Interaction of Heparin Active Factor and Egg-Yolk Lipoprotein.* (20879)

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The *in vivo* action of heparin on plasma lipids was initially observed by Hahn to be a clearing of a lipemic plasma of its light scattering components(1). Such action definitely suggested a transformation of the physical state of the plasma lipids. The work of Gofman and co-workers at this laboratory has shown that the different plasma lipids measured chemically could be accounted for in the spectrum of lipoprotein molecules(2). The action of heparin on the lipemic plasma indicated its probable involvement with this whole spectrum of lipid-bearing molecules. The action of *in vivo* heparin on the spectrum was observed by Graham and co-workers and subsequently studied by Lindgren and coworkers at this laboratory(3,4). Their investigations bring forth the observation that subsequent to an injection of heparin there is a uni-directional transformation of lipid-bearing molecules from the ultracentrifugally defined high S_f (molecules of high glyceride content) to the lower S_f values (which are correspondingly lower in glycerides). Such transformations could be produced *in vitro* by incubation of a lipemic serum with *in vivo* heparinized plasma. This report presents an investigation of the interaction of the active principle or factor of heparinized plasma with lipoprotein species isolated from egg yolk(5). The interaction was manifested by a reduction in the optical density of a solution containing a small amount of ELP (egg lipoprotein) maintained at a low ionic strength and pH 6.7. Addition of heparin in low concentrations to the dilute ELP solution increased the optical density. Thus, there was present in post heparin plasma an agent which altered the solubility characteristics of the ELP solution and which could not be reproduced by a simple *in vitro* addition of heparin.

Methods. The ELP was obtained by means of a 12-hour preparative ultracentrifugation at 30,000 RPM (Spinco preparative centrifuge and 30.2 rotor) of egg yolk contents diluted in the proportion one yolk to 18 ml phosphate buffer (pH 8.0; ionic strength 0.1). The lipoprotein forms a yellow concentrate in the uppermost zone of the centrifuge tube. This concentrate is carefully removed and when diluted one to 4 parts with the above buffer serves as a stock solution (concentration approx. 5%). An analytical ultracentrifugation (in density 1.0630 at 26°C at 52,640 RPM) determines the concentration and any sedimenting contamination, which is to be avoided, in the stock solution. Under the above conditions the lipoprotein preparation has a major component of approx. 25-30 S_f units with some faster species generally present. Infra-red chemical analyses by Freeman(6) give an average composition for the preparation as follows: Glyceryl ester, 67%; cholesterol (predominantly free), 6%; phospholipid (as lecithin), 16%; protein, 11%. For measurements on the interaction system of ELP with either pre-heparin serum‡ or post-heparin plasma§ the technic was to incubate

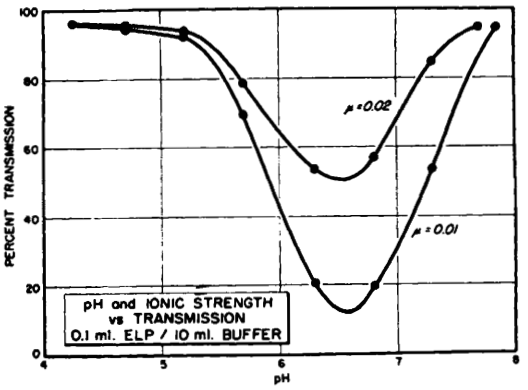

FIG. 1. pH and ionic strength vs. transmission. 0.1 ml ELP/10 ml buffer.

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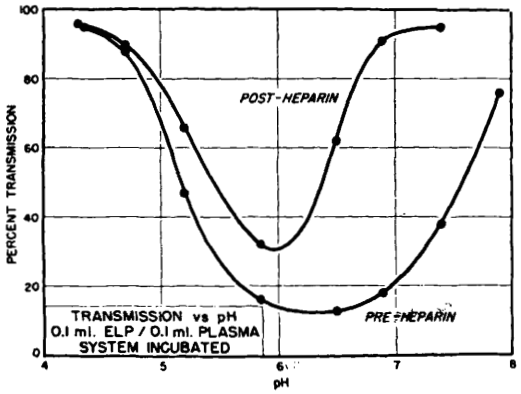

FIG. 2. Transmission vs. pH. 0.1 ml ELP/0.1 ml plasma, system incubated.

0.1 ml of a 3 to 5% ELP solution with 0.1 ml of serum or plasma at 37°C for a specified time. At the end of the incubation period the mixture was diluted with 10 ml of an appropriate buffer (depending on required pH value), stirred, and allowed to stand for 15 minutes. After this period the diluted mixture was stirred and the optical transmission at 650 mμ (Beckman spectrophotometer) was measured. The pH of the incubation mixture was controlled around pH 8.0 in order to operate in the neighborhood of maximal increase in transmission as shown in Fig. 5.

Results. As shown in Fig. 1 the ELP solubility is increased by an increase in ionic strength and is at a minimum in the region of pH 6.7. Fig. 2 shows that at a constant ionic strength of 0.01, there is a marked difference in solubility, when the effects of pre-heparin serum and post-heparin plasma on the ELP are compared. The shift in the point of minimum solubility, or what may be expressed alternatively, the increased solubility of the ELP in a given region of pH, at ionic strength 0.01, was observed only if one or more of the following conditions were met: 1) the post-heparin plasma was obtained from an individual whose blood contained an elevated level of lipoprotein species of high glyceride content. 2) the post-heparin plasma, obtained from a fasting individual normally low in the glyceride-containing lipoprotein species, was incubated with a lipemic serum prior to the addition of ELP. 3) a fasting post-heparin plasma, again from an individual low in glyceride-containing lipoproteins, was incubated with ELP before testing for activity. This condition is illustrated by comparison of Fig. 3 with Fig. 2 (incubated versus non-incubated systems in which plasma came from a fasting individual low in glyceride-containing lipoproteins). Thus, an essential requirement for the production of the shift in ELP solubility was a *prior* interaction—either *in vivo* or *in vitro* at 37°C—of heparinized plasma with lipoprotein species high in glyceride content.

The necessity for the presence of lipoproteins high in glycerides to enable post-heparin plasma to increase the solubility of ELP indicated that there may be the release of a labile lipid constituent which itself may effect the alteration. Such a constituent does not alter the pH of the system to a significant degree under the conditions of the solubility determination. Therefore, it might be supposed that a soluble agent with a very high surface activity such as a fatty acid soap is released during the interaction. Fig. 4 shows the effect of sodium oleate on ELP, and that it is analogous to the post-heparin plasma interaction effect shown in Fig. 2.

Data from rate determinations are shown in Figs. 6 and 7. The reaction was stopped with the addition of 10 ml of cacodylate buffer (ionic strength 0.01; pH 6.7). A plot

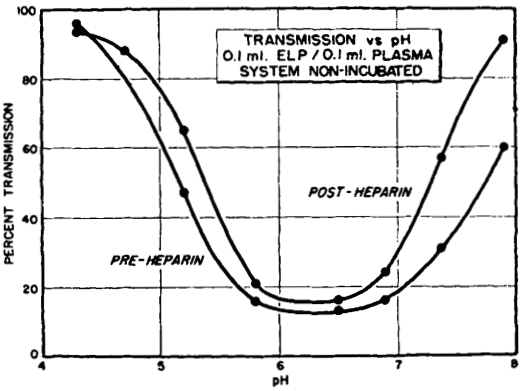

FIG. 3. Transmission vs. pH, 0.1 ml ELP/0.1 ml plasma, system non-incubated.

‡ Pre-heparin serum obtained from untreated whole blood from an individual prior to intravenous administration of heparin.

§ Post-heparin plasma obtained from whole blood drawn from an individual after intravenous administration of heparin.

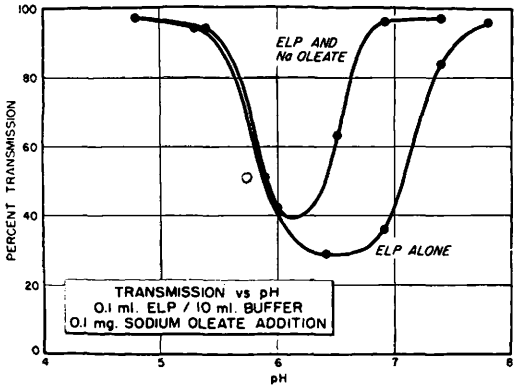

FIG. 4. Transmission vs. pH. 0.1 ml ELP/10 ml buffer, 0.1 mg sodium oleate addition.

of transmission versus varying concentrations of ELP in 0.1 ml in the presence of 0.1 ml preheparin serum is also shown in Fig. 6. This plot was obtained by adding 10 ml of the cacodylate buffer to the volumes of ELP and serum stated above, with no incubation. The transmission at any time interval was compared with a transmission obtained from the above concentration plot. Thus, the fraction of ELP still insoluble after an interval of incubation as evidenced by the % transmission is compared with a concentration of ELP necessary to give the same % transmission. By this procedure the rate curves plotted as changes in transmission against time can be reduced to comparatively linear curves expressed as concentration changes. Calibration is also possible with sodium oleate.

The duration of plasma activity *in vivo*

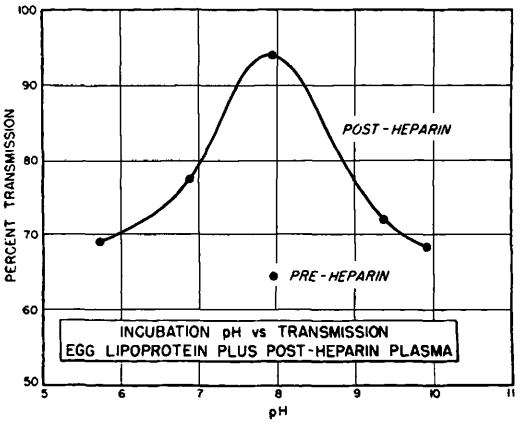

FIG. 5. Incubation pH vs. transmission. Egg lipoprotein plus postheparin plasma.

after a single 50 mg intravenous injection of heparin as measured under the conditions already described for rate studies, is shown in Fig. 8. Within 3 hours after the heparin injection the serum of this subject no longer had any demonstrable effect. Following intravenous injections of 100 mg the effect was observed to persist for 4 to 5 hours. Again, in Fig. 8, it should be noted that measurement of transmission without prior incubation of the ELP with the plasma reveals no change following the heparin injection because of the initial low level of glyceride in this subject's serum. Further chemical investigations at this laboratory have substantiated this

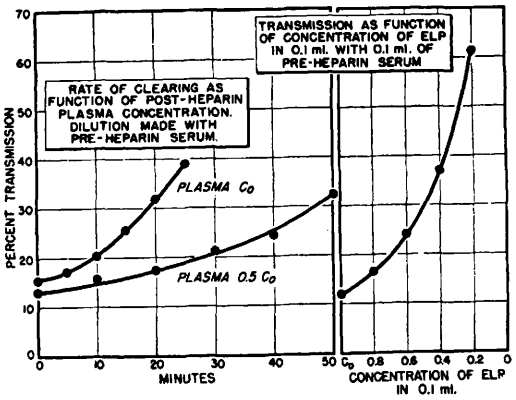

FIG. 6. Transmission as function of concentration of ELP in 0.1 ml with 0.1 ml of preheparin serum. Rate of clearing as function of postheparin plasma concentration. Dilution made with preheparin serum.

Undiluted postheparin plasma is designated C_o conc.; any dilution thereof with preheparin plasma or serum is designated as a fraction of C_o. Egg lipoprotein (ELP) stock sol. (whose actual conc. is obtained by analytical ultracentrifugation) is also designated C_o conc. and any dilution thereof with appropriate buffer is a fraction of that C_o conc.

hypothesis by showing that fatty acid is indeed liberated with a concomitant decrease in triglycerides when lipoproteins high in glyceride content are incubated with postheparin plasma(7). Further studies are in progress toward establishing the type of reaction scheme in the above system and its dependence on the concentration of the reactants.

Summary. 1. Egg lipoprotein under the conditions stated can be used as a qualitative

and semi-quantitative indicator for the lipoprotein-influencing properties of *in vivo* heparinized plasma. 2. The utilization of egg lipoprotein for this purpose requires prior contact of the active principle of post-heparinized plasma with lipoproteins of high neutral fat content. 3. Sodium oleate produces alterations in the egg lipoprotein system similar to those produced by post-heparin plasma. Chemical data showing that fatty acids are released from the glycerol ester fraction of the egg lipoprotein during incubation suggest that the egg lipoprotein solubility changes may be a secondary effect of the hydrolysis of glycerol esters by the active principle in post-heparinized plasma.

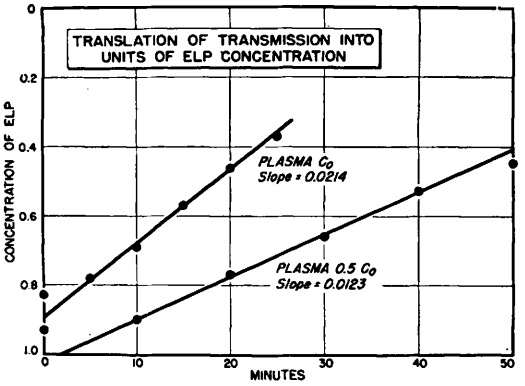

FIG. 7. Translation of transmission into units of ELP concentration.

Undiluted postheparin plasma is designated C_0 conc.; any dilution thereof with preheparin plasma or serum is designated as a fraction of C_0. Egg lipoprotein (ELP) stock sol. (whose actual conc. is obtained by analytical ultracentrifugation) is also designated C_0 conc. and any dilution thereof with appropriate buffer is a fraction of that C_0 conc.

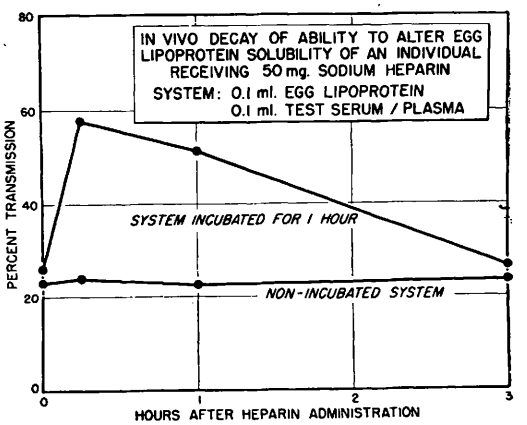

FIG. 8. *In vivo* decay of ability to alter egg lipoprotein solubility of an individual receiving 50 mg sodium heparin. System: .1 ml egg lipoprotein, .1 ml test serum/plasma.

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1. Hahn, P. F., *Science,* 1943, v98, 19.

2. Gofman, J. W., Lindgren, F. T., Elliot, H. A., Mantz, W., Hewitt, J., Strisower, B., and Herring, V., *ibid.,* 1950, v3, 166.

3. Graham, D. M., Lyon, T. P., Gofman, J. W., Jones, H. B., Yankley, A., Simonton, J., and White, S., *Circulation,* 1951, v4, 666.

4. Lindgren, F. T., Freeman, N. K., and Graham, D. M., *ibid.,* 1952, v6, 474, abst.

5. Nichols, A. V., Freeman, N. K., Shore, B., and Rubin, L., *ibid.,* 1952, v6, 457, abst.

6. Freeman, N. K., Lindgren, F. T., Ng, Y. C., and Nichols, A. V., *J. Biol. Chem.,* 1953, v203, 293.

7. Shore, B., Nichols, A. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

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