The Role of Heparin in Lipoprotein Metabolism

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RECENT STUDIES BY GOFMAN AND HIS COLLABORATORS (*1*) have suggested the existence of a direct relationship between the plasma levels of certain physically distinct lipoprotein moieties and the incidence of atherosclerotic lesions. These long-range correlative studies have recently been given considerable support by the acute studies of Bragdon (*2*). His experiments, involving the production of early lesions in experimental animals by infusion of ultracentrifugally fractionated hypercholesterolemic plasma, indicate that pathogenicity may be much more closely related to the levels of certain cholesterol-protein complexes than to the level of total plasma cholesterol itself.

Although accurate interpretation of these findings must await the accumulation of additional data, the tentative conclusions are of considerable interest to biochemist and clinician alike, since they suggest that a specific chemical defect may be an important etiologic factor in atherosclerosis. In common with several other laboratories, therefore, we have begun studies on the nature of the linkages that bind cholesterol and fats to protein molecules, and of the homeostatic mechanisms that control the relative concentrations of lipoprotein classes in plasma.

In 1943 Hahn (*3*) observed that heparin caused rapid clearing of alimentary lipemic plasma *in vivo*, but that this decrease in turbidity did not occur *in vitro*. Other heparinlike substances also produce such effects (*4*). It was recently found by Anderson (*5*) that this "clearing" phenomenon could be brought about *in vitro* by treating lipemic plasma with plasma withdrawn from donor animals shortly after intravenous administration of heparin. Anderson's results indicated that heparin administration activated, or stimulated the production of, a substance in plasma possessing "antichylomicronemic" properties.

The hypothesis that heparin may play a specific role in lipid metabolism and transport has recently been extended by the findings of Graham and his collaborators (*6*), who observed that heparin administration in rabbits and humans caused a marked reorientation in the distribution of low-density lipoproteins and retarded the formation of high levels of so-called S_f 10–50 lipoproteins during cholesterol feeding. This latter observation is of particular interest in view of the postulated relation between lipoproteins of this density class and the incidence and induction of atheromata (*1, 2*).

In order to facilitate a study of the mechanism of the heparin effect, experiments were undertaken to purify and define the components of the *in vitro* system described by Anderson (*5*). Since it was of primary importance from the standpoint of experimental simplification to be able to produce "clearing factor" in an isolated tissue system, preliminary studies were carried out to determine the richest sources of activity. Restricted perfusions of anesthetized rats and dogs with heparinized plasma indicated that the abdominal and thoracic regions were capable of producing clearing activity at high levels. Perfusion of isolated hind limbs in a similar manner produced no clearing factor.[1] The content of clearing factor was assayed by measuring the decrease in turbidity per unit time upon incubating the perfusate with alimentary lipemic plasma obtained from dogs previously fed a fatty meal. In these experiments, control perfusions with heparinized saline, before and after perfusion with heparinized plasma, did not yield active material. It appeared, therefore, that material in plasma was being converted to clearing factor under the influence of a tissue catalyst and in the presence of heparin.

The data obtained thus far indicate that, of the tissues tested, heart and lung are most active in carrying out this conversion. In these experiments, rat tissue minces were incubated at 38° C or at room temperature with pooled human plasma to which heparin had been added. After centrifugation, the supernatants were tested for clearing activity against lipemic dog serum, as described above. Although the nature of this tissue factor cannot be ascertained on the basis of present data, preliminary experiments indicate that cell-free saline homogenates of heart and lung tissue retain the ability to catalyze clearing factor production from plasma. The results of a typical experiment of this sort are presented in Fig. 1. The data demonstrate that each of the components—tissue extract, heparin, and plasma—must be present for the production of active material. A small but demonstrable formation of clearing factor was observed in certain plasma samples incubated with heparin in the absence of tissue factor, which suggests that tissue factor may occasionally exist in plasma in low concentrations.

With the *in vitro* incubation technique it was possible to test plasma and plasma fractions for their ability to act as precursor in clearing factor formation. Normal dog plasma and pooled human plasma were fractionated by the low-temperature alcohol

[1] This result is in contrast to the findings of Weld (*7*), who obtained clearing of lipemic blood perfused in this manner. We have also observed some clearing under these conditions, but only in cases where collateral circulation, connecting the hind limb and the abdominal region of the animal, was still partially operative.

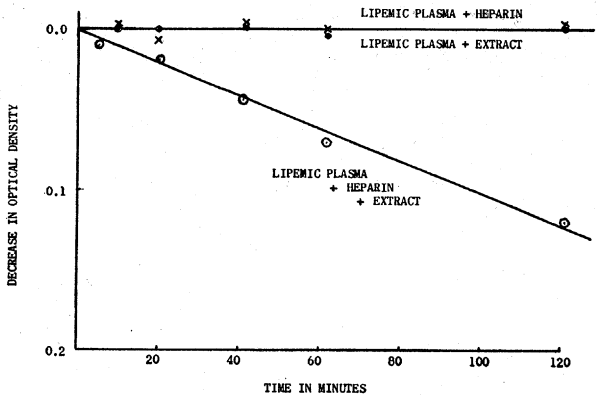

FIG. 1. The essential role of "tissue factor" in the clearing of lipemic plasma: Normal human plasma (0.4 ml) was incubated with 0.4 ml of a 4 : 1 saline homogenate of rat heart in the presence and absence of heparin (0.25 mg). In the upper control, heart extract was replaced by 0.25 mg heparin in 0.15 *M* NaCl. After 15 min at 37° C, the tubes were centrifuged, 0.5 ml of dog alimentary lipemic serum was added to 0.5 ml of the supernatant, and the rate of decrease in turbidity determined.

methods of Cohn, Edsall, and their collaborators (*8*), following their procedures 10 and 6 + 9.

The lyophilized fractions were then incubated with minced heart or lung tissue in the presence of heparin. After centrifugation, the supernatants were tested for clearing factor activity. Although a completely sharp fractionation of precursor substance was not obtained in these preliminary experiments, the data in Fig. 2 clearly indicate that the bulk of this material is localized in Fraction IV–1.

The fractionation characteristics of the clearing factor itself were determined, for the most part, on samples of plasma derived from humans, rats, cats, or dogs which had received intravenous heparin.[2] The activity produced by such injections was roughly proportional to the amount of heparin administered.

In Fig. 3 is presented a typical assay for clearing factor activity, in which rough proportionality between the volume of active plasma used and the change in turbidity is demonstrated. Upon low temperature alcohol fractionation, clearing activity was found to be localized, for the most part, in Fraction III–1, 2, 3 (Fig. 4), although Fractions III–0 and I exhibited considerable activity as well. It is felt that the activity found in Fraction III–0 is not comparable with that in other fractions since, as will become evident, this fraction contains still another component of the complete clearing system in particularly high concentration. The purification of the clearing factor by the single alcohol fractionation employed (Method 6 + 9) is about fourteenfold over the original plasma on a protein-nitrogen basis. Its abundant presence in Fraction III–1, 2, 3 confirms and extends the results of Graham *et al.* (*6*), who reported that the factor fell in the ultracentrifugal globulin fraction.

[2] Rabbits of the NIH strain were not good sources of clearing factor, nor was the lipemic plasma of cholesterol-oil-fed rabbits significantly changed by clearing factor from other sources in respect to either lipoprotein pattern or turbidity. The positive ultracentrifugal results of the Donner Laboratory group may well be explained on the basis of species variation (personal communication from J. W. Gofman).

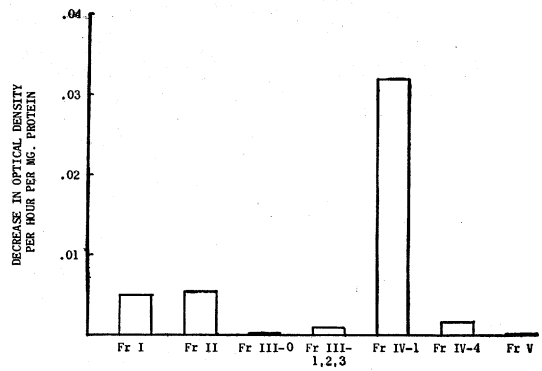

FIG. 2. The distribution of clearing factor-precursor in normal human plasma: A solution of each fraction in 0.15 *M* NaCl, at a concentration equivalent to that in whole plasma, was incubated with a tissue-factor preparation for 15 min in the presence of heparin, and centrifuged. The supernatant was tested against lipemic dog plasma for clearing factor activity.

Clearing factor is destroyed by heating at 60° C for 3 min, high levels of salt, and chymotrypsin digestion (Fig. 5, *A*). Its activity in clearing the turbidity of lipemic plasmas is maximal at approximately 40° (Fig. 5, *B*). The optimal pH for the clearing reaction is about 7.4. Clearing factor (as well as precursor (Fraction IV–1) and the tissue factor described above) withstand dialysis and lyophilization. The properties of clearing factor suggest that it may be enzymatic in nature, although further kinetic and equilibrium data are necessary to establish this point conclusively.

In order to simplify the test system for the assay of clearing factor activity, experiments were carried out in which the purified factor, Fraction III–1, 2, 3, was allowed to act on lipoproteins prepared by flotation from lipemic plasma, rather than on the plasma itself. The lipoprotein substrate was prepared by ultracentrifugation, at 80,000 *g* for one hour, of alimentary lipemic dog plasma layered under 0.85 per cent saline. Surprisingly, no decrease in turbidity was observed without the simultaneous addition of the in-

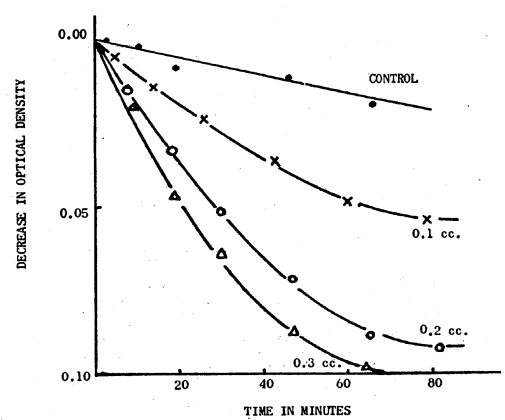

FIG. 3. The decrease in turbidity of lipemic serum as a function of clearing factor concentration: Plasma from a 17-kg dog given 25 mg of heparin intravenously 10 min before bleeding was used as a source of clearing factor; 0.0, 0.1, 0.2, and 0.3 ml were added to 0.5 ml lipemic human plasma, and total volumes adjusted to 1.0 ml with 0.15 *M* NaCl.

franatant fraction of plasma. These findings suggested the involvement of an additional "coprotein" acting in the capacity of acceptor or stimulator in the clearing system. Alcohol fractionation of plasma resulted in the partial purification of this coprotein to the extent of about fivefold. The data summarized in Fig. 6 indicate the essential role of this component in the clearing reaction. The distribution of coprotein among various plasma fractions is shown in Fig. 7, and it will be seen that the bulk of this material was found in Fraction III–0. Subsequent ultracentrifugal purification has demonstrated that coprotein activity resides in the nonlipoprotein portion of this fraction.

A tentative scheme, based on the fractionation studies described above, is presented in Fig. 8. In this scheme, a soluble tissue substance is depicted as catalyzing the conversion of a component of plasma, Fraction IV–1 to clearing factor, in the presence of added heparin (reaction A). The role of heparin in this reaction is obscure at present, although the possibility that heparin occurs as a tightly bound prosthetic group of clearing factor is suggested by the finding that active Fraction III–1, 2, 3 contains levels

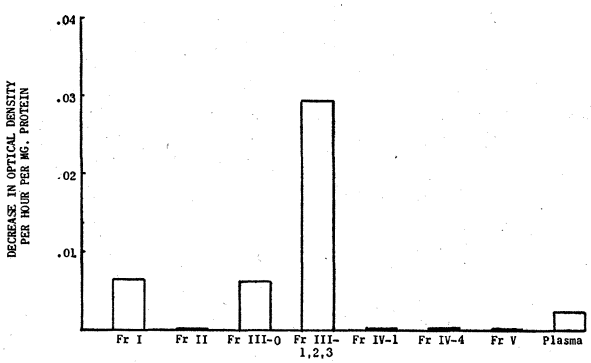

FIG. 4. The distribution of clearing factor in the plasma of an 18-kg dog which had received 500 mg of heparin 15 min before bleeding.

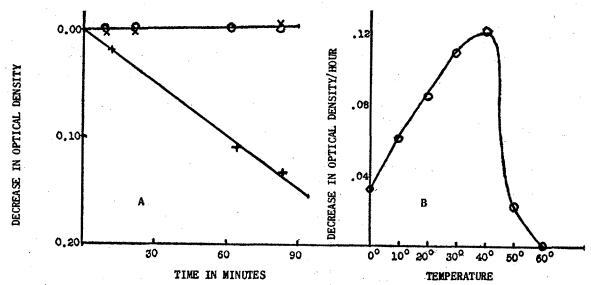

FIG. 5. A solution of each fraction in 0.15 *M* NaCl at a concentration equivalent to that in whole plasma was tested for clearing factor activity against alimentary lipemic dog serum. *A*, the action of chymotrypsin on clearing factor: Lower curve, untreated clearing factor (5 mg); upper curve, 0, clearing factor (5 mg) with 0.2 mg chymotrypsin; upper curve, *X*, 0.2 mg chymotrypsin. The proteins were dissolved in 1 ml 0.15 *M* NaCl. All tubes were buffered to pH 7.9 with phosphate buffer, final concentration 0.003 *M*. All were incubated at 37° C for 1 hr, and tested for clearing factor activity against alimentary lipemic dog serum.

B, the activity of clearing factor as a function of temperature. All samples were placed in a 37° C bath for 3 min before reading in the colorimeter, since lipemia turbidity varies with temperature.

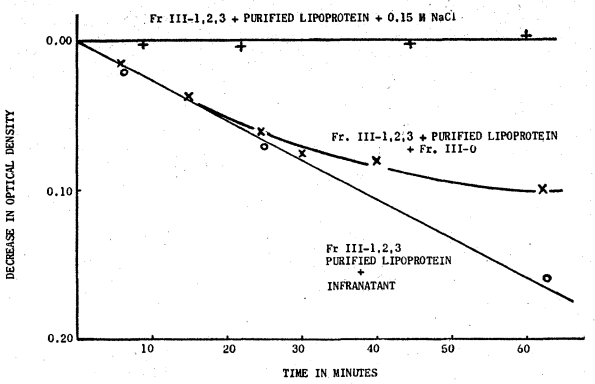

FIG. 6. The involvement of coprotein in the clearing of lipemic plasma: Five ml of dog alimentary lipemic serum was layered under 5 ml of 0.15 *M* NaCl and centrifuged at 80,000 *g* for 1 hr. The top 1 ml, containing very low-density lipoproteins, was separated by the tube-slicing technique, and the bottom 2 ml of infranatant was withdrawn. The following determinations were set up: Middle curve, 5 mg purified clearing factor (Fraction III–1, 2, 3) + 14.2 mg purified coprotein (Fraction III–0) + water, 0.9 ml; lower curve, 5 mg purified clearing factor + infranatant, 0.5 ml, + 0.15 *M* NaCl, 0.4 ml; upper curve, 5 mg purified clearing factor + 0.15 *M* NaCl, 0.9 ml. After warming, 0.1 ml of the purified lipoprotein was added to each, and the rate of decrease of turbidity determined.

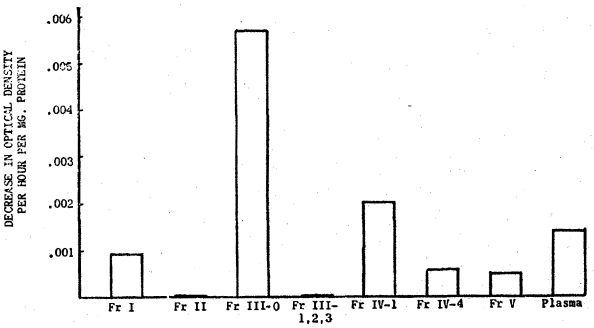

FIG. 7. The distribution of coprotein in normal human plasma: Normal human plasma fractions were dissolved in 0.15 *M* NaCl at a concentration twice that in the original plasma. To 0.5 ml of these fractions was added 0.5 mg of purified clearing factor in 0.4 ml 0.15 *M* NaCl, and after warming to 37° C, 0.1 ml of purified lipoproteins prepared by layering 5 ml of dog alimentary lipemia serum under 5 ml of 0.15 *M* NaCl, centrifuging for ½ hr at 80,000 *g* in the Spinco preparative ultracentrifuge, and discarding all but the top 1 ml of solution. The turbidity was recorded at various times after the addition of lipoprotein.

of bound heparin far in excess of that predicted by uniform distribution of the injected material in the total plasma space.[3]

In reaction B, clearing factor is pictured as catalyzing the physical or chemical redistribution of plasma lipids in such a way that turbidity is decreased, or, as in the ultracentrifugal studies of Graham *et al.*, and in certain of our own, a reorientation of plasma lipoproteins is brought about. The involvement of "coprotein" in this reaction suggests an acceptor role for this substance, although certain alternative explanations cannot be ruled out.

[3] The authors wish to express their sincere thanks to E. Cronkite, of the National Naval Medical Center, for carrying out the heparin assays.

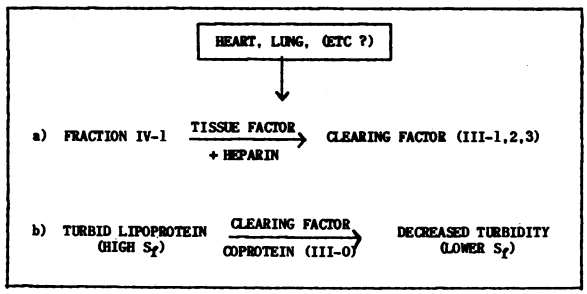

FIG. 8.

Since substantial quantities of clearing factor precursor and tissue factor exist in normal animals, the over-all data suggest that clearing factor may exist normally in plasma in low concentrations, dependent on available heparin supplies. This conclusion is supported by the finding that normal control rats, which are the most responsive of the experimental animals tested for ability to produce clearing factor upon heparin administration, frequently exhibit easily demonstrable clearing activity. The complete absence of atheromata in rats is well known (although early lesions have recently been induced in these animals by direct transfusion of human and rabbit low-density lipoproteins).[4]

The studies described in this paper are based for the most part on assays by the turbidity-decrease method. To relate the reactions outlined in Fig. 8 to the "lipoprotein reorientation" described by Graham and his collaborators, we have carried out ultracentrifugal tests on lipoprotein preparations before and after treatment with purified clearing factor. The results indicate that lower S_f lipoproteins accumulate at the expense of higher S_f components, in accordance with the findings of the Donner Laboratory group.

The data presented in Fig. 9 illustrate this reorientation phenomenon brought about by the action of purified clearing factor on the lipoproteins of a 62-year-old male individual with proved myocardial infarction. Three ml of serum was incubated with 40 mg of Fraction III–1, 2, 3 (active clearing factor) for 5 hours at 37° with gentle agitation. A control sample was run without clearing factor.

At the end of the incubation period the volume of the samples was brought to 10 ml and a density of 1.063 by the addition of NaCl solution. The low-density lipoproteins were separated by centrifugation at 79,640 *g* for 18 hours. The top 1.3 ml of each sample was removed by the tube-slicing technique and analyzed simultaneously in the 12-mm regular and wedge cells of the analytical Spinco ultracentrifuge to insure identical conditions during centrifugation. Photographs were taken at the intervals and speeds indicated in the legend of Fig. 9. The upper patterns in each case are from the treated sample.

Figure 9, *B* indicates that a decrease in the concentration of the S_f 30–150 class of lipoproteins has occurred in the sample treated with clearing factor. The patterns in Fig. 9, *C* indicate a decrease in the S_f 20–30 class (visible in the lower pattern as an inverted peak) and an increase in the S_f 10–20 lipoproteins in the treated sample. The concentration and distribution of the "normal" S_f 3–10 lipoproteins remain unchanged (Fig. 9, *D*).

[4] J. Bragdon and E. Boyle. Presented at the April meeting of the American Association of Pathologists and Bacteriologists.

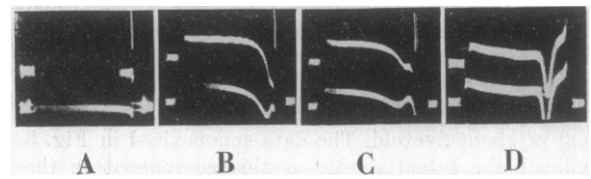

FIG. 9. The effect of clearing factor on the ultracentrifugal lipoprotein pattern: The upper pattern represents the sample treated with purified clearing factor, and the lower the control. Conditions: 18° C; density of solvent, 1.063; acceleration time, 6 min; inclined slit angle, 60°. Air bubble menisci are on the left of the pattern, components migrating from right to left against the centrifugal field. *A*, 10,000 rpm during acceleration; exposure time, 10 sec. *B*, zero minutes after reaching maximum speed, 56,100 rpm; exposure time, 1 sec. *C*, 2 min; exposure time, 1 sec. *D*, 30 min; exposure time, 1 sec.

During the incubation described above, a considerable decrease in the turbidity of the treated sample was observed during the first hour. In the course of the subsequent 4-hour period, however, a marked increase in turbidity took place. The appearance of the control sample did not change throughout the incubation. The patterns in Fig. 9, *A* (photographed, during acceleration, at a rotor speed of 10,000 rpm) clearly show the presence of a large quantity of low-density material released in the sample treated with clearing factor. This material had migrated out of the optical system at 20,000 rpm.

The accurate interpretation of these findings must await further study. Nevertheless, the ultracentrifugal results combined with the turbidity changes observed suggest that a component of the complete clearing system, probably coprotein, present during the first hour of incubation was exhausted during the subsequent 4-hour period. This explanation is supported by the finding that, in the experiment described above, a direct assay indicated the complete absence of coprotein in the ultracentrifugal infranatant of the sample treated with clearing factor, but considerable quantities of coprotein in the untreated control. The results presented here are being extended in the direction of further purification of the components of the clearing system and toward a more detailed study of the mechanism of lipid labilization both *in vivo* and *in vitro* under the influence of these purified materials.

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