_2$ solution to 1 ml. of test mixture.

concentration of 2.74 μeq./ml. at the start of the experiment and despite this relatively high concentration of FFA, the recalcification time was 19 minutes. Incubation for 1 hour did not change the recalcification time or the FFA content significantly.

The results of these experiments appeared to rule out the lipid substrate or the products of its chemical hydrolysis as the factor responsible for the changes

in coagulation observed during clearing. They suggested strongly that the process of clearing itself was an essential feature of the phenomenon.

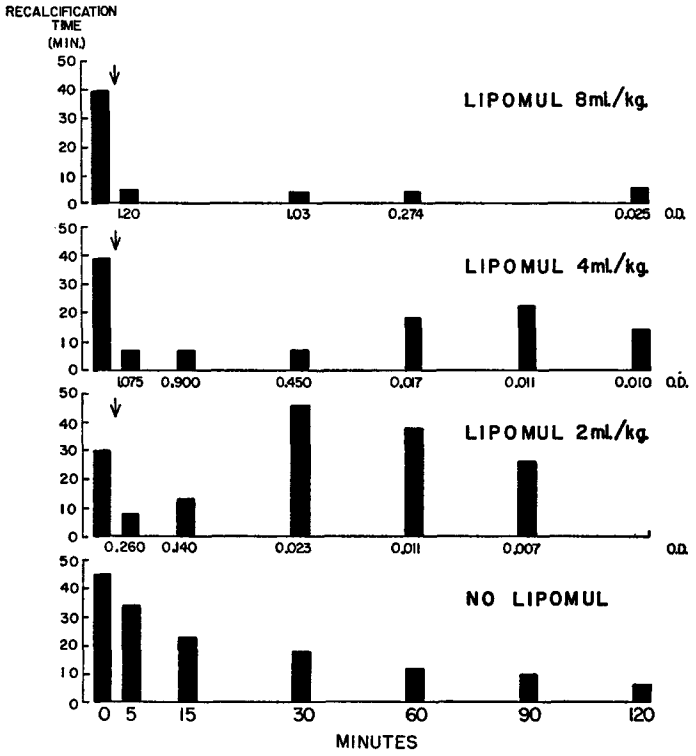

FIG. 3. Changes in the recalcification time of rabbit plasma after the intravenous administration of heparin and lipomul.

Each animal received heparin, 7.5 mg. per kg. at the start of the experiment.

Injection of lipomul indicated by the arrow.

Reduction of the Recalcification Time during the Clearing of Lipids in Vivo

The intravenous injection of heparin into hyperlipemic animals markedly accelerates the disappearance of the hyperlipemia, as was first noted by Hahn (2). It seemed of interest to study the recalcification time of plasma obtained from hyperlipemic animals during the course of accelerated clearing following heparin administration in order to ascertain whether the changes in blood coagulation already noted *in vitro* take place *in vivo* under these circumstances.

Ten rabbits were given an intravenous injection of heparin, 7.5 mg. per kg. A blood sample was obtained from each rabbit 15 minutes later, and then lipomul, a cottonseed oil emulsion, was administered intravenously to the animals. Two animals each received 2 and 4 ml. of lipomul per kg., respectively; four animals received 8 ml. per kg.; and two animals received

none. All animals were bled at intervals of 5, 15, 30, 60, 90, and 120 minutes thereafter. Each blood sample was immediately chilled to halt lipoprotein lipase activity, and the ensuing manipulations were carried out at 4°C. The plasma was separated and diluted with imidazole buffer, six parts of buffer to four parts of plasma, with a final pH of 6.9. The optical density of each of these mixtures was measured. An aliquot of each was then warmed to 37°C. in a water bath, recalcified, and the recalcification time measured. Sufficient lipomul was added to the first plasma sample (collected before the lipomul was injected) to produce an optical density similar to that of the sample obtained 5 minutes after the lipid was injected to control the effect of the lipid itself on the recalcification time. Two of the rabbits that received 8 ml. of lipomul per kg. were also given an intravenous injection of triton WR1339 in saline, 650 mg. per kg., immediately prior to the injection of lipomul. This surface active agent has been shown to prevent completely the clearing of lipids by lipoprotein lipase *in vitro* (10), and also to inhibit markedly the rate of removal of lipids from the circulating blood (12).

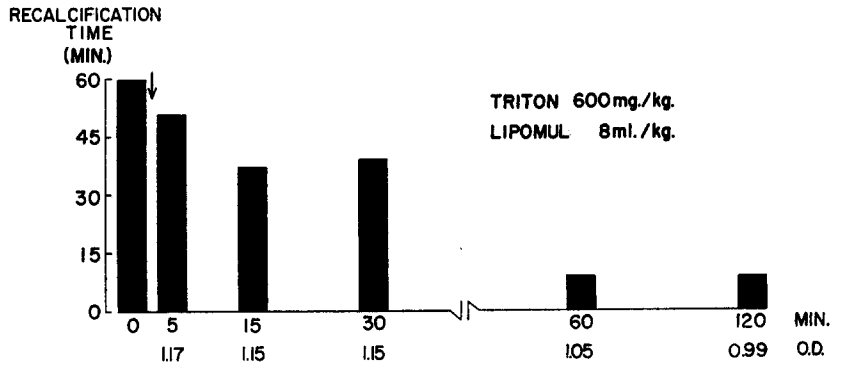

FIG. 4. The recalcification time of rabbit plasma at intervals after the intravenous administration of heparin, followed by lipomul and triton.

Each animal received heparin, 7.5 mg. per kg. at the start of the experiment.

Arrow indicates injection of lipomul and triton mixture.

The results of this experiment are illustrated in Figs. 3 and 4. Fig. 3 shows a striking reduction in the prolonged recalcification time of the heparin-containing rabbit plasma during the time that the turbidity of the plasma was being rapidly reduced. Within 5 minutes after intravenous administration of the lipid, the prolonged recalcification time was reduced 75 per cent regardless of the dose of the lipid. Thereafter, the duration of the effect was dependent upon the duration of the hyperlipemia, which in turn was a function of the dose. At a dose of 2 ml. per kg., the recalcification time was reduced to 8 minutes within 5 minutes after the injection of the lipid, but rose again at 15 minutes; by 30 minutes, the turbidity was completely cleared and the recalcification time rose to a level higher than the baseline value. When the dose of lipid was doubled, the effect was prolonged for almost an hour, but rose rapidly as soon as the lipid disappeared. With the extremely large dose of 8 ml. per kg., the effect persisted throughout the duration of the physiological activity of the heparin. The shortening of the recalcification time of the heparin-containing plasma *in vivo* appeared to parallel closely the period of rapid reduction in the turbidity.

The findings in the two animals that were given triton WR1339 in addition to lipomul, are in sharp contrast to this. Fig. 4 summarizes the data from one of these animals. Throughout the duration of this experiment the turbidity of the plasma was maintained at the initially

high level and no significant reduction took place in the recalcification time of the plasma. Indeed, the recalcification times of these animals were comparable to those obtained when the rabbits were given heparin alone. Thus, although a large quantity of lipid was present in the plasma, there was no reduction in the prolonged recalcification time, presumably related to the fact that lipolysis *in vivo* was inhibited by the presence of triton.

Studies of the Mechanism Whereby the Recalcification Time Is Reduced

The reduction in recalcification time noted in these experiments could have been brought about either by inactivation of heparin, or by alteration of one or more of the phases of blood coagulation to circumvent the anticoagulant activity of the heparin. In the foregoing *in vivo* experiments the recalcification time was reduced only during the clearing of the hyperlipemia and rose rapidly as soon as the lipid was gone from the blood stream. This finding lent credence to the first alternative, namely that the heparin was inactivated temporarily during the removal of lipids, but became effective once again when the clearing process was over. Preliminary experiments suggested that the factor responsible for lowering the recalcification time during clearing was heat-sensitive, while heparin is known to be resistant to heat. The hypothesis that heparin is reversibly inactivated during clearing was therefore investigated in an experiment in which the factor responsible for the reduction in recalcification time was destroyed by heat, and the cleared mixture retested for the presence of anticoagulant.

In this experiment postheparin plasma, ediol, and imidazole buffer were mixed in the same proportions as heretofore utilized and divided equally between two tubes. Both mixtures incubated in a water bath at 37°C. for 30 minutes. In each case the optical density fell and the recalcification time was reduced from 20 minutes to 3 minutes. At this time, one tube that had cleared for 30 minutes was placed in a water bath at 56°C., and the other was kept at 37°C. Each tube was then incubated for an additional 30 minutes. Then 0.1 ml. of fresh normal rabbit plasma was added to 0.9 ml. of each of these mixtures to replace any normal heat-labile clotting factor destroyed by incubation at the higher temperature, and these mixtures were recalcified. The results are summarized in Table V. Clearing for 30 minutes at 37°C. reduced the recalcification time from 20 minutes to 3 minutes. The recalcification time was still 3 minutes after incubation for an additional 30 minutes at 37°C., but was greater than 30 minutes when the mixture was incubated at 56°C. for the second 30 minute incubation period. Thus it appeared that incubation for 30 minutes at 56°C. destroyed the factor or factors responsible for the reduction in recalcification time seen during clearing and permitted the re-emergence of the anticoagulant effect of heparin.

Further evidence that this was due to the reappearance of effective heparin, and not to the heat treatment, was afforded when protamine sulfate, a specific heparin antagonist, was added to each mixture. The prolonged recalcification time of the mixture incubated at 56°C. was promptly returned to normal by this substance. The recalcification time of the mixture incubated solely at 37°C. was actually slightly prolonged, presumably because protamine sulfate in the absence of any heparin with which it can combine acts as a weak anticoagulant.

In sum, this experiment suggests that a factor, or factors, released during clearing inactivates the anticoagulant property of heparin without destroying

it permanently. The agent responsible for inactivating heparin is sensitive to heat, and when its effectiveness is nullified by heat, the anticoagulant effect of heparin re-emerges.

TABLE V

Reversal by Heat of the Clearing-Induced Reduction in Recalcification Time of Postheparin Plasma

Time of incubation	Recalcification time*	
	Clearing mixture incubated at 37°C.	
	Tube A‡	Tube B‡
min.		
0	20 min.	20 min.
30	3 min.	3 min.
	Temperature of incubation maintained at 37°C.	Temperature of incubation increased to 56°C.
60	3 min.	>30 min.
	Protamine sulfate, 50 mg. added to both tubes	
	4 min.	2 min.

* 0.25 ml. of 0.05 M $CaCl_2$ added to 1 ml. aliquots of test mixtures for determination of recalcification time.

‡ Each tube contained 4 ml. PHP, 1 ml. ediol 1–100, 5 ml. imidazole buffer.

DISCUSSION

It is well known that the intravenous injection of heparin into animals produces both a marked prolongation of the blood clotting time and calls forth a lipolytic enzyme into the blood stream. The present experiments indicate that when a lipid emulsion is added *in vitro* to plasma from such an animal, prolongation of blood clotting due to the presence of heparin is reduced progressively toward normal, coincident with and dependent upon hydrolysis of the lipid by the enzyme. Furthermore, injection of lipid emulsions intravenously into rabbits that previously had received a large dose of heparin resulted in a similar reduction in the recalcification time of their plasma during the period that the lipid was circulating in the bloodstream, suggesting that both lipolysis and inactivation of heparin take place *in vivo* as well.

The precise mechanism whereby the prolonged clotting time is reduced is not known, but in general terms it could be accomplished in one of two ways. First, a product of lipolysis may accelerate the first or second phase of normal blood coagulation sufficiently to overwhelm or by-pass the antithrombin ac-

tivity of the heparin. In support of this possibility is the fact that several specific lipid substances have been shown to have powerful thromboplastic properties (13). However, the recalcification time of the plasma after clearing was never found to be shorter than normal, and studies of the thromboplastin generation time and the one-stage prothrombin time of clearing mixtures thus far have failed to indicate any acceleration of normal blood clotting (14). Alternatively, the recalcification time may be reduced by a product of lipolysis which unites with heparin and inactivates its anticoagulant properties in a manner similar to that of protamine sulfate or toluidine blue (15, 16). The present experiments indicate only that during clearing heparin is rendered ineffective as an anticoagulant by a process which is readily reversible. They cast no light on the mechanism by which this effect is accomplished.

The significance of an inhibitor of the anticoagulant effects of heparin developed during the course of lipolysis is not at the moment clear. It could conceivably act as a feed-back mechanism to maintain homeostasis of blood coagulation should excessive amounts of endogenous heparin be released. It is possible also that the inhibitor of heparin plays a role in control of the clearing reaction, for the studies of Engelberg indicate that a factor is released during clearing which limits the extent of lipolysis (17). Both these effects, the inhibition of the anticoagulant properties of heparin and of the enzymatic activity of lipoprotein lipase, could be brought about by a single substance produced during clearing. In this respect, such a substance would act in a manner similar to other known heparin antagonists such as protamine sulfate and toluidine blue which also inhibit lipoprotein lipase.

There has been considerable interest recently in the effects of dietary fat and blood lipids on normal blood coagulation. Some investigators have reported shortening of the whole blood clotting time in human beings and experimental animals during postprandial hyperlipemia (18). Since the effect of clearing appears to be due to a reversible inactivation of heparin which in no case reduced the recalcification time below the normal range, it is unlikely that the phenomenon reported in these experiments plays a role in these other observations where the clotting time during alimentary lipemia was significantly lower than normal.

SUMMARY

The clearing of lipid emulsions by postheparin plasma from rabbits or human beings was accompanied regularly by a sharp reduction in the recalcification time of the plasma. No change in blood coagulation took place when the activity of lipoprotein lipase, the clearing enzyme, was inhibited by means of heat or the action of a surface-active agent. In control studies, the recalcification time of postheparin plasma was not altered by the addition of glycerol, free fatty acids, or the products of hydrolysis of a lipid emulsion. Taken together, the findings indicated that a heat-labile factor was released during the

course of clearing which was capable of inactivating reversibly the anticoagulant properties of heparin.

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