Title

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EVIDENCE FOR LIPOLYTIC ACTION BY HUMAN PLASMA OBTAINED AFTER INTRAVENOUS ADMINISTRATION OF HEPARIN

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The action of heparin in vivo has become a focal point in the study of serum lipoproteins and their relationship to the development of atherosclerosis. It was observed by Hahn[1] that intravenous injection of heparin could effectively clear lipemic blood in humans. More recently, it has been shown by Graham, et al.[2] in this laboratory, using ultracentrifugal techniques, that drastic alterations in the serum lipoprotein distribution could also be produced by heparin in vivo. These effects have not been observed when heparin was allowed to react with serum in vitro; but plasma obtained from a human subject 15-30 minutes after the administration of heparin can evidently interact with certain classes of lipoproteins on incubation to cause changes in their ultracentrifugal pattern and other observable effects. For brevity, such plasma will be referred to as post-heparin plasma. Attempts to isolate or concentrate an active component by chemical fractionation of post-heparin plasma have been reported by other workers,[3] and ultracentrifugal fractionation is being carried out in this laboratory toward a similar goal.

The most pronounced transformations that occur as a result of reaction with post-heparin plasma involve glyceride-containing lipoproteins of the lower density classes. These classes tend to diminish or disappear, whereas the lipoproteins of higher density may increase in quantity. Another type of effect, which can be correlated with the ability of such plasma to cause lipoprotein changes, is the reduction in turbidity of a solution of egg yolk lipoprotein when incubated with the plasma under specified conditions. This has been developed as a test for heparin-induced activity, and is being reported in detail elsewhere.[4]

As an initial step in investigating the nature of these reactions, we have carried out chemical studies of the lipid changes brought about in certain lipoproteins by post-heparin plasma. The essential results of these studies are the subject of this paper.

EXPERIMENTAL PROCEDURE

Blood was withdrawn from normal human subjects before and after injection of 100 mg of sodium heparin (Lederle). Pre-heparin blood was allowed to clot and the serum centrifuged at low speed to remove any residual blood cells. Post-heparin blood was centrifuged at 4000 rpm to obtain clear plasma.

Egg lipoprotein (ELP) was prepared by a centrifugal isolation method described elsewhere.[4] The concentration of this preparation (ELP syrup, usually about 25 percent lipoprotein) was determined in the analytical centrifuge, and an appropriate dilution made with phosphate buffer (pH = 8.0, ionic strength = 0.1). The final lipoprotein concentration is about 5 to 10 percent.

In general, 1 ml of diluted ELP was incubated with 2 or 3 ml of serum or plasma at 37°C for periods ranging from 1/2 hour to 8 hours. The incubations were performed with parallel sets of samples, one set containing pre-heparin serum, the other post-heparin plasma from the same individual. At the end of the desired time interval, the reaction was stopped by the addition of 3 ml of methanol and 3 ml of water with stirring. The contents of the reaction tube were quantitatively transferred to a glass-stoppered 100 ml glass cylinder for extraction of lipids.

The mixture was extracted twice with 20 ml portions of ethyl ether to remove most of the lipids. The residual aqueous phase was next acidified to pH 2 by gradual addition of 6 N HCl, 3 ml of methanol were added, and two more extractions were made with ethyl ether. All of the ether extracts were combined and the solvents were removed by evaporation. The guiding principles in this procedure, wherein most of the lipids are extracted prior to acidification, were to achieve quantitative removal with minimum hydrolysis of lipids to yield fatty acids. While we have demonstrated that hydrolysis of glycerides

and cholesteryl esters is negligible under the usual conditions of extraction from acid or alkaline media, the situation with regard to phospholipids remains uncertain. Our precautions are concerned mainly with guarding against possible hydrolysis of this class of compounds, and are prompted in part by inconsistent results obtained in earlier experiments.

In order to titrate free fatty acids, the dried extract was dissolved in about 60 ml of low-boiling petroleum ether and the solution filtered into a 100 ml glass-stoppered cylinder. Removal of any trace of the hydrochloric acid which may have remained was accomplished by washing the solution four times with distilled water. (This was found to be sufficient to reach the same pH value as a similar system to which no hydrochloric acid had been added. Petroleum ether is preferred for this purpose since hydrochloric acid is removed from it more easily than from ethyl ether.) The solvent was evaporated, the residue was dissolved in about 40 ml of ethyl alcohol, and the solution boiled for one minute to expel carbon dioxide. The hot solution was titrated with sodium hydroxide (0.01 N in 90 percent ethanol aqueous) to a phenolphthalein end point. This procedure gave satisfactory agreement with titration at room temperature under a nitrogen atmosphere, and the end point determination was checked with a Beckman Model H pH meter (glass electrode). Simplicity of operation dictated the choice of the procedure described. The fatty acids were calculated from the titration as oleic acid (molecular weight = 282). The selection of oleic acid as a reference standard is arbitrary, and is based on the assumption that the fatty acids being measured are predominantly long chain. This follows from the observation that they are semi-solid when isolated, and have the infrared spectral characteristics of long-chain fatty acids. The presence of a minor fraction of low molecular weight acids would not invalidate our basic conclusions. The estimated error in titration is about ± 0.5 mg.

INFRARED ANALYTICAL METHODS

The methods of lipid analysis by infrared spectrophotometry will be reported elsewhere in greater detail, but the essential outline of the procedures

as applied in this investigation will be given here. The spectral measurements were made with a Baird Associates Model B double beam recording spectrophotometer equipped with a sodium chloride prism. The absorption cell had a capacity of 0.15 ml and an optical path of 0.9 mm.

The identity of the titrable material as fatty acid was confirmed by its infrared spectrum, and in many instances quantitative infrared measurements were carried out in parallel with titrations. In general, the absolute values for fatty acid were slightly lower than those obtained by titration, but the differences resulting from pre- and post-heparin incubations were substantially the same by both methods. The infrared measurements were carried out by dissolving the isolated fatty acids in small measured volumes of carbon disulfide and recording the spectrum of the solution. The peak absorbance (logarithm of the reciprocal transmittance) of the carboxyl absorption band at 5.9 microns was measured and converted to fatty acid concentration by means of a calibration curve (absorbance vs. concentration) which had been determined previously with pure oleic acid. In cases where neutral material (esters) remained as a contaminant of the separated fatty acids, its presence could be detected in the spectrum and an appropriate correction applied.

In analyzing the total lipid extract from an incubation mixture to determine fat and in some instances other lipids as well, a separation was carried out by means of a silicic acid adsorption column in the manner described by Borgstrom.[5] The lipid mixture was dissolved in petroleum ether, put on the adsorption column, and eluted in three fractions by the following sequence of solvents: I-5 percent chloroform in petroleum ether (combined with petroleum ether from original solution); II-chloroform; III-methanol. The principal lipids are distributed in these three fractions as follows: I-cholesterly esters; II-fat (triglycerides), free fatty acids, unesterified cholesterol; III-phospholipids. The eluted solutions were evaporated to dryness, the residues were redissolved in measured volumes of carbon disulfide, and spectra were recorded of the resulting solutions. In each of these three spectra, the ester carbonyl absorption band at 5.8 microns can be used to measure a different lipid component. (In Fraction III, it represents the sum of lecithins and cephalins, but

sphingomyelins do not contribute to this band.[6] If free fatty acids are present in Fraction II in detectable amounts, a correction can be made. Unesterified cholesterol requires no correction for amounts which can conceivably be present. It has been shown by experiment that partial glycerides, which may be presumed to be present as hydrolysis products in certain cases, do not accompany triglycerides in the chloroform eluate, but remain on the solumn and are partially eluted by methanol.

The reference materials used for intensity calibrations were: I-cholesteryl sterate, synthesized by direct esterification;[7] II-edible olive oil (commercial); III-egg lecithin, reprecipitated several times with acetone.

Statistical evaluation of these methods is not complete, but on the basis of column recoveries, instrumental error, etc., we believe that they are accurate to within about ± 10 percent. In a series of duplicate analyses of various lipoproteins, the triglyceride values disagreed by 0.0, 1.1, 10.0, 2.5, and 1.2 percent. To a considerable extent we have relied on the reproducibility in several experiments and comparison with controls as criteria of the validity of the observed differences.

RESULTS AND DISCUSSION

Some typical graphs, Figs. 1, 2 and 3, illustrate the essential findings with respect to fatty acid release and other lipid changes in the type of incubation experiment described. The liberation of fatty acids from lipoprotein substrates by the action of post-heparin plasma, as contrasted with a minimal effect with pre-heparin plasma, has been observed in many other such experiments. Lipemic serum or an appropriate ultracentrifugal fraction thereof containing low density lipoproteins can also serve as a substrate for the reaction. The source of the fatty acids is principally the triglycerides as evidenced by a decrease in their amount which corresponds roughly to the amount of fatty acids released. The phospholipid (sphingomyelin not included) curves (Figure 3) show no divergence greater than the experimental error. Analysis of the cholesteryl esters are somewhat erratic but indicate no hydrolysis. Complete hydrolysis of the total amount present would account for only about 25 percent of the free fatty acid observed.

The release of fatty acids could not be effected with pre-heparin serum by adding heparin prior to incubation. Nor was any reaction observed with post-heparin plasma which had first been heated to 58°C for ten minutes. In order to explore the enzymatic nature of the process further, some experiments were performed with various inhibitors of the type commonly employed in metabolic studies. The following substances at concentrations of 0.02 M in the incubation mixture showed less than 10 percent inhibition (as determined by titration): sodium cyanide, sodium fluoride, sodium azide, sodium iodoacetate, and sodium oxalate. Data with 2,4-dinitrophenol were uncertain because of interference with the measurements. Sodium arsenite at the same concentration caused 60-70 percent inhibition throughout a period of 1-24 hours.

Further studies of fatty acid release in this type of system are now in progress, with these and other inhibitors as well as activators, in an effort to develop the kinetic and enzymatic relationships.

The process of lipoprotein alteration that is initiated in the body by heparin is certainly more complex than the straightforward lipolysis we have observed in vitro. Nevertheless a selective breakdown of triglycerides is consistent with the disappearance of part or all of the faster-floating species of lipoproteins, in which fat is a predominant component. If hydrolysis of this fat is accompanied by removal of the products from the lipoprotein, the residual portion of the molecule may simply appear as a higher density species in the ultracentrifuge pattern. Thus the lipolysis reaction could conceivably be a direct cause of the observed lipoprotein transformations; but the experimental results thus far are not sufficient to demonstrate such a relationship.

The clearing of turbidity in solutions of egg lipoprotein on incubation with post-heparin plasma could also be explained by lipolysis. This reaction yields products--fatty acids and partial glycerides--which are more hydrophilic than the original material. And the resulting change in the colloidal character of the system would tend toward a greater degree of solubilization.

SUMMARY

Plasma from human subjects who have received intravenous heparin a short time previously, when incubated with certain lipoproteins for period of 4 to 8 hours at 37°C, has been found to cause partial hydrolysis of the glyceride component of the lipoprotein with concomitant release of fatty acids. Sera from the same individuals before heparin administration did not cause measurably real hydrolysis under the same conditions.

Heating the plasma before incubation prevents the reaction, but of a number of common enzyme inhibitors tried, only sodium arsenite is effective.

Our results are consistent with--and may at least partially explain--certain other lipoprotein changes which may be classed as heparin effects, e.g., the observed redistribution of lipoproteins shown by the ultracentrifuge, and the clearing of turbidity in egg lipoprotein solutions.

ACKNOWLEDGMENT

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FIGURE CAPTIONS

Fig. 1 - Lipid changes on incubation of egg lipoprotein (1 ml) with 2 ml of pre-heparin serum or post-heparin plasma from Subject No. 1. (Triglycerides as olive oil; fatty acids as oleic.)

Fig. 2 - Lipid changes on incubation of egg lipoprotein (1 ml) with 3 ml of pre-heparin serum or post-heparin plasma from Subject No. 2. (Triglycerides as olive oil; fatty acids as oleic; cholesteryl esters as stearate.)

Fig. 3 - Lipid changes on incubation of egg lipoprotein (1 ml) with 3 ml of pre-heparin serum or post-heparin plasma from Subject No. 3. (Triglycerides as olive oil; phospholipids as lecithin -- sphingomyelin not included in measurement; fatty acids as oleic.) In this particular experiment the cholesteryl esters were not separated from the triglyceride fraction and presumably contribute a small constant error in these curves.

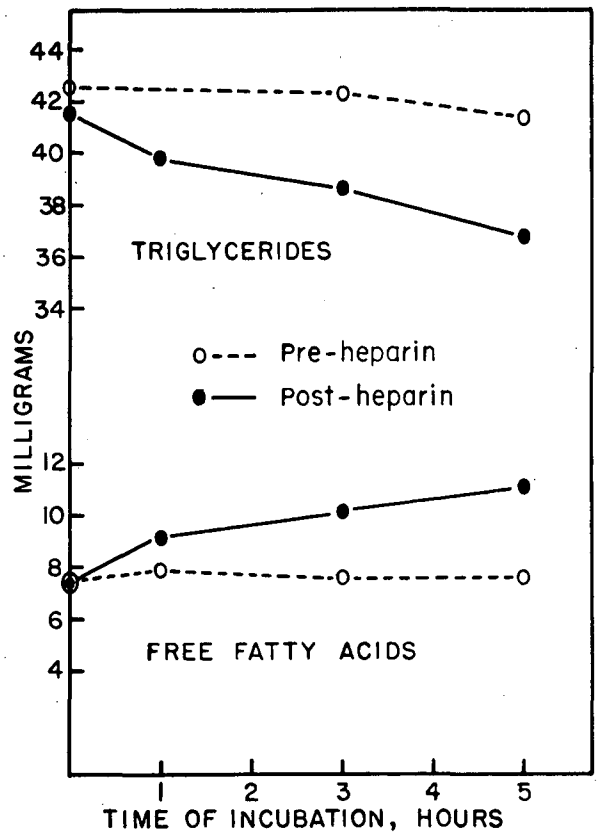
44
42
40
38
36
34
12
10
8
6
4
MILLIGRAMS
TRIGLYCERIDES
Pre-heparin
Post-heparin
FREE FATTY ACIDS
1
2
3
4
5
TIME OF INCUBATION, HOURS

Fig. 1

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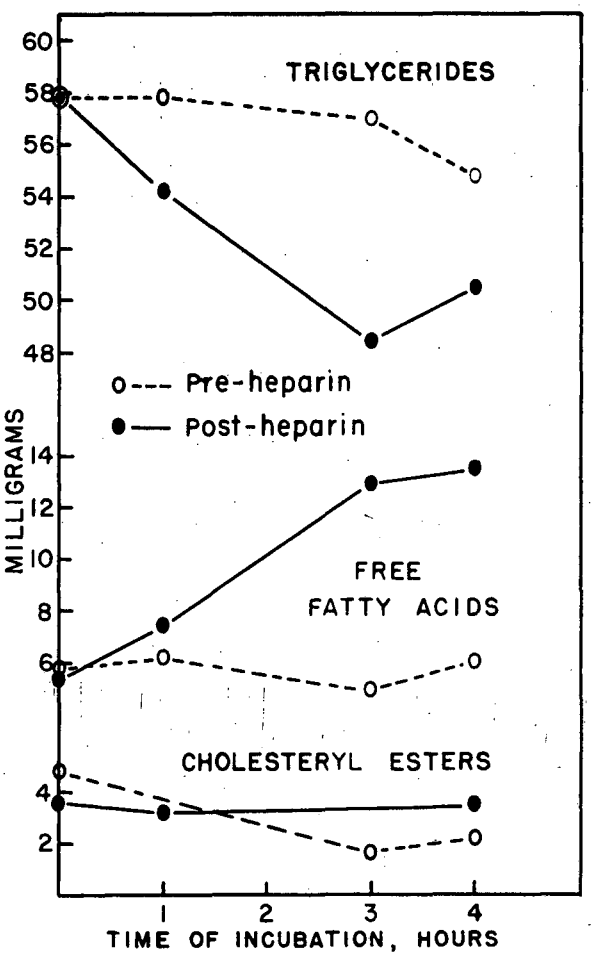
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54
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o--- Pre-heparin
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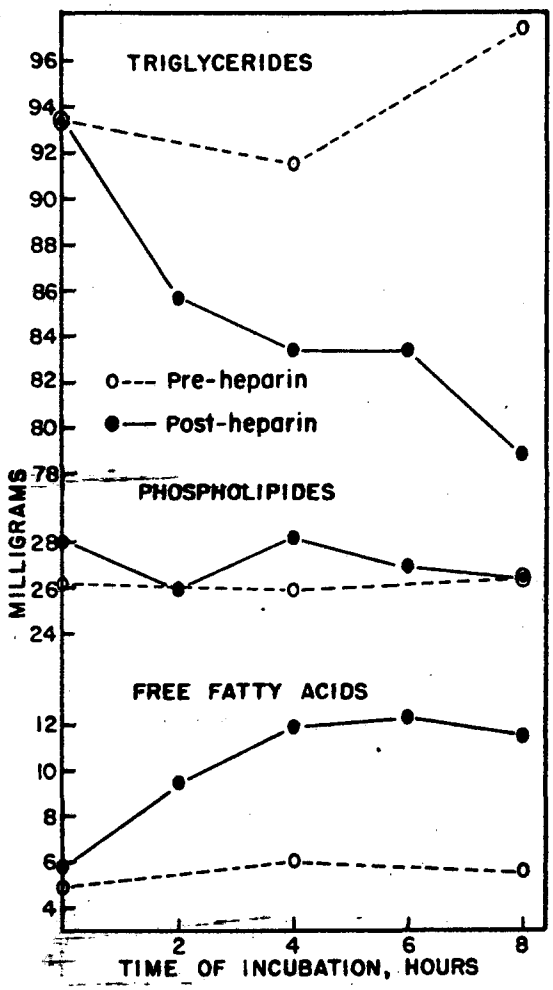
MILLIGRAMS
14
12
10
8
6
FREE
FATTY ACIDS
CHOLESTERYL ESTERS
4
2
1
2
3
4
TIME OF INCUBATION, HOURS

Fig. 2

MU-5328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28
26
24
FREE FATTY ACIDS
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MILLIGRAMS
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TIME OF INCUBATION, HOURS

Fig. 3

MU-5329