Post-heparin phospholipase

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SUMMARY Egg phosphatidyl ethanolamine is degraded to lysophosphatidyl ethanolamine and fatty acids by a phospholipase in post-heparin plasma at rates of 10 to 30 μmoles/hr per ml plasma. The optimal system for measuring this enzyme contained albumin and $(NH_4)_2SO_4$, and the optimal pH was 8.8–9.1. Only slight degradation of egg or beef lecithin occurred.

Post-heparin and pancreatic phospholipase differ in heat stability; in the inhibiting effects of EDTA, $HgCl_2$, and diethyl-*p*-nitrophenyl phosphate; in the requirement for deoxycholate or albumin; and in an apparent specificity of the post-heparin enzyme for the ethanolamine phosphatide.

THE APPEARANCE OF a "clearing factor" or lipoprotein lipase in the blood plasma after intravenous heparin has been the subject of extensive investigations (1, 2). Shore and Shore (3, 4) presented evidence in recent studies that heparin may release more than one enzyme or more than one form of an enzyme involved in the stepwise degradation of triglycerides of chylomicra or low density lipoproteins. In earlier studies Shore and co-workers (5) found no degradation of the phospholipids of egg lipoproteins by post-heparin plasma.

Rizack (6) proved that there was an in vivo increase of free fatty acids in blood plasma after intravenous injection of heparin by inhibiting lipoprotein lipase with diethyl-*P*-nitrophenyl phosphate (para-oxon) in the blood samples drawn. However, there was no demonstrable concurrent decrease in the concentration of plasma triglycerides, perhaps because the expected changes in triglycerides were too small to measure accurately. We had observed that the incubation of normal plasma for 4 hr resulted in the conversion of approximately 10% of the lecithin to lysolecithin (7). Thus it seemed possible that the increased amount of free fatty acid in post-heparin plasma could arise from phospholipids as well as from triglycerides, and that heparin might release a phospholipase into the plasma.

In this report we present evidence for the presence in post-heparin plasma of a phospholipase capable of degrading egg phosphatidyl ethanolamine (8), provide a means of quantifying the activity of this enzyme, and describe properties which distinguish it from those of pancreatic phospholipase A. The generic term phospholipase is used for the post-heparin enzyme because we do not know specifically which fatty acid is removed, α' or β.

METHODS

Preparation of Phospholipids

Pure egg PE[1] and lecithin were prepared essentially by the methods of Rhodes and Lea (9). The silicic acid columns were prepared with 100–200 mesh silicic acid. The silicic acid was activated at 110° and used without agents promoting solvent flow. The separation and purity of phospholipids in eluates were determined by TLC (10) and by chromatography on silicic acid-impregnated paper (11).

The eluate containing the PE also contained an acidic phospholipid with an R_F on TLC similar to that of lysolecithin. This was removed on DEAE cellulose by the method of Rouser et al. (12). The DEAE cellulose (Distillation Products) was first treated with 0.5 N NaOH, washed with water until neutral, and then alternately washed with methanol and chloroform twice before applying the sample.

Beef liver lecithin was obtained by suitable column separation after extraction of beef liver as described by

[1] Abbreviations used: Phosphatidyl ethanolamine, PE. Lysophosphatidyl ethanolamine, LyPE. Amounts of PE or LyPE determined from phosphorus content are designated as micrograms of PE–P or micrograms of LyPE–P respectively. Glycerylphosphoryl ethanolamine, GPE. Fatty acid(s), FA. Disodium ethylenediamine tetraacetate, EDTA. Tris[hydroxymethyl]aminomethane, Tris. Diethylaminoethyl cellulose, DEAE cellulose. Thin-layer chromatography, TLC.

Hanahan and co-workers (13). This lecithin moved as a single spot, with the R_F of egg lecithin, on TLC and silicic acid-impregnated paper chromatograms.

Organic solvents were not deoxygenated. Oxidized products of phospholipids which may have been formed would be removed from the PE preparation in the passage through DEAE cellulose (12). Further purification of the egg lecithin by passage through DEAE cellulose did not increase its rate of reaction with post-heparin plasma.

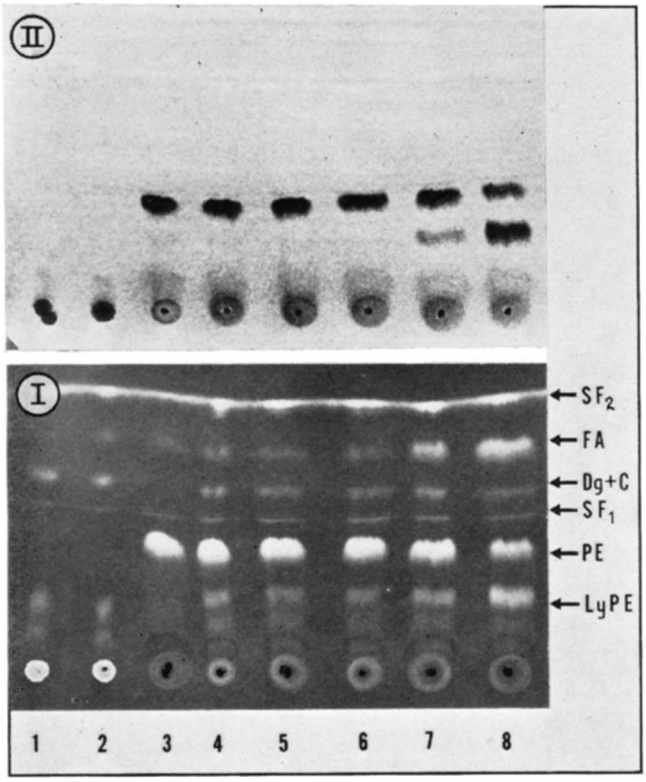

FIG. 1. Chromatographic demonstration of action of post-heparin phospholipase on PE.

I and II are chromatograms of identical samples. Both I and II were developed and sprayed with dichlorofluorescein as described under Methods. II was also sprayed with ninhydrin reagent. 3 μl of pre- and post-heparin plasma were applied directly at positions 1 and 2 respectively.

Lipid extracts were made of the standard incubation system, which contained 800 μg PE–P and 0.002 M EDTA, and were applied at positions 3 through 8 with the following variations: position 3, the use of 1.0 ml 0.9% NaCl in place of plasma and incubation for 40 min before lipid extraction; position 4, the use of 1.0 ml pre-heparin plasma and immediate lipid extraction without incubation; positions 5 and 6, use of 1.0 ml pre-heparin plasma of subjects A and B respectively and incubation for 40 min before lipid extraction; positions 7 and 8, use of 1.0 ml post-heparin plasma of subjects A and B respectively and incubation for 40 min before lipid extraction.

Lipid extracts applied at positions 3 through 8 were prepared by the addition of 1.0 ml of each incubation mixture to ethanol–ether (3:1) at a total volume of 25 ml. 20 ml of the filtered extract was evaporated and the lipids were redissolved in 2.0 ml of chloroform–methanol (2:1). 50 μl aliquots were applied to the plates.

Abbreviations: LyPE, lysophosphatidyl ethanolamine; PE, phosphatidyl ethanolamine; SF_1, first solvent front which contains unidentified or irreversibly adsorbed lipid as well as monoglyceride; Dg + C, isomeric diglycerides and free cholesterol; FA, fatty acids; SF_2, second solvent front containing esterified cholesterol with triglyceride just below or in the solvent front.

Blood Collections after Intravenous Heparin

Blood was collected before and 18 min after injection of 5000 USP units (50 mg) of heparin (Upjohn). Measures to prevent in vitro lipolysis (6) were not used. Four healthy adult human males were used as subjects. Subject A and subject B were used in all experimental studies presented. The post-heparin plasmas of these two subjects gave markedly different responses with our method of measuring post-heparin phospholipase. The plasma of subject A degraded PE to LyPE at an approximate rate of 10 μmoles/hr per ml plasma with no evidence of further degradation of LyPE. The plasma of subject B degraded PE to LyPE at a rate of 25 to 30 μmoles/hr per ml plasma, and a small amount of GPE was formed. The enzymatic activities in the plasmas of the other two subjects were similar to the activity found with the plasma of subject A. These responses were observed in freshly obtained plasmas used within 30 min or in plasma preserved at $-20°$. They were never observed in pre-heparin plasma.

In some experiments heparin was also used as the post-heparin plasma anticoagulant, 0.5 mg/ml whole blood. During the course of this study the activities of the enzyme in serum and plasma of subjects A and B were compared. Plasmas were collected using 0.5 mg heparin per ml or the anticoagulants EDTA, oxalate, and citrate (11). In these comparisons the tubes containing the blood were allowed to stand at room temperature for 5 hr. Then the serum or plasma was separated. The heparinized plasma had 90% of the activity of the other plasmas or serum.

While subjects A and B were frequently used as donors of individual post-heparin plasma samples, they also contributed on several occasions to large post-heparin pools of blood plasma or serum which were kept frozen for later use. Pool A consisted of post-heparin serum from subject A. Pool B consisted of post-heparin plasmas from subject B. These plasmas were obtained with EDTA, oxalate, or citrate as anticoagulants.

Standard Incubation System for Measurement of Post-Heparin Phospholipase

All measurements of the enzyme were made at 38°. Post-heparin plasma (1.0 ml) was incubated with at least 800 μg PE–P. The PE was dissolved in 5.0 ml of an aqueous solution containing 400 mg of albumin and sufficient $(NH_4)_2SO_4$ to give a concentration of 0.075

M in the final volume of 6.0 ml. This solution was prepared by using 2.0 ml of the albumin solution described below and 0.45 ml of 1 M $(NH_4)_2SO_4$, adjusting with NaOH to pH 9.2 in a final volume of 5.0 ml. Other agents, such as EDTA, when used, were incorporated into this solution prior to adjustment of pH. A fresh solution of bovine serum albumin (Armour No. 2293 fraction V) was prepared for each experiment by dissolving in the proportion of 2 g/10 ml water. This solution was used as containing 200 mg protein per ml.

Quantification of Post-Heparin Phospholipase

The amounts of LyPE and PE in the incubation system were determined immediately after the addition of the plasma and at intervals thereafter. The phosphorus content of these phospholipids was measured after their chromatographic separation on and elution from silicic acid-impregnated paper as previously described (11). The experimental error of the phospholipid phosphorus assay was approximately ±5%. The respective increase or decrease of LyPE–P and PE–P was used as a measure of action of the enzyme. The PE area was clearly delineated above the plasma lecithin on the chromatograms. This endogenous plasma lecithin component was not degraded. The LyPE area, as analyzed in this study, included the plasma sphingomyelin and lysolecithin. If the concentrations of these latter two components fell within the ranges found for them in 10 normal subjects studied previously (11), they would have contributed from 28–41 μg of phosphorus to the incubations in the present experiments.

Lipid extracts (11) were made for the studies of Fig. 1 and 5. In other studies a technique, previously described (14), involving the direct application of samples to silicic acid-impregnated paper, was used without prior extraction of lipids. Thus 15 to 25 μl aliquots of the incubation system were applied directly to the paper. The two techniques were not compared directly. Analogous studies with the two approaches as well as the consistency of the results indicated that the direct application of samples to the paper effectively halted enzymatic action. The technique of direct application permits a proportionate reduction in total volume of the incubation system to 0.6 ml. Such reductions were used whenever feasible.

Thin-Layer Chromatography

The Mallinckrodt silicic acid plates were prepared as described previously (10). However, in Fig. 1 of this study the pertinent phospholipids and neutral lipids in the sample under study were demonstrated on the same plate by developing the chromatogram with two solvents. The chromatogram was developed for 6 min in chloroform–methanol–water (80:35:4 v/v/v). After drying at room temperature the chromatogram was again developed for 6 min using petroleum ether–ethyl ether–acetic acid (80:15:1). Lipids were visualized by spraying the plate with 0.2% 2′,7′-dichlorofluorescein in ethanol. A solution of 0.25% ninhydrin in lutidine–acetone (1:9) was also used to demonstrate PE and LyPE. Qualitative studies of the activity of the post-heparin enzyme were made by application of lipid extracts (Fig. 1) or by direct application of 10–20 μl aliquots of the incubation mixture to the plate. All quantitative assays were made by the procedure described in the previous section.

RESULTS

Preliminary Experiments Indicating the Presence of Post-Heparin Plasma Phospholipase Activity

In a previous study (7) we found that pre- and post-heparin plasmas were equally active in converting endogenous lecithin to lysolecithin during incubation of the plasmas. Attempts to demonstrate the presence of phospholipase activity in post-heparin plasma by the addition of pure egg lecithin to a variety of incubation systems were uniformly unsuccessful. However, when post-heparin plasma was incubated for 16–18 hr in the presence of an egg yolk emulsion, the consistent appearance of LyPE and FA with a concurrent decrease in PE was observed. The lecithin component was unaffected. A significant decrease of the relatively large triglyceride component was not apparent, but a significant change, expressed as a percentage of the total, may have been too small to detect. The appearance of LyPE was not observed with pre-heparin plasma. These results led to the use of chromatographically pure egg PE as substrate to demonstrate post-heparin plasma phospholipase activity.

TLC Demonstration of Reactions of Post-Heparin Plasma with PE

Incubations with the standard system (EDTA added, to a concentration of 0.002 M) were used in chromatograms I and II of Fig. 1 to show the conversion of PE to LyPE and FA using post-heparin plasma of subjects A and B at postions 7 and 8 respectively. The pre-heparin plasmas of these subjects (positions 5 and 6) showed no phospholipase activity. Position 4 represents a control in which a lipid extract of unincubated pre-heparin plasma in the standard system was made immediately and applied to the plate. Position 3 represents another control in which the incubated mixture was as in positions 5–8 except for the replacement of plasma by 0.9% NaCl. The FA component above position 3 was derived

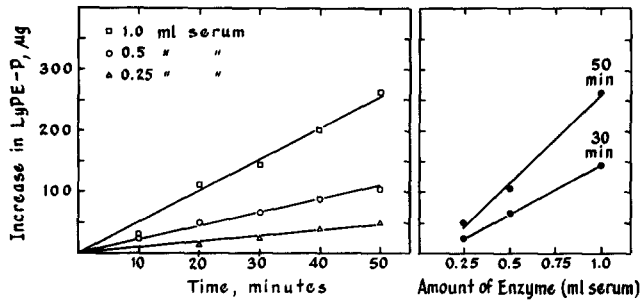

FIG. 2. Post-heparin plasma phospholipase activity as a function of time of incubation and enzyme concentration.

1200 µg PE–P were used in the standard incubation system. 1 ml of a solution containing 1.0, 0.5, or 0.25 ml of serum pool A appropriately diluted with 0.9% NaCl was added. The increase in LyPE–P with time is plotted on the left for the different amounts of serum used. The increase of LyPE–P as a function of enzyme concentration at two time intervals is plotted on the right.

from the Armour albumin. Purely pre- and post-heparin plasma were applied directly at positions 1 and 2 respectively.

Effects of Enzyme Concentration, Time of Incubation, and Substrate Concentration

The data shown in Fig. 2 indicate that over a 50 min period the rate of enzyme action is linear as a function of time at any given concentration of enzyme, and that over the range of enzyme concentration used, it is a linear function of enzyme concentration.

Studies using varying amounts of PE substrate with plasma pool B and serum pool A as sources of enzyme are shown in Figs. 3 and 4, respectively. 400 µg of PE–P did not provide a substrate concentration sufficient for maximum enzyme activity. It is apparent from Fig. 3 that during the first 6 min of incubation the decrease in PE–P is greater than the increase in LyPE–P. While this initial difference in the change of each component remained essentially constant thereafter, there was a gradual decrease in the sum of the actual amounts of PE–P and LyPE–P measured (approximately 10% in 30 min), because of the further breakdown of LyPE. This latter phenomenon was accompanied by the gradual appearance of GPE as demonstrated by TLC. No evidence of GPE production was detected with serum pool A, and the sum total of PE–P plus LyPE–P remained constant during a 30 min incubation.

Effect of pH and of Ammonium Sulfate

The optimum pH of the assay system is shown in Fig. 5 to be in the range of 8.8–9.1. This optimum was the same in the absence of EDTA. For convenience in measurements, the solution in which the PE was dissolved was adjusted to pH 9.2. After addition of the plasma, the pH of the complete assay system was usually approximately 9.05. The pH decreased less than 0.1 unit during a 20 min incubation period in which approximately 250 µg of LyPE–P was formed.

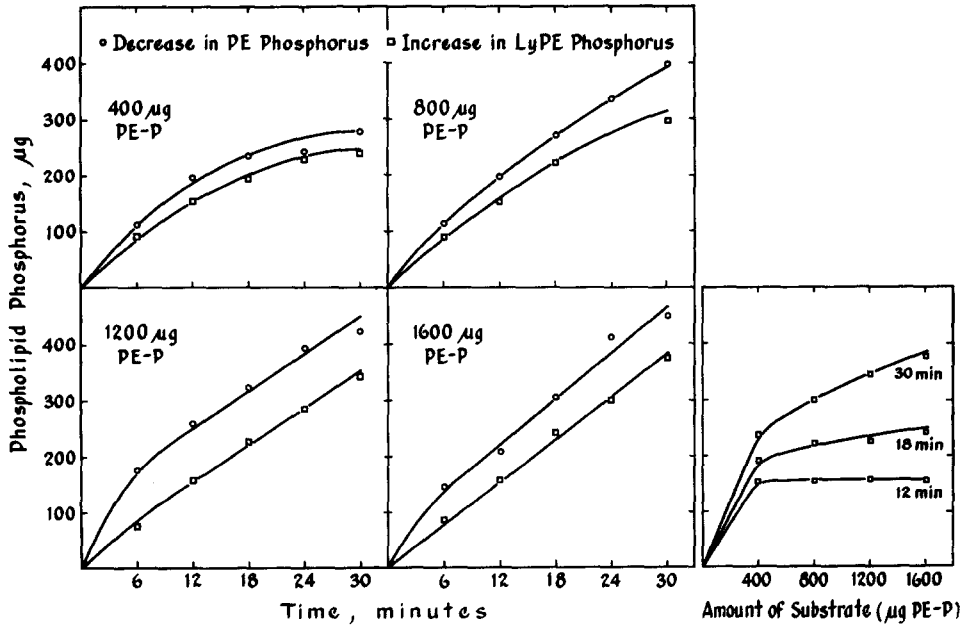

FIG. 3. Effect of substrate concentration on the enzyme activity of plasma pool B.

400, 800, 1200, and 1600 µg PE–P were used in the standard incubation system and incubated with 1.0 ml of plasma pool B for 30 min.

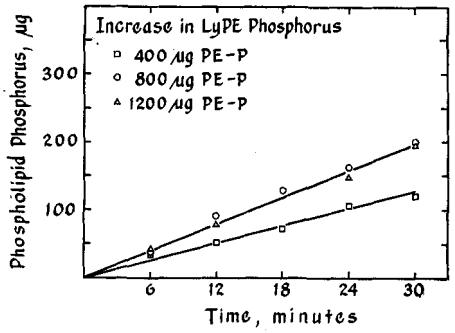

FIG. 4. Effect of substrate concentration on the enzyme activity of serum pool A.

400, 800, and 1200 µg PE–P were used in the standard incubation system and incubated with 1.0 ml of serum pool A for 30 min.

The optimum $(NH_4)_2SO_4$ concentration used in the standard incubation system (0.075 M) was determined from the data shown in Fig. 6.

Effect of Albumin as a FA Acceptor

The dependence of enzymatic activity on albumin concentration in the absence of $(NH_4)_2SO_4$ is shown in Fig. 7. When this experiment was repeated in the presence of 0.075 M $(NH_4)_2SO_4$ the 30 min increments in LyPE–P were 282, 335, and 350 µg with 100, 200, and 400 mg albumin, respectively, indicating less dependence of enzymatic activity upon the amount of albumin in the presence of $(NH_4)_2SO_4$.

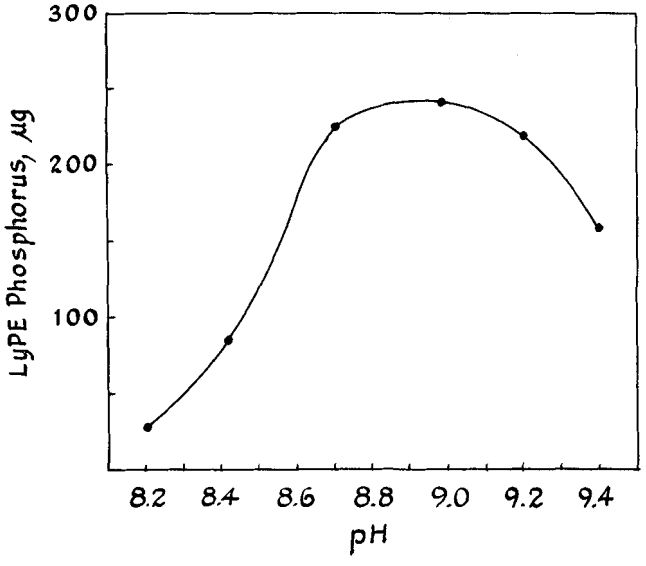

FIG. 5. Effect of pH on post-heparin plasma phospholipase activity.

800 µg PE–P were used in the standard incubation system in the presence of 0.005 M EDTA. Post-heparin plasma (heparinized) of subject B was used. pH measurements were made immediately after the addition of the plasma. LyPE–P formed during a 20 min period of incubation was plotted.

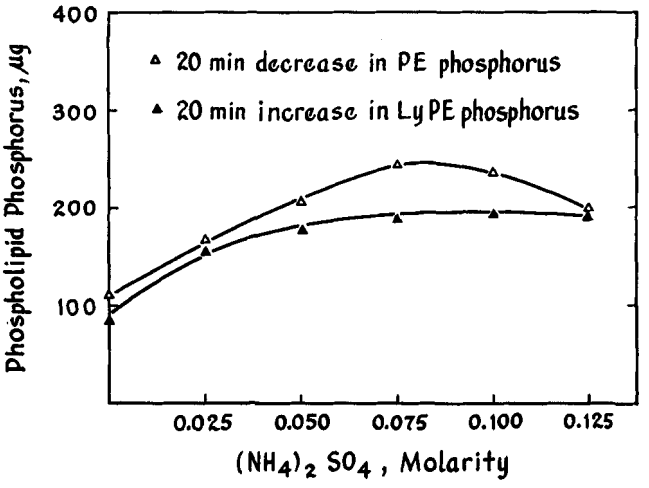

FIG. 6. Effect of $(NH_4)_2SO_4$ concentration on post-heparin plasma phospholipase activity.

The amounts of $(NH_4)_2SO_4$ in the standard incubation system were varied to obtain the molar concentrations of the figure. Post-heparin plasma (heparinized) of subject B was used with 800 µg PE–P. The respective changes in PE–P and LyPE–P during a 20 min incubation period are plotted.

Only slight phospholipase activity was obtained in buffers without added albumin. The activity was greater in glycine than in boric acid or tris buffers. A comparative study of the reaction in glycine–NaOH buffer alone, glycine–NaOH buffer plus 400 mg albumin, and the standard incubation system gave LyPE–P increments of 26, 153, and 256 µg, respectively. When the pH adjustment of the latter two systems was made with NH_4OH instead of NaOH, further increments of 12 and 22% respectively were observed.

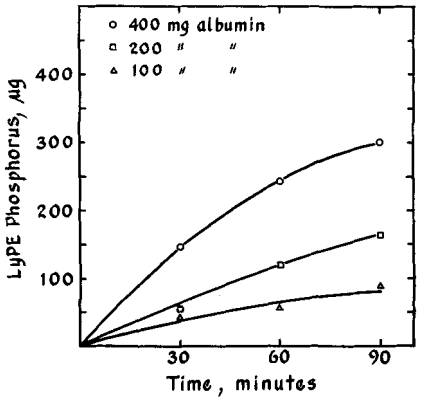

FIG. 7. Effect of albumin concentration on post-heparin plasma phospholipase activity.

The amount of albumin in the standard incubation system ($(NH_4)_2SO_4$ omitted) was varied as shown in the figure. 1200 µg PE–P were used with plasma pool B as the enzyme source. LyPE–P formed during a 90 min incubation period was plotted.

Effect of Other Variables

Quantitative studies with added EDTA (0.002–0.005 M) were made using albumin alone or albumin plus $(NH_4)_2SO_4$ as in the standard incubation system. Enzymatic activity was the same with or without EDTA. EDTA also did not prevent the appearance of GPE in incubations with the post-heparin plasma of subject B.

The marked activation of phospholipase A of pancreatic origin by sodium deoxycholate (15, 16) was not observed with the post-heparin enzyme. Studies were made adding varying amounts of this bile salt to the standard system as well as one containing boric acid or glycine buffers.

The post-heparin enzyme was completely inhibited by para-oxon at a concentration of 0.01 mM or greater. Activity was destroyed by heating at 55° for 20 min when the plasma was diluted 1:1 with either water or boric acid–glycine buffer at pH 8.45 (15).

In the presence of 0.002 M $HgCl_2$ post-heparin enzyme activity was 150% of the control.

Lecithin as the Substrate for the Post-Heparin Enzyme

Egg and beef liver lecithin before and after passage through DEAE cellulose were used in qualitative studies of enzyme action, utilizing the standard incubation system and TLC as in Fig 1. Over an incubation interval of 6 to 7 hr only a slight increase of FA derived from lecithin was observed. A component with the R_F of lysolecithin was noted in only a few of these incubations. This suggests that any lysolecithin formed is rapidly degraded by a lysophospholipase.

DISCUSSION

Shore et al. (5) were unable to detect any phospholipase activity when human post-heparin plasma was incubated with egg lipoproteins for a period of 8 hr. Our demonstration of extensive degradation of chromatographically pure PE by human post-heparin plasma evolved from earlier studies in which such plasma alone was allowed to act on whole egg yolk for much longer periods of time. A later observation that use of whole egg yolk as a substrate (even in the sensitive standard incubation system) resulted in only a slow rate of degradation of lipoprotein-bound PE suggests that the form of the substrate in the egg yolk or the presence of other substances interferes with the phospholipase activity.

The post-heparin phospholipase is similar to post-heparin lipoprotein lipase in its requirement for albumin as a fatty acid acceptor, the inhibition by para-oxon, and the optimal pH. Conceivably the same enzyme might be acting on both the phospholipid and the glyceride substrates. We have not as yet undertaken a study of the two incubation systems with the object of searching for specific differences in enzymes. The inability of Shore et al. (5) to detect any phospholipase activity upon incubating egg lipoproteins with post-heparin plasma under conditions in which they could show lipase activity favors the concept of a post-heparin phospholipase distinct from the post-heparin lipase. It is premature to relate the post-heparin phospholipase to the many studies of the effects of intravenous heparin on the lipids of blood or on fatty acid transport, effects which have heretofore understandably been ascribed solely to the action of heparin-released lipase. However, the possible importance of a phospholipase cannot be discounted.

We were unable in this study to demonstrate the presence in plasma of a phospholipase capable of degrading either lipoprotein-bound lecithin of whole egg yolk or chromatographically pure lecithin prepared from egg yolk or beef liver. While the conversion of endogenous plasma lecithin to lysolecithin was observed during incubation of pre- or post-heparin plasma as such (7), it was not detected during incubation of diluted plasma in the standard assay system. Thus, although the conversion of endogenous plasma lecithin to lysolecithin during incubation of whole plasma provided the stimulus for this study, no relationship of this observation to the activity of the apparently PE-specific post-heparin plasma enzyme was demonstrated.

We have considered two possible explanations for the failure of the post-heparin plasma enzyme to degrade egg lecithin: one, the physicochemical properties of the incubation system; the other, differences in the fatty acid composition of egg lecithin and PE. Bangham and Dawson (17) have shown that the electrokinetic characteristics of the substrate and enzyme profoundly influence the degradation of PE and lecithin by phospholipase D of *Clostridium perfringens* α-toxin. Perhaps the post-heparin plasma enzyme is similarly affected. Hawke (18) has demonstrated marked differences in fatty acid composition of egg PE and lecithin. The possible effect on the activity of the post-heparin plasma enzyme of these differences in fatty acid composition and in positional location of unsaturated and saturated fatty acids remains to be determined.

There are a number of major differences between the properties of human post-heparin plasma phospholipase and those of human pancreatic phospholipase A (15, 16). Inactivation at 55° of the post-heparin enzyme is in marked contrast to the heat stability of the pancreatic enzyme. The post-heparin enzyme was not affected by 2–5 mM EDTA whereas the pancreatic enzyme was completely inhibited by 1–2 mM EDTA. While $HgCl_2$ augmented the activity of the post-heparin enzyme,

it depressed the activity of the pancreatic enzyme somewhat. The post-heparin enzyme was completely inhibited by 0.01 mM paraoxon, whereas the pancreatic enzyme was activated slightly with a concentration of 0.1 mM. The marked activation of the pancreatic enzyme with desoxycholate was not observed with the post-heparin enzyme. Magee et al. (16) found that albumin (10 mg/ml) increased the activity of the pancreatic enzyme to 160% of the control. Use of larger amounts of albumin has not been reported. Finally, with respect to substrate specificities, we have so far been able to show only that the post-heparin enzyme will degrade PE, whereas the pancreatic enzyme degrades both lecithin and PE (15, 19).

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