PARTIAL PURIFICATION OF CLEARING FACTOR OF POSTHEPARIN HUMAN PLASMA

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The rather extensive work done in recent years on the mechanism of the lipemia-clearing phenomenon induced by heparin *in vivo* has resulted in the demonstration of the clearing factor (CF) as a new type of lipase, which is active only against protein-bound neutral fats[1,2]. In spite of this advance, the isolation of the active enzyme has not, thus far, been successful; the number of attempts has also been relatively few. ANFINSEN *et al.*[3] showed that the clearing factor occurs in Fraction III-1,2,3 in the ethanol fractionation scheme of Cohn, and they could thus achieve about a 14-fold purification of the activity. Later, ANFINSEN AND QUIGLEY[4] reported a purification up to 100- to 200-fold by a relatively laborious procedure, based on a complex formation between the CF and low density lipoproteins (enzyme–substrate complex). Earlier studies in our laboratory[5] have revealed that the clearing activity was precipitated quantitatively from plasma at a low ionic strength and at pH 5 to 6, along with 1/20 to 1/25 of the plasma proteins. The finding was confirmed somewhat later by HOOD *et al.*[6]. Using this preparation, HOLLETT AND MENG[7] have recently reported a further purification (up to 1500-fold) of the activity by the use of ammonium sulphate fractionation. With this procedure, however, the yield remains very low (16% recovery), and the method is also questionable owing to the known inactivation of CF at high ionic strength.

A new approach to the problem of CF purification was suggested by the demonstration of NILSSON AND WENCKERT[8] in which was shown that heparin is adsorbed to known prothrombin adsorbents and can be eluated from these by citrate, and that a natural heparin-like anticoagulant occurs in plasma and accompanies prothrombin through the various stages of its purification. These findings led us to assume that CF, because of its probable heparin content, might behave similarly. This appeared, in fact, to be the case, and the principles of a simple and effective purification procedure thus achieved have been outlined in a preliminary communication[9].

In the present paper a more detailed account of the purification procedure and some further developments are given. In its present stage it allows the purification of the activity of postheparin human plasma up to *ca.* 700-fold in a simple and rapid way and with a good yield.

METHODS AND PREPARATIONS

Postheparin plasma was derived from normal human donors 10 to 15 min after an i.v. administration of 75 mg of sodium heparin (Vitrum). Because this amount of heparin proved to be inadequate to inhibit the separation of some fibrin during storage of the plasma completely, 1/30 volume of 0.1 *M* sodium oxalate was added to the blood, and the small precipitate formed in

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the plasma was removed by centrifugation. The clearing activity of oxalated plasma is about 10% lower than that of untreated postheparin plasma, apparently owing to adsorption of the activity to the calcium oxalate precipitate. In spite of this, oxalate was found the most convenient anticoagulant for this purpose. Citrate cannot come into question, heparin disturbs possible analyses of the composition of the active preparation, and plasma containing EDTA loses its activity rather rapidly. Stored at $+4°$ C oxalated plasma retains its activity unchanged for 8 to 10 days.

Assay of clearing activity was performed by measuring the decrease of optical density during 30 min at 37° C in a mixture containing 0.5 ml of the solution to be tested, plus 0.5 ml of 0.25% (v/v) emulsion of pure triolein (stabilized with Tween 80 that was prepared each day) in physiological saline, 0.3 ml of protein solution (either normal human serum or postheparin plasma adsorbed with calcium phosphate), and 1.2 ml of physiological saline. In connection with the experiments, the observation was made that citrate ion increases the clearing activity to a considerable extent, and, therefore, the citrate concentration was equalized within each assay series by the addition of a proper amount of sodium citrate solution. The activities are expressed as decrease of optical density per 30 min measured at 650 mμ and with 1-cm light path. The original postheparin plasma samples used had an activity of 0.200 to 0.250.

Calcium phosphate gel was prepared from anhydrous calcium chloride and trisodium phosphate according to the directions of QUICK AND STEFANINI[10]. The true concentration of each preparation was checked by dry weight determination. The gel was stored as 0.2 *M* stock, from which a 1:4 dilution (0.05 *M*) was made before use. In the course of the experiments it became clearly evident that aged gels gave much better results than freshly prepared ones, which tended to dissolve during washing of the adsorbate. Therefore, the gel should preferably be prepared 1 to 2 months before use (*cf.* SINGER AND KEARNEY[11]).

The grade of purification was estimated by determining the tyrosine content by a modified method of LOWRY *et al.*[12] and converting the values to protein by means of a standard curve prepared with bovine serum albumin. This method can, of course, give approximate values only, but it is adapted for the present purpose because of its simple and rapid performance. The specific activity is expressed as clearing per mg of protein.

EXPERIMENTAL

The essential steps in the purification procedure are adsorption of the activity from plasma with calcium phosphate, washing of the adsorbate with sodium oxalate, and elution with sodium citrate. To determine the optimal conditions for each stage the following series of experiments was performed.

Amount of $Ca_3(PO_4)_2$. The minimum quantity of calcium phosphate needed for maximal recovery varied somewhat in different plasma samples even when derived from the same donor. Highest recoveries, generally 80–90%, were obtained by the use of 0.05 to 0.10 ml of 0.2 *M* gel per 1 ml of plasma when the elution was made directly from unwashed adsorbate. The protein content of the eluate is then 1/40 to 1/50 of the original plasma, and its specific activity is 0.180 to 0.200 per mg of protein (about 0.004 in postheparin plasma). Higher grades of purification are achieved at the expense of the yield by the use of smaller amounts of gel. If the adsorbate is washed before elution, the optimal gel/plasma ratio is somewhat higher than on direct elution, apparently owing to a slight solubilization of the gel by the washing solution. In different experiments this ratio varied between 0.10 to 0.15 ml of 0.2 *M* $Ca_3(PO_4)_2$ per 1 ml of plasma. Freshly prepared gel should be used in still greater amounts to obtain maximal recovery.

Effect of time, temperature, pH and ionic strength on the adsorption. All these experiments were made by adsorbing the activity from 1 volume of plasma with 0.1 volume of 0.2 *M* Ca phosphate and eluting, without washing, with 0.05 *M* Na citrate.

The dependence of the recovery percentage on the adsorption time and temperature are shown in Fig. 1. A rather efficient adsorption occurs immediately on mixing of the gel and plasma, but maximal recovery is achieved by increasing the adsorption

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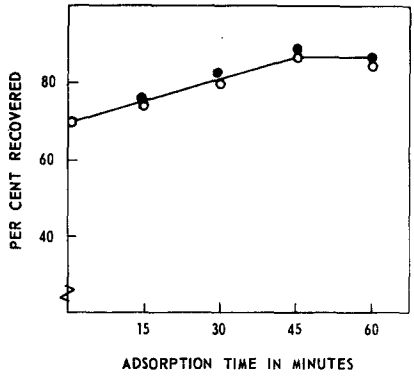

Fig. 1. Effect of adsorption time and temperature on the recovery of CF in eluate. Amount of $Ca_3(PO_4)_2$: 0.1 ml, 0.2 *M*, per 1 ml of plasma. Direct elution with 0.05 *M* sodium citrate. ○: adsorption at room temperature; ●: adsorption at 4° C.

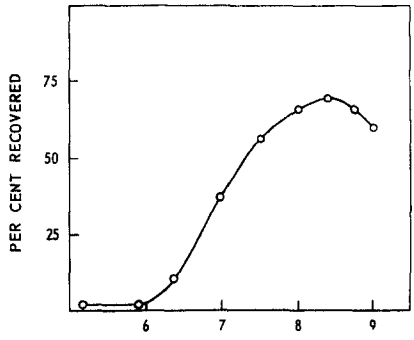

Fig. 2. Effect of pH on the adsorption of CF from postheparin plasma on $Ca_3(PO_4)_2$. Procedure identical to that in Fig. 1.

time up to 45 min. There is no essential difference between the effectiveness of adsorption at room temperature and at 4° C. In the following experiments a 45-min adsorption at 4° C was used throughout.

The effect of pH on adsorption is apparent from Fig. 2. Only the recovery percentage is presented since the protein content of the eluates is largely independent of the pH of the adsorption mixture. The optimum pH range is 8.0 to 8.7, *i.e.* any adjustment of the pH of plasma before adsorption is unnecessary.

Dilution of plasma before adsorption with physiological saline increases both the recovery percentage and the specific activity of the eluate, the latter effect being apparently only due to a decreased attachment of protein to the vessel walls. The optimal range of dilution is 1:3 to 1:5 (Table I). The effect of ionic strength (NaCl) on the adsorption is illustrated in Fig. 3. Both the yield and the specific activity are maximal at the range between 0.15 and 0.30. Thus, adjustment of ionic strength is also unnecessary.

TABLE I

DEPENDENCE OF RECOVERY AND PURIFICATION GRADE OF CF IN THE CITRATE ELUATE ON THE DILUTION OF POSTHEPARIN PLASMA BEFORE ADSORPTION

Dilution	*% recovery*	*Spec. activity clearing/mg/30 min*
Undiluted	70	0.130
1:1	77	0.175
1:3	81	0.210
1:5	78	0.200
1:9	65	0.160

Removal of inactive protein from the adsorbate. A considerable proportion of the protein adsorbed to the gel can be removed by washing with water or various salt solutions without any significant elution of the CF (Table II). Of the various single salt and buffer solutions tried, sodium oxalate proved to be definitely superior in

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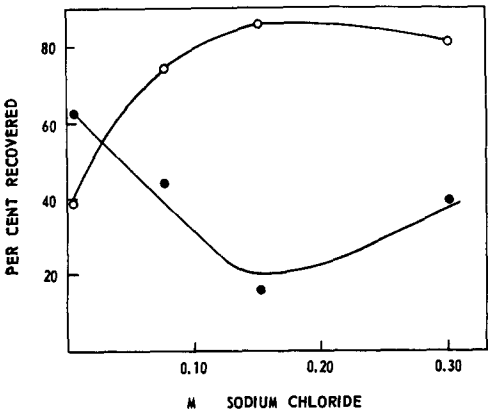

Fig. 3. Effect of ionic strength on the adsorption of CF from postheparin plasma on $Ca_3(PO_4)_2$ gel. Adjustment was made with addition of NaCl. Otherwise, the procedure is identical to that in Fig. 1. ○ : activity of citrate eluate; ● : activity of plasma after adsorption.

TABLE II

REMOVAL OF PROTEIN FROM $Ca_3(PO_4)_2$ ADSORBATE OF POSTHEPARIN PLASMA ON WASHING WITH VARIOUS SOLUTIONS

Elution of clearing activity to 0.05 *M* sodium citrate.

Washing with	*Percentage recovery of clearing activity*	*Protein concentration ratio eluate/plasma*	*Specific activity (clearing/ mg/30 min)*
Direct elution	85	1:45	0.170
Water	60	1:70	0.210
0.9% NaCl	65	1:80	0.260
Na oxalate 0.1 *M*	70	1:180	0.650
NaH_2PO_4 0.1 *M*	70	1:70	0.250
Na_2HPO_4 0.1 *M*	0	—	—

this respect. The dependence of the purification grade on the concentration of oxalate in the washing solution is seen in Fig. 4. By treatment with 0.2 *M* sodium oxalate only 1/300 to 1/400 of the protein, but 60–80% of the clearing activity of the starting plasma, was recovered in the citrate eluate, which has a specific activity of the order of 1,500. On repeated washing with oxalate additional inactive protein is removed from the gel, and an eluate containing about 60% of the activity and purified 600- to 700-fold over the postheparin plasma can thus be obtained (specific activity of

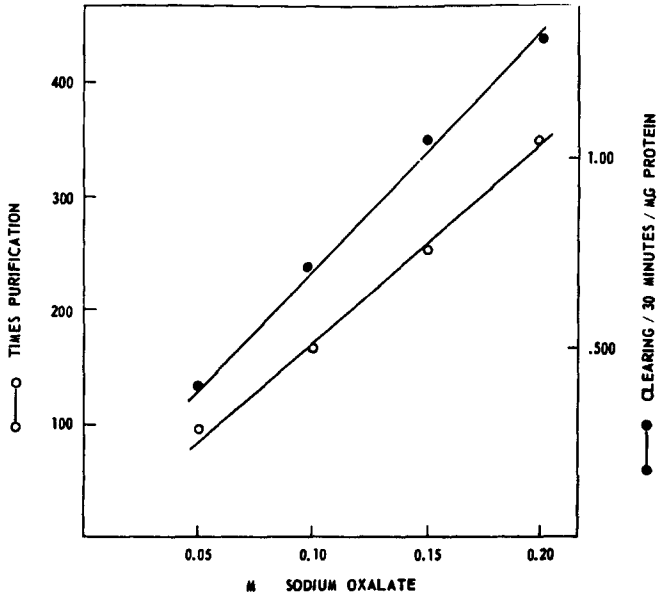

Fig. 4. Dependence of the purification grade of CF in citrate eluate on the concentration of oxalate in washing solution. Adsorption on 0.1 volume of 0.2 *M* $Ca_3(PO_4)_2$, elution with 0.05 *M* sodium citrate.

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the order 2,000). Washing with double original volume twice for 20 min at +4° C produces the maximal effect.

A further purification after this step was attempted by the low pH–low ionic strength precipitation of the eluate previously freed from citrate by dialysis, but no precipitation occurred.

Elution. CF is highly soluble in the presence of citrate-ion concentrations as low as 0.005 *M*. On elution of the activity from the calcium phosphate, maximal recovery is obtained with solutions from 0.03 to 0.08 *M*, higher concentrations being somewhat less effective (Fig. 5). The high degree of solubility of the enzyme into citrate solutions is also revealed in the volume of the latter needed for effective elution. Thus, the recovery is equally good on elution with 1/6 as with double the original plasma volume. This is advantageous from the point of view of further purification, since impractically large fluid volumes can be avoided. A maximal elution occurs at once on mere suspension of the gel into citrate solution and, therefore, centrifugation can be done immediately to obtain a clear or slightly opalescent active supernatant.

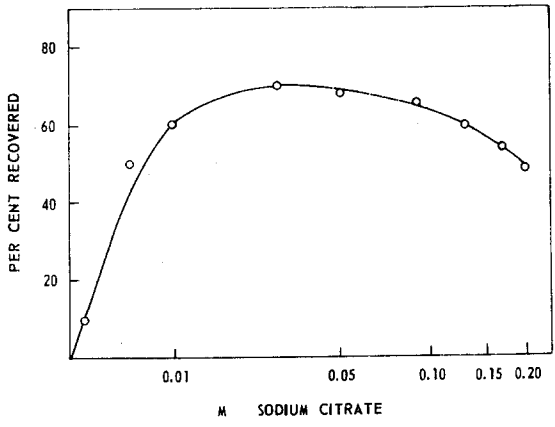

Fig. 5. Elution of clearing activity from $Ca_3(PO_4)_2$ gel with sodium citrate solutions.

Purification procedure. One volume of postheparin plasma is diluted with 3 volumes of physiological saline, and 0.10 to 0.15 volumes of 0.2 *M* calcium phosphate gel (diluted) are added. The optimal gel/plasma ratio varies somewhat for different plasma samples, and, therefore, if an exact ratio is desired it should be determined separately for each plasma. After gentle shaking for 45 min the adsorbate is spun down by centrifugation. The supernatant is discarded if not used as protein solution in the CF assay. The walls of the centrifuge tube are carefully dried, *e.g.* with a cotton plug. Then the adsorbate is suspended into 2 volumes of 0.2 *M* sodium oxalate and the tube is shaken gently for 15 min. The precipitate is separated by centrifugation and the washing is repeated. Finally, the gel is eluted with a desired volume of 0.05 *M* sodium citrate. All operations are preferably carried out at 4° C.

DISCUSSION

The CF preparation obtained by the method described is far from pure (containing prothrombin among other proteins), but further purification is being attempted by

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the preparative-zone electrophoresis technique. These additional steps are needed before any statement can be made concerning the composition of the enzyme.

The adsorption and solubility properties of CF strongly suggest that heparin forms an integral part of its structure. Evidence for a similar composition of the lipoprotein lipase of rat tissues was recently provided by KORN[13], who has demonstrated a partial inactivation of the enzyme by heparinase. The heparin moiety is thus essential for the lipolytic activity, probably in the stage of formation of the enzyme–substrate complex (see below). Application of the present procedure to the purification of the tissue enzyme is under trial.

The relation of prothrombin to the CF activity is an interesting problem worthy of further investigation. In this connection a tentative explanation for the clot-promoting effect of lipemia presents itself. It has been shown by NILSSON AND WENCKERT[8] that the link between prothrombin and the heparin-like anticoagulant is weak enough to break on dialysis and that the release of the latter is followed by an activation of prothrombin to thrombin. On the other hand, the studies of BROWN[14] and of LAURELL[15] suggest the presence of heparin–chylomicron complexes in lymph and blood, *i.e.*, an affinity of heparin to these lipid particles. Hence, it can be assumed that the appearance of chylomicra in the blood causes a dissociation of the heparin–prothrombin complex, and thus leads to an accelerated generation of thrombin.

SUMMARY

Like prothrombin, the lipemia-clearing activity of postheparin plasma is adsorbed on tricalcium phosphate gel and can be eluted with citrate. A considerable proportion of inactive protein adsorbed can be washed out with sodium oxalate. Clearing factor preparations with a specific activity of about 500 times greater than that of postheparin plasma can thus be obtained simply and rapidly. The optimal conditions in each stage of the procedure have been determined.

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Received September 14th, 1957