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Berkeley, California

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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 this spectrum was initially observed by Graham and co-workers and subsequently studied by Lindgren and co-workers at this laboratory.[3,4] Their investigations bring forth the observation that subsequent to an injection of heparin there is a unidirectional 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 transformation could be produced in vitro by incubation of a lipemic serum with in vivo heparinized plasma. There is thus an active principle in heparinized plasma that may be studied in vitro with a prescribed lipoprotein-containing medium. To this end the interaction of this active principle or factor was studied with lipoprotein species isolated from egg yolk.[5] These, too, have been shown capable of a limited transformation into lower S_f species by Lindgren at this laboratory.

The egg yolk lipoprotein was obtained by means of a 12-hour preparative ultracentrifugation at 30,000 (Spinco preparative centrifuge and 30.2 model rotor) of egg yolk contents diluted in the proportion of 1 yolk to 18 ml phosphate buffer (pH 8.0 ; ionic strength 0.1). The lipoprotein concentrates into the upper portion of the centrifuge tube, forming a gel on top and a layer of syrup below the gel. The syrup is removed and when diluted with the above buffer is used as the initial lipoprotein concentrate. The centrifugation is repeated if there

is contamination of the initial egg lipoprotein concentrate with any sedimenting protein, as determined by an analytical centrifugation of the diluted syrup in a medium of density 1.0630 at 26°C. In addition, under these conditions, the lipoprotein species float and allow their concentrations to be determined.

The lipoprotein preparation thus isolated has a major component of approximately 25-30 S_f units (in the above solution density of 1.0630 at 26°C.) with some faster species generally present. Infrared chemical analyses by Freeman at this laboratory give an average composition of the lipoproteins of the syrup as follows:

Neutral fat (measured as glyceryl ester)	67%
Cholesterol (predominantly free)	6%
Phospholipid (measured as lecithin)	16%
Protein	11%

Investigations on the system of egg lipoprotein and serum or plasma revealed a distinct difference in the behavior of the preheparin serum* and postheparin plasma** upon dilution of the system with distilled water. A volume of 0.1 ml of preheparin serum plus 0.1 ml of a 3 to 5% solution of egg lipoprotein was markedly turbid upon dilution to a volume of 10 ml, whereas when 0.1 ml of postheparin plasma was used with 0.1 ml of the above egg lipoprotein solution, the system was clear upon dilution. Dilution of 0.1 ml of the egg lipoprotein solution alone resulted in a turbid solution. Heparin added in concentrations approximating those in the plasma of individuals receiving 100 mg or less of sodium heparin caused turbidity in excess over the corresponding control not containing added heparin. Thus there was something in the postheparin plasma that altered the solubility characteristics of the egg lipoprotein and that could not be reproduced by a simple addition of heparin to the system. This initiated a study to ascertain the solubility characteristics of the egg lipoprotein and serum or plasma system. The relative extent of solubility or insolubility in a given system, as evidenced by its turbidity, was measured by light transmission in a Beckman spectrophotometer at a wave length of 650 μ.

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* Preheparin serum: serum obtained from untreated whole blood from an individual prior to intravenous administration of heparin.

** Postheparin plasma: plasma obtained from whole blood drawn from an individual after the intravenous administration of heparin.

The dependence of the solubility of the egg lipoprotein (designated by ELP in figures) alone on ionic strength and pH can be seen in Fig. 1. An increase in ionic strength increases the lipoprotein solubility, while the pH relationship corresponds to isoelectric curves typical of many protein systems. There is thus a point of minimum solubility in the region of pH 6.7 with increasing solubility to either side.

In investigating the interaction of the egg lipoprotein with heparinized active factor, the technique generally employed was to incubate 0.1 ml of 3 to 5% egg lipoprotein solution with 0.1 ml of serum or plasma at 37°C. for a specified time. The mixture was then diluted with 10 ml of an appropriate buffer, stirred, and allowed to stand for fifteen minutes. The diluted mixture was then stirred again and the optical transmission at 650 μ was measured immediately. The pH of the incubation mixture was controlled around pH 8.0 by the buffer used in the preparation of the egg lipoprotein solution. It was subsequently found, as shown in Fig. 5, that this is an optimum value, and that small variations in pH do not seriously alter the results.

By varying the pH of the buffer in which the incubated mixture is diluted for the turbidity measurement, the curves shown in Fig. 2 were obtained. The ionic strength was maintained constant at 0.01. In the pH-versus-%-transmission curve for the egg lipoprotein - preheparin serum system a broader curve is obtained than with the egg lipoprotein alone. In the curve of pH versus % transmission for the egg lipoprotein - postheparin plasma system there is a marked shift of the point of minimum solubility to the acid side of the same point for the egg lipoprotein alone under the same conditions.

The shift in the point of minimum solubility, or what may be expressed alternatively, the increased solubility of the egg lipoprotein in a given region of pH, at the ionic strength of 0.01, was observed only if one or more of the following conditions were met:

1) the postheparin plasma was obtained from an individual whose blood contained an elevated level of lipoprotein species of high glyceride content.

2) the postheparin plasma, obtained from a fasting individual normally low in the glyceride-containing lipoprotein species, was incubated at 37°C. with a lipemic serum prior to the addition of egg lipoprotein.

3) a fasting postheparin plasma, again from an individual low in glyceride-containing lipoproteins, was incubated at 37°C. with egg lipoprotein before testing for turbidity. This condition is illustrated by comparison of Fig. 3 with Fig. 2(the incubated versus the nonincubated system in which the plasma came from a fasting individual low in glyceride-containing lipoproteins).

Thus, an essential requirement for the production of the shift in egg lipoprotein solubility characteristics, as studied in this system, depended on a prior interaction--either in vivo or in vitor at about 37°C. -- of heparinized plasma with lipoprotein species high in glyceride content.

The necessity for the presence of lipoproteins high in glycerides for the ability of the postheparin plasma system to increase the solubility of the egg lipoprotein indicated that there may be the release of a labile lipid constituent which itself may effect the solubility alteration. Such a constituent does not alter the pH of the system to a significant degree under the conditions of the solubility determination (i.e., 0.1 ml egg lipoprotein added to 10 ml of buffer of 0.01 ionic strength), but does change the solubility of the egg lipoprotein. Therefore it might be supposed that a soluble agent with a very high surface activity per unit weight, such as a fatty acid soap, is released during the incubation. A pH-versus-%-transmission curve obtained when 0.1 mg of sodium oleate was added to 0.1 ml of egg lipoprotein and 10 ml of buffer is shown in Fig. 4. The effect of the oleate was analogous to that exhibited by the postheparin plasma as shown in Fig. 2.

Since a prior incubation could form some agent responsible for the shift in solubility characteristics, it was pertinent to study the rate at which the shift occurred. This rate was studied by incubating at 37°C. 0.1 ml of a 3 to 5% egg lipoprotein solution in phosphate buffer (pH 8.0; ionic strength 0.1) with 0.1 ml of postheparin plasma. The reaction was stopped by an addition of 10 ml of cacodylate buffer of pH 6.7 and 0.01 ionic strength. As may be seen in Fig. 2, this pH provides for maximal turbidity of the original egg lipoprotein solution as well as a maximal range of solubility change. These conditions were chosen as optimum for studying the production of an agent which increases the egg lipoprotein solubility. (Moreover, these conditions are the ones employed in assaying for heparin-induced activity in plasma generally.) The transmission at any time interval of incubation was compared with a transmission obtained from a plot of transmission versus varying concentrations of unincubated egg lipoprotein preparation used in the previous incubation. Thus, the fraction of egg lipoprotein still insoluble after an interval of

incubation, as evidenced by the percent transmission, is compared with a concentration of egg lipoprotein necessary to give the same percent transmission. By this procedure the rate curves plotted as transmission (Fig. 6) can be reduced to the linear curves of Fig. 7, which are expressed in concentration terms. Calibration is also possible by utilizing the effect of a known amount of sodium oleate on the percent transmission.

The duration of plasma activity in vivo after a single 50-mg intravenous injection of heparin, as measured by its effect on the solubility of egg lipoprotein under the conditions already described for rate studies, is shown in Fig. 8. Within three hours after the heparin injection the serum of this subject no longer had any demonstrable effect on the egg lipoprotein solution. Following an intravenous injection of 100 mg this effect on egg lipoprotein was observed to persist for four to five hours.

Again, referring to Fig. 8, it should be noted that when the transmission is measured without prior incubation of the egg lipoprotein with the plasma, no change is noted following the heparin injection because of the initial low level of glycerides in this particular subject's serum. This, plus the fact that the percent transmission of a nonincubated system containing postheparin plasma is proportional to the amount of the high-glyceride-containing lipoprotein molecules present, indicates that the glycerides may well contribute the component that is directly responsible for the effect observed.

Further chemical investigations at this laboratory have substantiated this 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 plastma.[6] A dependence upon the concentration of heparinized plasma in the interaction is indicated in the rate studies reported above. 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 semiquantitative 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 postheparinized plasma with lipoproteins of high neutral fat content.

3. Sodium oleate produces alterations in the egg lipoprotein system similar to those produced by postheparin 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 postheparinized plasma.

FIGURES

1. pH and Ionic Strength vs. Transmission
   0.1 ml. ELP/10 ml. Buffer.

2. Transmission vs. pH
   0.1 ml. ELP/0.1 ml. Plasma
   System Incubated.

3. Transmission vs. pH
   0.1 ml. ELP/0.1 ml. Plasma
   System Non-Incubated.

4. Transmission vs. pH
   0.1 ml. ELP/10 ml. Buffer
   0.1 mg. Sodium Oleate Addition.

5. Incubation pH vs. Transmission
   Egg Lipoprotein plus Postheparin Plasma.

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.

7. Translation of Transmission into Units of ELP Concentration.

8. In Vivo Decay of Ability to Alter Egg Lipoprotein Solubility
   of an Individual Receiving 50 mg. Sodium Heparin.

   System: 0.1 ml. Egg Lipoprotein
   0.1 ml. Test Serum/Plasma

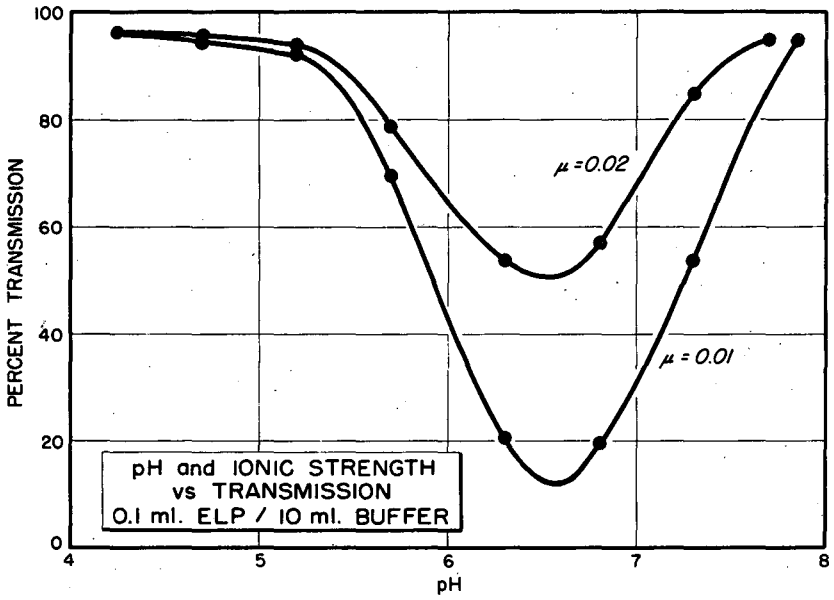
100
80
60
40
20
0
PERCENT TRANSMISSION
4
5
6
7
8
pH
μ = 0.02
μ = 0.01
pH and IONIC STRENGTH
vs TRANSMISSION
0.1 ml. ELP / 10 ml. BUFFER

Fig. 1

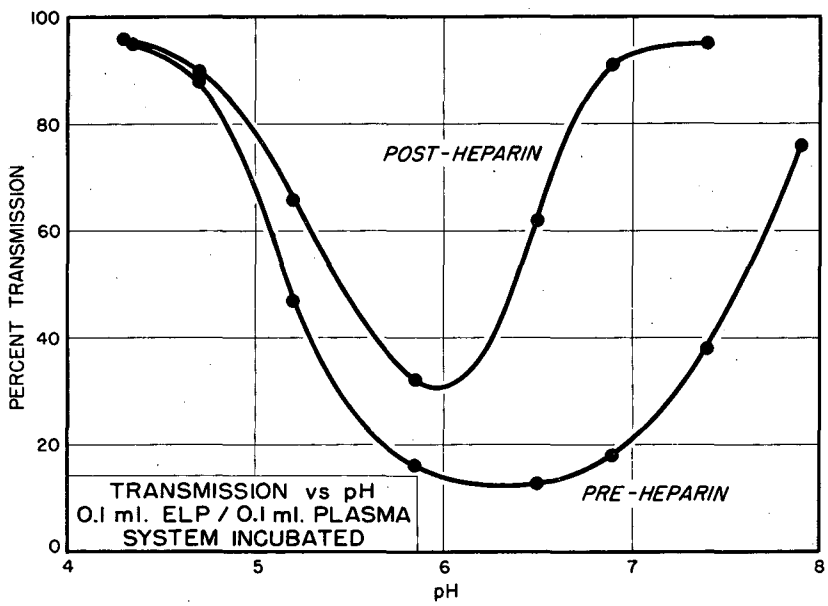
100
80
60
40
20
0
PERCENT TRANSMISSION
POST-HEPARIN
PRE-HEPARIN
TRANSMISSION vs pH
O.1 ml. ELP / O.1 ml. PLASMA
SYSTEM INCUBATED
4
5
6
7
8
pH

Fig. 2

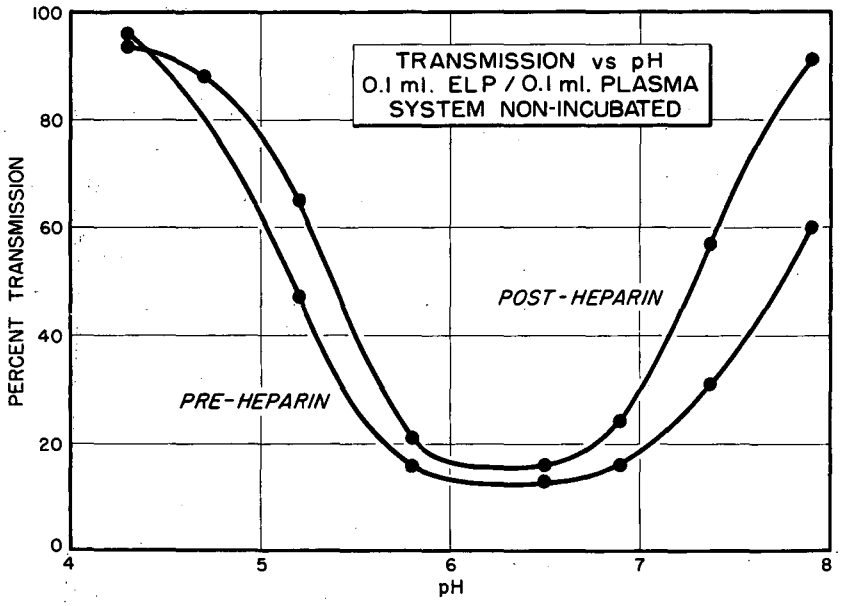
TRANSMISSION vs pH
0.1 ml. ELP / 0.1 ml. PLASMA
SYSTEM NON-INCUBATED
POST-HEPARIN
PRE-HEPARIN
PERCENT TRANSMISSION
100
80
60
40
20
0
4
5
6
7
8
pH

Fig. 3

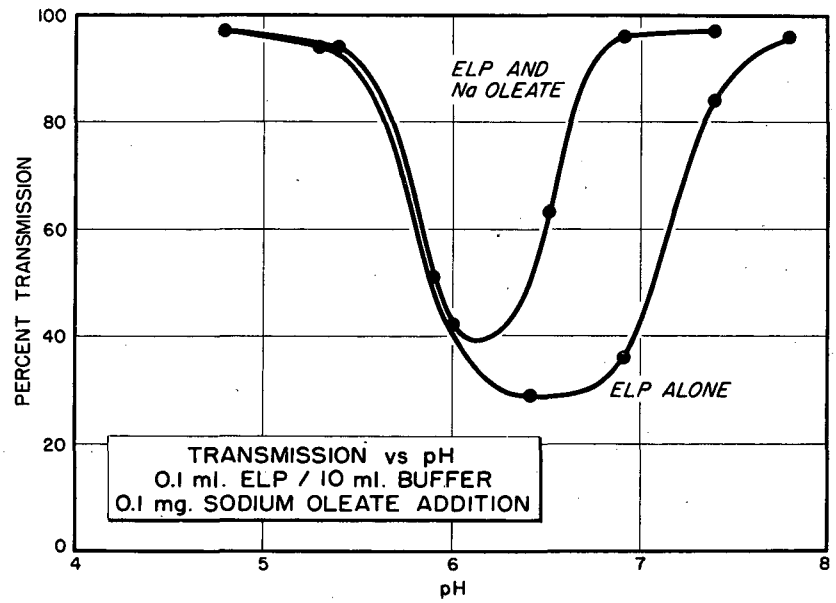
100
80
60
40
20
0
PERCENT TRANSMISSION
4
5
6
7
8
pH
ELP AND
Na OLEATE
ELP ALONE
TRANSMISSION vs pH
0.1 ml. ELP / 10 ml. BUFFER
0.1 mg. SODIUM OLEATE ADDITION

Fig. 4

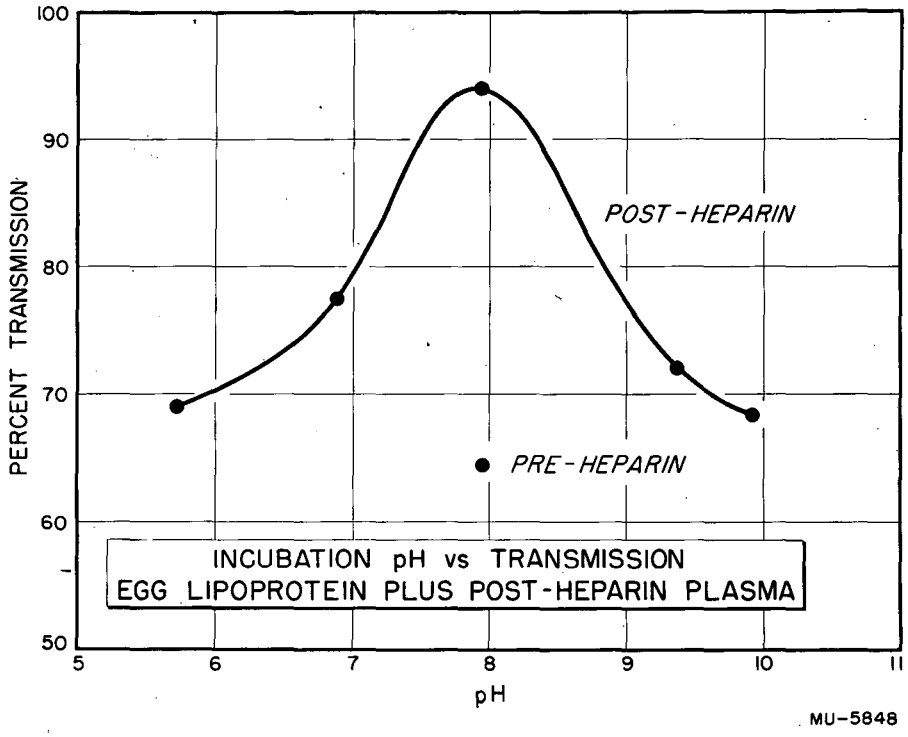
100
90
80
70
60
50
PERCENT TRANSMISSION
POST-HEPARIN
PRE-HEPARIN
INCUBATION pH vs TRANSMISSION
EGG LIPOPROTEIN PLUS POST-HEPARIN PLASMA
5
6
7
8
9
10
11
pH
MU-5848

Fig. 5

LEGEND FOR FIGS. 6 AND 7

Undilated postheparin plasma is designated C_o concentration; any dilution thereof with preheparin plasma or serum is designated as a fraction of C_o. The egg lipoprotein (ELP) stock solution (whose actual concentration is obtained by analytical ultracentrifugation) is also designated C_o concentration and any dilution thereof with appropriate buffer is a fraction of that C_o concentration.

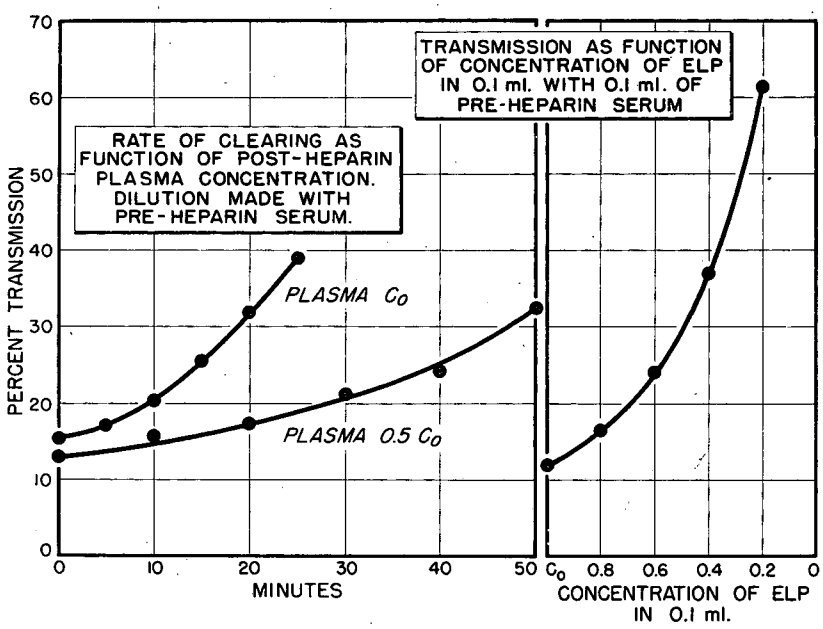
TRANSMISSION AS FUNCTION OF CONCENTRATION OF ELP IN 0.1 ml. WITH 0.1 ml. OF PRE-HEPARIN SERUM
RATE OF CLEARING AS FUNCTION OF POST-HEPARIN PLASMA CONCENTRATION. DILUTION MADE WITH PRE-HEPARIN SERUM.
PLASMA C_o
PLASMA 0.5 C_o
PERCENT TRANSMISSION
70
60
50
40
30
20
10
0
0
10
20
30
40
50
MINUTES
C_o 0.8 0.6 0.4 0.2 0
CONCENTRATION OF ELP IN 0.1 ml.

Fig. 6

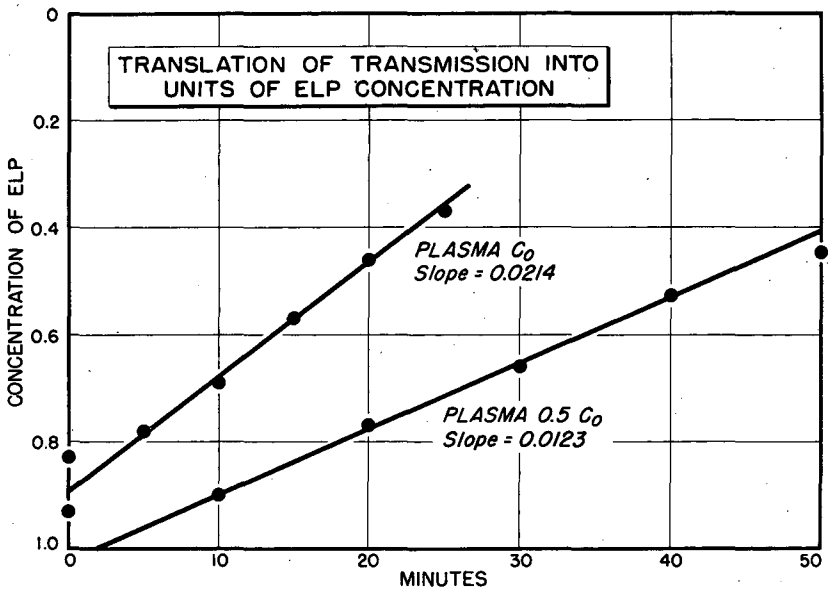
TRANSLATION OF TRANSMISSION INTO
UNITS OF ELP CONCENTRATION
PLASMA C_o
Slope = 0.0214
PLASMA 0.5 C_o
Slope = 0.0123
CONCENTRATION OF ELP
0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
MINUTES

Fig. 7

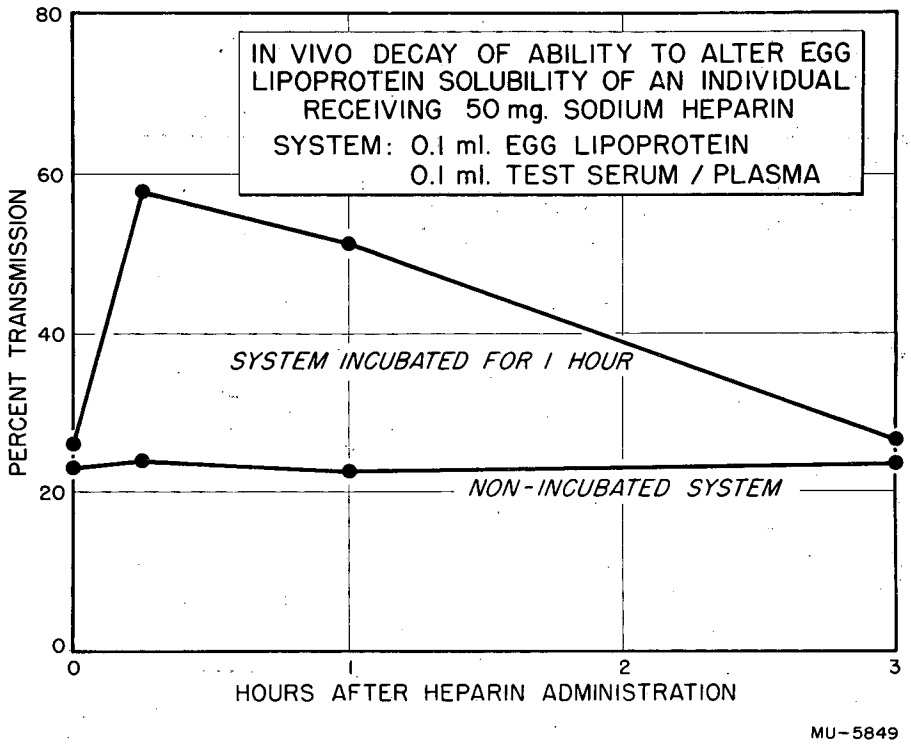
IN VIVO DECAY OF ABILITY TO ALTER EGG LIPOPROTEIN SOLUBILITY OF AN INDIVIDUAL RECEIVING 50 mg. SODIUM HEPARIN
SYSTEM: 0.1 ml. EGG LIPOPROTEIN
0.1 ml. TEST SERUM / PLASMA
SYSTEM INCUBATED FOR 1 HOUR
NON-INCUBATED SYSTEM
PERCENT TRANSMISSION
80
60
40
20
0
0
1
2
3
HOURS AFTER HEPARIN ADMINISTRATION
MU-5849

Fig. 8

ACKNOWLEDGMENT

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6. B. Shore, A. V. Nichols and N. K. Freeman. Proc. Soc. Exp. Biol. and Med. 83, (2), 216, 1953