

STUDIES ON THE NATURE AND THE PURIFICATION OF THE
COAGULASE-REACTING FACTOR AND ITS RELATION
TO PROTHROMBIN*

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Although considerable progress has been achieved in the study of the coagulation of plasma by staphylocoagulase, the precise identity of the coagulase-reacting factor (CRF) has remained in doubt. The claims linking CRF with various factors involved in the clotting of blood have been recently summarized elsewhere (1). Questions pertaining to the possible relation of CRF to prothrombin have remained particularly challenging. To be sure, prothrombin and CRF activities have been dissociated by repeated Seitz (asbestos) filtration (2-4). While this observation establishes that CRF may exist independently of prothrombin activity, it does not preclude the possibility that other factors eliminated by the filtration procedure, including prothrombin itself, may function similarly. The prime objective of the present report has therefore been twofold:

1. To purify and characterize CRF as recovered from human plasma after prothrombin activity has been reduced to a minimum by serial Seitz filtration.
2. To study directly the CRF activity of highly purified preparations of human prothrombin.

Materials and Methods

Staphylocoagulase.—Highly purified products were used, to the extent that on analytical ultracentrifugation, over 95 per cent of the material formed a single symmetrical peak. The method of purification has been described elsewhere (5). The concentration of the coagulase was adjusted so that it clotted an equal volume of non-inhibitory citrated human plasma within 17 to 20 seconds.

Prothrombin.—Four purified human prothrombin preparations were furnished by Dr. Walter H. Seegers. Several of these lots were prepared from 20 liter plasma pools of citrated blood bank discards, shipped in the frozen state from Atlanta to Detroit. The techniques of purification are referred to in recent studies by Seegers and his associates (6). According to the data of Seegers, the potency of these products had such values as 24,400 units/mg. of tyrosine for lot 541216 and 20,700 units for lot 550503.

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Plasma.—Outdated or VDRL-positive citrated human blood bank discards were used, excluding those showing significant inhibition of clotting by coagulase when compared to a non-inhibitory plasma control.

Filtration of Plasma.—Pools of 1 liter were filtered through 5 sterilizing Seitz asbestos pads, 14 cm. in diameter, collecting the filtrates in suction flasks under a vacuum. Unusually turbid plasmas were first passed through a Seitz clarifying pad. Before use, all filters were thoroughly washed until the oxalate test for calcium ions turned negative.

Titration of CRF.—The appropriate plasma or CRF preparations were diluted in physiological saline containing 2 mg./ml. of bovine albumin (Armour). To each dilution of CRF in a volume of 0.2 ml., 0.2 ml. of bovine fibrinogen (Armour fraction I) was added, at a concentration of 250 mg. per cent. 0.2 ml. of coagulase was then blown into the tube as the stop watch was started. The end point was taken as the first wisp of fibrin or the first appearance of floccules with more slowly reacting mixtures. Observations were made at room temperature with a loop magnifying 5 times, using a fluorescent light source. The clotting times of the various dilutions were plotted, and the per cent activity extrapolated arithmetically. Controls included the clotting times of mixtures of coagulase and fibrinogen as well as of plasma dilutions and fibrinogen.

Prothrombin Time.—Minor modifications of the one step method of Ware and Stragnell (7) were adopted. For the prothrombin conversion, either thromboplastin (Difco, Detroit) and calcium, or simplastin (Warner-Chilcott, New York) with bovine serum as the source of accelerator globulin, were employed.

Nitrogen Determinations.—The micro-Kjeldahl technique was used as described by Kabat and Mayer (8), substituting for the indicator a mixture of methyl red and brom-cresol green.

Paper Electrophoresis.—The determinations were made in a Spinco Model R Durrum type apparatus using veronal buffer at a pH of 8.6, ionic strength of 0.075, and the runs were generally made at 5 ma. for 16 hours, as is customary for the electrophoresis of serum.

Ultracentrifugation.—The preparations were sedimented in a Spinco analytical ultracentrifuge at 59,780 R.P.M., or 259,700 g. Photographs were taken at 16 minute intervals, and runs generally were terminated within 2 hours. The lack of sufficient material limited the observations to obtaining uncorrected sedimentation rates only, and the molecular weights were approximated from the charts of Svedberg and Pedersen (9), and Lundgren and Ward (10).

Preparation of Absorption Columns.—Chromatographic apparatus of varying dimensions, supplied by Scientific Glass Co., was employed. For processing 2 liter batches of plasma, columns were used which were 660 mm. in length, 80 mm. in diameter, and had a joint size of 71/40. 500 gm. of hyflo grade of celite (Johns-Manville, New York) and 110 ml. of aluminum hydroxide gel (amphogel, without flavor, Wyeth and Co., Philadelphia) were thoroughly mixed with 3 liters of distilled water, the mixture was introduced into the column, and packed solidly by tapping gently with a flanged glass rod as the water was collected in a suction flask under negative pressure maintained by an oil vacuum pump. The column was further washed by passing through it several liters of distilled water. The plasma at 6–8°C. was then carefully layered on the column to minimize any disturbance of the surface, and only columns were processed further which permitted an even progression of the plasma front and which lacked any significant faults or cracks. All fractions were collected under negative pressure in suction flasks at a room temperature of about 21.5°C., and tested for activity.

RESULTS

The purification of CRF.—Munro and Munro (11) have used phosphate buffer elution from amphogel in the purification of prothrombin, and preliminary studies have indicated that this aluminum hydroxide preparation

may also be useful in the purification of CRF (12). Working with plasma lots of 100 ml., purification of the order of 125-fold was attained, but the products proved quite labile, and at times significantly active as prothrombin as well. It became necessary to modify the procedure so that larger lots of plasma could be processed which might make feasible higher concentrations thus favoring its stability.

After various grades of celite were tested, hyflo proved most acceptable as the filler, and different amounts of filler and amphogel were first tested in smaller lots. The ratio of 500 gm. of hyflo to 110 ml. of amphogel was adopted for the processing of 2 liter batches of plasma. The scheme of purification of CRF on celite-amphogel columns is herewith summarized.

1. Filter 2 liters of plasma through five successive washed sterilizing Seitz pads, using 1 liter of plasma per pad 14 cm. in diameter.
2. Layer carefully the plasma on the column prepared as described under Materials and Methods.
3. Discard the plasma eluate.
4. Add 2 liters of 0.1 M phosphate buffer at a pH of 8. Discard eluate.
5. Add 1 liter of 0.2 M phosphate buffer at a pH of 8. Discard eluate.
6. Add 1 to 2 liters of 0.2 M phosphate buffer at a pH of 8.6 and collect fractionally the eluates, adjust pH to 7.0, and check each sample separately for CRF activity. Select the most purified and active sample for further processing.

The course of a representative column purification of CRF is summarized in Tables I *a* and I *b*, the former presenting the clotting times obtained, and the latter expressing the results in terms of yield and purification. The 5 X Seitzed plasma is taken as the base line (sample 1), and itself involves a loss of some 50 per cent of CRF and over 95 per cent loss of prothrombin of the unfiltered plasma. The greatest removal of inactive nitrogenous impurities takes place in samples 2 and 3, with only approximately 15 per cent of the CRF sacrificed. The use of 0.2 M phosphate buffer at pH 8 serves essentially as a washing step, with little further release of nitrogen or activity. Upon the introduction of 0.2 M phosphate buffer at a pH of 8.6, however, significant CRF activity is released. While all three fractions tested separately showed CRF activity, the second lot (5 *b*) possessed the greatest activity, and the highest purification factor of 173-fold. There was, however, a slight concentration of prothrombin activity as well, estimated at about 10 per cent of the prothrombin content of the original unfiltered plasma.

In the course of carrying out column purifications on 20 different plasma pools, it became evident that many inherent and technical factors may influence the results significantly. For example, raising the amount of amphogel to 130 ml. led to a great delay in the elution of active CRF with 0.2 M buffer at a pH of 8.6; indeed, not until 900 ml. of buffer was used did a subsequent sample exhibit a high order of purification of some 210-fold. It is quite probable that with the aid of constant positive pressures and an automatic fraction collector, the yield and purification might be further improved.

Attention has already been drawn to the need of carefully packing the column, and to the fact that the development of cracks and an uneven plasma advance may lead to unpredictable results. The outcome has also been prejudiced by some operations taking considerably longer than the 5 to 6 hours

TABLE I a
Purification of CRF on Hyflo-Amphogel Column

Sample		CRF clotting times at final plasma dilutions below					Prothrombin* times
		1:30	1:60	1:120	1:240	1:480	
		sec.	sec.	sec.	sec.	sec.	sec.
1	5 × Seitzed plasma	32	49	101	195	305	79
2	Plasma eluate	335	510				90
3	0.1 M phosphate pH 8.0	205	350				75
4	0.2 M phosphate pH 8.0	>420					85
5 a	0.2 M phosphate pH 8.6	42	76	157			85
5 b	0.2 M phosphate pH 8.6	32	50	74			60
5 c	0.2 M phosphate pH 8.6	65	135				87

* 100 per cent prothrombin, 28 seconds; 50 per cent prothrombin, 35 seconds; 25 per cent prothrombin, 45 seconds; 10 per cent prothrombin, 55 seconds; 1 per cent prothrombin, 85 seconds.

TABLE I b
Purification of CRF on Hyflo-Amphogel Column

Sample		Volume	CRF	Total CRF units (volume × per cent CRF)	CRF yield	N/ml.	CRF per mg. N	Purification factor
1	5 × Seitzed plasma	2000	100	200,000	100	8.65	11.5	—
2	Plasma eluate	2000	5	10,000	5	7.96	0.6	—
3	0.1 M phosphate pH 8 eluate	2000	10	20,000	10	4.05	2	—
4	0.2 M phosphate pH 8 eluate	1200	<3	3,600	1.8	0.06	50	—
5 a	0.2 M phosphate pH 8.6 eluate	350	50	17,500	8.75	0.14	357	31
5 b	0.2 M phosphate pH 8.6 eluate	380	100	38,000	19.0	0.05	2000	173
5 c	0.2 M phosphate pH 8.6 eluate	380	35	13,000	6.5	0.03	1166	101

generally sufficient to process 2 liters. This delay was at first attributed to an excessively tight packing of the columns, but actually was traced to the plugging of the fritted disk of the inner member of the chromatographic assembly. This difficulty may be best overcome by thoroughly cleansing the disks before each use, or by employing perforated disks protected by glass wool.

Since the best products attained by the column procedure had nitrogen

values in the range of 0.03 to 0.06 mg. per ml. they were too dilute for direct further study on paper electrophoresis and on analytical ultracentrifugation. These fractions also proved to be quite labile on standing, even when refrigerated. Various techniques were, therefore, explored to concentrate the product, and to attain further purification. Prolonged rotation dialysis against 25 per cent dextran in the cold bath at 0°C. achieved excellent concentration, with little loss of activity, and the removal of some cold precipitable impurities. Such concentrated products were then further purified by the fractional addition of ammonium sulfate. Subsequently, it was found feasible to treat the CRF directly with ammonium sulfate at 0°C., provided the final column eluates were processed at once. Substantial impurities were first cut off by adding ammonium sulfate crystals to 33 per cent saturation, and allowing the precipitate to form in the cold for about 6 hours. This fraction contained fibrinogen, and relatively little CRF. The final product was generally precipitated by adding ammonium sulfate to a final concentration of 45 to 50 per cent. The precipitates which formed in the cold then redissolved readily in a small volume of saline, and formed the final active product. However, this step often involved a considerable loss of CRF. Such procedures generally resulted in an additional purification factor of some 40- to 50-fold relative to the initial filtered plasma. Thus, in the case of sample 5*b* of Table I *a* and I *b* the ammonium sulfate treatment raised the purification factor from 172-fold to 210-fold, and concentrated the sample 16-fold. A 40 per cent loss of CRF yield, however, was incurred. These products were now sufficiently concentrated and purified to render possible direct studies by paper electrophoresis and by analytical ultracentrifugation to serve as a basis of comparison with purified human prothrombin.

Paper Electrophoresis of Purified CRF.—On paper electrophoresis, the purified CRF preparations invariably stained the area between the beta and the gamma globulin zones when tested under the conditions applied to the separation of serum proteins. This finding is expressed graphically in Fig. 1, and represents the tracing of the CRF superimposed on the tracing of human serum, as recorded by the Spinco calibrated recording photometer (analytrol). To determine the relation of CRF activity to the protein-stained zone, duplicate strips were left unstained, and on the completion of the migration, were dried and sectioned at 1 cm. intervals. These strips were then eluted with a constant volume of phosphate buffer, and the CRF activity was found to correspond to the zone of maximal staining between the beta and the gamma globulins.

Analytical Ultracentrifugation of Purified CRF.—Although subject to some variation depending on concentration, all samples exhibited a major symmetrical peak which in three separate lots gave uncorrected sedimentation rates of 2.75, 2.99, and 3.07 respectively. In addition, a heavier and much smaller

component was found, with sedimentation rates of 6.37, 6.68, and 6.95 respectively. It may thus be estimated that the major component has a molecular weight approximating 30,000, while the minor component approximates molecular weights between 110,000 and 160,000. (Fig. 3).

Comparison between Human Prothrombin and Purified CRF

Previous tests of purified bovine prothrombin of Seegers (2) failed to indicate any significant reactivity with staphylocoagulase. Since bovine plasma is deficient in CRF, the non-reactivity of the bovine preparations may represent a species effect, and need not apply to human or rabbit prothrombin, derived from plasmas high in CRF content. Direct studies of highly purified

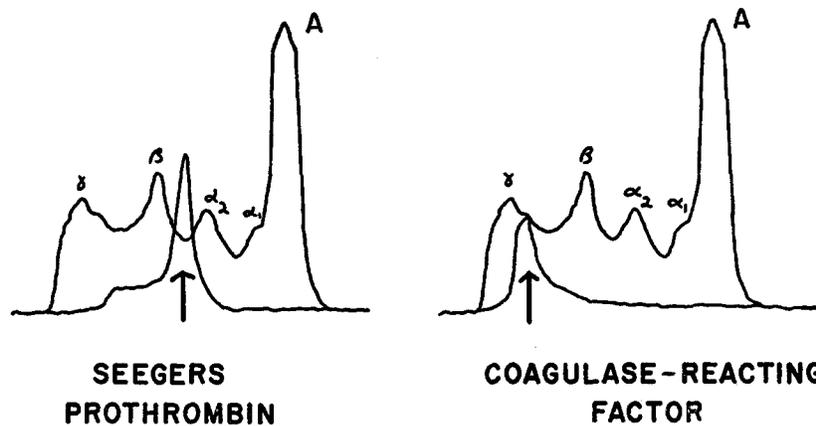


FIG. 1. Paper electrophoresis of purified CRF and of purified Seegers human prothrombin, superimposed on human serum, photometer (analytrol) representation. A, albumin peak; α_1 , α_2 , β , and γ are the respective globulin peaks.

and active human prothrombin were therefore undertaken to furnish a basis for comparison with the purified CRF.

The four human prothrombin preparations of Seegers proved to be highly reactive with coagulase. In Fig. 2, the effect of coagulase on prothrombin is compared to the conversion of prothrombin by means of thromboplastin, calcium, and accelerator globulin in the form of bovine serum. At this level of coagulase potency, the coagulation proceeded as rapidly upon the addition of coagulase as upon the addition of the battery of accelerators and cofactors implicated in the physiological conversion of prothrombin to thrombin.

Although purified prothrombin was highly reactive with coagulase, its characteristics on paper electrophoresis differed sharply from those established by the purified CRF (Fig. 1). The prothrombin peak fell between the α_2 and the β serum peaks when 0.01 ml. of a 2 per cent solution was used.

When the amount was doubled, the prothrombin peak tended to overlap the α_2 position; indeed with one preparation it was shifted more towards the α_1 position. In no instance, however, did any of the purified human prothrombin preparations localize in the zone between the beta and the gamma position in which the purified CRF consistently fell. Buffer elution of unstained strips again indicated a close correspondence between activity and the zones corresponding to the maximal protein staining.

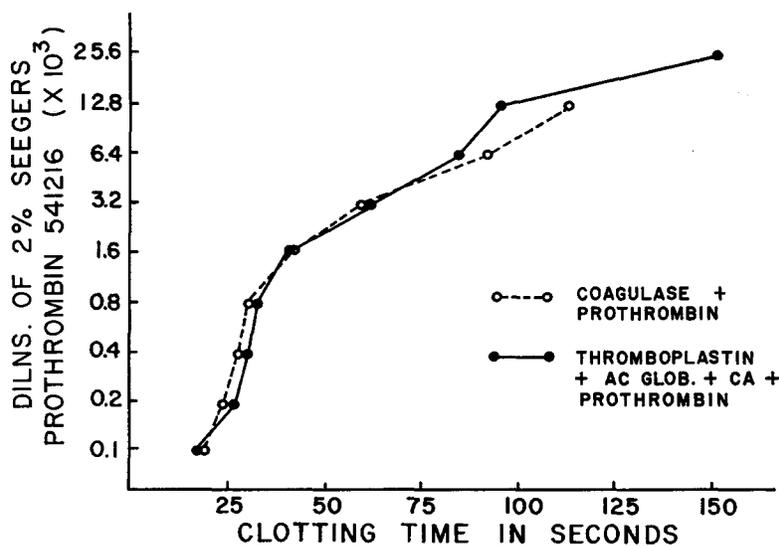


FIG. 2. The action of coagulase on purified human prothrombin of Seegers compared to the "physiological" conversion of prothrombin.

Controls: prothrombin 1:100 + fibrinogen, 480 seconds; prothrombin 1:200 + fibrinogen, 900 seconds; prothrombin 1:100 + fibrinogen + thromboplastin + calcium, 142 seconds; prothrombin 1:100 + ac. glob. + fibrinogen, 480 seconds; ac. glob. + fibrinogen + thromboplastin + calcium, 142 seconds; coagulase + fibrinogen, 15 minutes.

On analytical ultracentrifugation, the human prothrombin preparations examined did not possess the homogeneity and consistency reported by Lamy and Waugh (13) for purified bovine prothrombin preparations of Seegers. These authors reported a sedimentation constant of 4.85, and a molecular weight of 62,700. This finding was at variance with an estimated molecular weight of 140,000 found with other preparations and referred to by Seegers and Ware (14). In the present study, the major human prothrombin component gave uncorrected sedimentation rates of such values as 6.2 and 6.38, corresponding closely to the higher molecular weight range reported for bovine prothrombin, while a minor component fell in the range reported by Lamy and Waugh. Regardless of the question of the purity of these preparations

versus the possibility of complexing of the prothrombin molecule, none of the values corresponded to those of the principal CRF component. (Fig. 3).

CRF Activity of Prothrombin Derivatives

The observation that CRF activity is associated with plasma products very low in prothrombin (purified CRF) as well as with highly purified prothrombin itself, raises the question of the possible relation of these preparations to each other. It becomes pertinent to determine whether the prothrombin molecule might be deliberately altered to cause it to lose its prothrombin

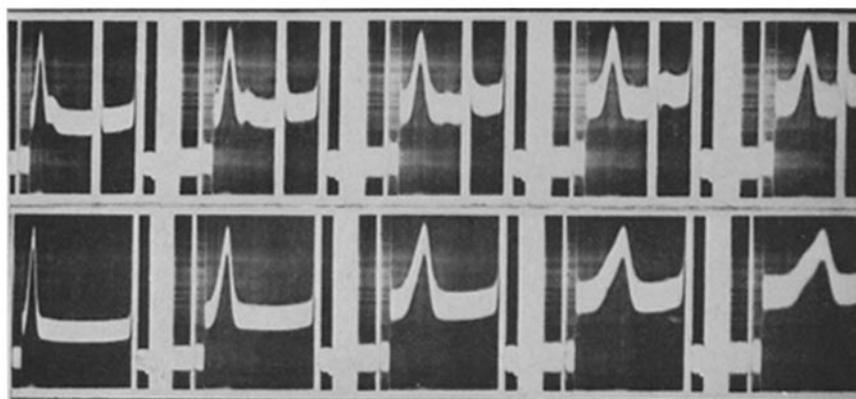


FIG. 3. Ultracentrifugal patterns of purified CRF and human prothrombin. Top picture: Purified CRF in saline (separation cell), $s = 2.99$ (large peak) 6.68 (small peak). Bottom picture: Purified human prothrombin, 1 per cent, in saline (standard analytical cell); $s = 6.20$ (large peak) and 4.93 (small peak).

function, and yet not abolish its function as CRF. It has already been established (2) that the conversion of prothrombin to thrombin, involving the breakdown of prothrombin into many smaller units, does not eliminate CRF activity. When the thrombin is sufficiently diluted so that its direct reaction with fibrinogen does not obscure the effect of coagulase, it is still possible to demonstrate a very significant acceleration of clotting upon the addition of the staphylococcal product.

Further support for the concept that prothrombin activity *per se* is not essential for the exhibition of CRF activity was recently furnished by the study of a preparation of "autoprothrombin," prepared as follows by Dr. Takeshi Abe in Dr. Seegers' laboratory (15). Prothrombin was first incubated with platelet factor 3 and calcium, followed by the addition of purified accelerator globulin. The product was then ultracentrifuged to remove the platelet factor, and the material shell frozen and stored in the deep freeze overnight.

After thawing, the material was dialyzed against cold distilled water, and lyophilized. This procedure caused a drop of prothrombin activity from 25,800 units per ml. to one of 256 units per ml., and the thrombin activity remained very small. When this preparation was reacted with coagulase however, it showed excellent CRF content, although the speed of clotting at higher dilutions was slower than with the unmodified prothrombin from which it has been derived (Table II).

TABLE II
CRF Clotting Times of Purified Prothrombin and Its "Autoprothrombin"

Prothrombin dilution of					"Autoprothrombin" dilution of			
1:300	1:600	1:1200	1:2400	1:4800	1:300	1:600	1:1200	1:2400
<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>	<i>sec.</i>
26	30	36	50	91	26	32	61	132

Prothrombin 1:300 plus fibrinogen and no coagulase: no clot in 30 minutes;
 "Autoprothrombin" 1:300 plus fibrinogen and no coagulase: clot in 100 seconds;
 Coagulase plus fibrinogen control: clot in 25 minutes.

DISCUSSION

The identity of CRF has been in question since this factor was first proposed by Smith and Hale (16). In the opinion of many observers (1-4, 16, 17), the key to the problem has been the relation of CRF to prothrombin. Other principal contenders, such as fibrinogen, have been essentially eliminated from consideration. The arguments for and against relating CRF to prothrombin have been recently summarized (1). The present study has sought to resolve an apparent inconsistency: the view that prothrombin and CRF are identical, and the separation of prothrombin and CRF activity by repeated Seitz filtration.

By direct test, it has been established that 4 separate highly purified and active human prothrombin preparations of Seegers effectively function as CRF. When, however, prothrombin activity is virtually eliminated, as by converting prothrombin to autoproteithrombin or to thrombin, CRF activity is not likewise eliminated. Evidently, therefore, the whole prothrombin molecule is not essential for CRF function.

The purification of CRF after Seitz filtration herewith reported lends further support to the existence of CRF independent of prothrombin activity. Duthie and Lorenz (17) made the intriguing suggestion that the filtration procedure does not actually remove prothrombin, but so alters it that it no longer functions as prothrombin, but yet operates as CRF. The present data, however, have indicated that the purified CRF recovered has a distinctive

mobility on paper electrophoresis, and that its principal component has a sedimentation rate differing from that of any of the prothrombin components. It is tempting to speculate that CRF is a part of the prothrombin molecule, with a molecular weight estimated to be about half that of the minor human prothrombin molecule, and half that of the single bovine prothrombin component reported by Lamy and Waugh (13). However, there is no direct proof that this CRF is derived from prothrombin, nor is there certain knowledge that this fraction exists in native plasma or is split off during the filtration procedure.

Both the purified CRF and the purified human prothrombin presented a major and a minor component on ultracentrifugation. It is possible that the heavier components represent an aggregation of smaller units, or polymerization, rather than unrelated impurities. While it is noteworthy that the components fit into a pattern of multiples of molecular weights of approximately 35,000, similar relations have been earlier claimed for many unrelated proteins as well (18). It is, therefore, not possible at the present time to appraise with any degree of assurance the significance of the minor peaks observed. Direct evidence for the association of activity and the maximal protein concentration has been obtained, however, by buffer elution of paper strips after electrophoresis, both with prothrombin and with CRF.

The present observations therefore lend themselves to a unifying hypothesis of the nature of CRF. It is suggested that the capacity to react with coagulase, or CRF activity, resides in some groups of the prothrombin molecule, and these groups are not involved in prothrombin function as such. The whole molecule will exhibit both prothrombin and CRF activity, but a part of the molecule, no longer functioning as prothrombin is implicated in CRF activity, and is preserved in such derivatives of prothrombin as thrombin and auto-prothrombin, and possibly the purified CRF of Seitz-filtered plasma as here described. This hypothesis explains why prothrombin and CRF activities may parallel each other closely, as noted following dicumarol (4, 19) or phenylindanedione (19) administration, or diverge partially or completely, as occurs after Seitz filtration.

SUMMARY

The coagulase-reacting factor (CRF) of human plasma has been purified, concentrated, and largely freed of prothrombin activity by Seitz filtration, absorption on hyflo-amphogel columns, controlled phosphate buffer elution, and fractional ammonium sulfate precipitation.

The purified CRF preparations localized between the beta and the gamma globulin position on paper electrophoresis, and the principal of two components observed on analytical ultracentrifugation gave sedimentation rates between 2.75 and 3.07.

Highly purified prothrombin preparations of Seegers effectively react with staphylocoagulase to lead to the coagulation of plasma.

The electrophoretic and ultracentrifugal properties of the human prothrombin preparations examined differ significantly from those of the purified CRF.

Derivatives of prothrombin, involving a loss of prothrombin activity, such as thrombin and "autoprothrombin," do not correspondingly lose in CRF function.

The hypothesis is proposed that CRF activity is resident in some component or components of the prothrombin molecule not involved in prothrombin function *per se*, and that therefore prothrombin and CRF activity may either parallel each other when the molecule is intact or diverge when the smaller units only are involved.

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