

## MECHANISM OF ACTION OF FACTOR D OF THE ALTERNATIVE COMPLEMENT PATHWAY\*

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Factor D, also referred to as C3 proactivator convertase, constitutes the activating enzyme of the C3 convertase of the alternative pathway (2). As such, it is an essential component of initiation and amplification of the pathway (3, 4). The enzyme cleaves factor B, or C3 proactivator, into the activation fragment Ba (30,000 daltons) and the active site bearing fragment Bb (62,000 daltons) (5, 6). This reaction proceeds only when factor B is in Mg<sup>++</sup>-dependent association with C3b and results in the formation of C3b,Bb which is endowed with C3 cleaving activity (2, 7).

Unlike other alternative pathway components, factor D has escaped unequivocal elucidation as to its mode of action, probably because it is a trace protein in human serum and its function is mimicked by certain tryptic enzymes which are unrelated to complement. Uncertainty persists as to whether factor D (*a*) is absolutely required for C3/C5 convertase formation, (*b*) is physically incorporated into this multimolecular enzyme, and (*c*) occurs in plasma and serum in zymogen form, in which case the mode of activation is unknown.

We wish to report that factor D occurs in human plasma and serum only as an active enzyme and that it is absolutely required for C3/C5 convertase formation under physiological conditions without becoming a subunit of this complex enzyme. Thus, factor D, which is responsible for the first enzymatic event of the alternative pathway, necessitates no activation and is subject to no consumption. Its function depends entirely upon proper presentation of its substrate. It may be of biomedical significance that the enzyme that plays an essential role in the initiation and amplification of the alternative pathway is available in the circulation of the host in active form only.

### Materials and Methods

*Materials.* The following materials were purchased from the sources indicated: Bolton-Hunter reagent (<sup>125</sup>I), Amersham Corp., Arlington Heights, Ill.; diisopropylfluorophosphate (DFP),<sup>1</sup> phenylmethylsulfonyl fluoride (PMSF) and crystallized bovine serum albumin from

\* Publication 1586. Supported by U. S. Public Health Service grants AI 07007 and HL 16411. Presented in part at the American Society of Biological Chemists and American Association of Immunologists Joint Meeting, Atlanta, Georgia, June, 1978 (1).

‡ Supported by Fellowship IG58 of the Delegation à la Recherche Scientifique et Technique, France.

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<sup>1</sup> *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; DTT, dithiothreitol; E<sub>R</sub>, rabbit erythrocytes; GPS, guinea pig serum; GVB<sup>++</sup>, VB<sup>++</sup> containing 0.1% gelatin; GVBE, VB containing 0.1% gelatin and 0.01 M EDTA; NF, nephritic factor; P, properdin; PMSF, phenylmethylsulfonyl fluoride; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; TAME, *p*-tosyl-L-arginine methyl ester HCl; VB, isotonic veronal-buffered saline, pH 7.4; VB<sup>++</sup>, VB containing 1.5 × 10<sup>-4</sup> M CaCl<sub>2</sub> and 5 × 10<sup>-4</sup> M MgCl<sub>2</sub>; Z, lysis.

Calbiochem, San Diego, Calif.; bovine trypsin, Worthington Biochemical Corp., Freehold, N.J.; ampholine carrier ampholytes, LKB Instruments, Rockville, Md.; EGTA, Eastman Kodak Co., Rochester, N. Y.

**Buffers.** VB: isotonic veronal-buffered saline, pH 7.4; VB<sup>++</sup>: VB containing  $1.5 \times 10^{-4}$  M CaCl<sub>2</sub> and  $5 \times 10^{-4}$  M MgCl<sub>2</sub>; GVB<sup>++</sup>: VB<sup>++</sup> containing 0.1% gelatin; GVBE: VB containing 0.1% gelatin and 0.01 M EDTA; DGVB<sup>++</sup>: half isotonic VB<sup>++</sup>, pH 7.4, containing 2.5% sucrose and 0.1% gelatin; Mg-EGTA: 0.1 M MgCl<sub>2</sub>, 0.1 M EGTA adjusted to pH 7.0 and used in blood at the final concentration of  $5 \times 10^{-3}$  M EGTA; Mg-GVB: VB containing  $1.2 \times 10^{-3}$  M MgCl<sub>2</sub> and 0.1% gelatin.

**Purified Components.** Highly purified human C3 (8), factor B (5), and properdin (P) (9) were isolated as described. Factor D was purified according to Medicus et al. (10) with modifications. Briefly, human serum containing 0.01 M EDTA was subjected to a four-step procedure, including chromatography on QAE Sephadex A50 (pH 8.5), CM52 (pH 6.0), and Bio-Rex 70 (pH 7.0). Factor D was eluted at 9, 16, 20 mS, respectively, with a linear salt gradient. Further purification was obtained by gel filtration on Sephadex G75. 1,600 ml of serum yielded 0.5 mg of factor D. Highly purified human  $\alpha$ -thrombin (2,200 U/mg) was kindly provided by Dr. J. H. Griffin of the Department of Immunopathology, Scripps Clinic and Research Foundation. Plasminogen was isolated as described (11) and kindly provided by Dr. J. P. Gorski of this Department. 1 mg of plasminogen was activated by 30,000 U of streptokinase (Hyland Diagnostics Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) during 30 min at 37°C in VB.

**Preparation of Factor D Immune Adsorbent.** The IgG fraction (40% ammonium sulfate precipitate) of rabbit anti-factor D antiserum (12) was coupled to cyanogen bromide-activated Sepharose utilizing the method of March et al. (13).

**Preparation of C3b Coupled to Sepharose.** Sepharose 4B gel was coated with human C3b according to Medicus (14). In brief, cobra venom factor coupled to cyanogen bromide-activated Sepharose was incubated in presence of Factors B, D, and Mg<sup>++</sup> and then C3 was added after washing.

**Preparation of Sheep Erythrocytes Bearing C3b (EC3b).** EC3b<sub>try</sub> was prepared as described (9). Additional C3b was then deposited on the cells by means of the nephritic factor stabilized C3 convertase as reported (15). The number of C3b molecules per cell estimated by using trace amounts of <sup>125</sup>I-C3 varied between 100,000 and 150,000.

#### Assays for Factor D

**HEMOLYTIC ACTIVITY ASSAY.** 50  $\mu$ l of a mixture of P (3  $\mu$ g/ml), factor B (15  $\mu$ g/ml), and EC3b ( $2 \times 10^8$ /ml) in GVB<sup>++</sup> was added to 5  $\mu$ l of sample containing factor D activity and incubated for 5 min at 37°C. The C3/C5 convertase activity was measured by the extent of hemolysis produced during 10 min incubation at 37°C in 1 ml of guinea pig serum (GPS) diluted 1:100 in GVBE (GPE-EDTA). Under these conditions, the C3/C5 convertase activity was directly proportional to factor D concentration between 1 ng and 10 ng/ml.

**RADIOIMMUNOASSAY (RIA).** The packed factor D immune adsorbent was suspended in 3 vol of GVB<sup>++</sup>. 160  $\mu$ l of the suspension was added to plastic tubes containing 40  $\mu$ l of a preincubated mixture of the unlabeled protein to be tested and 40 ng of <sup>125</sup>I factor D. After 30 min at 37°C and 1 h at 4°C, the unbound material was removed by three washings in cold GVB<sup>++</sup> and the radioactivity of the pellet determined. The specific binding (percent) was calculated according to the following formula:

$$\frac{\text{radioactivity (cpm) of experimental pellet} - \text{radioactivity (cpm) of control pellet}}{\text{radioactivity (cpm) of input (40 ng } ^{125}\text{I factor D)}}.$$

The control pellet was prepared from a reaction mixture that contained 1.2  $\mu$ g of unlabeled factor D (30-fold excess over the amount of labeled factor D).

**Assays for Esterolytic Activity.** The synthetic esters listed in Table I were dissolved at a concentration of 0.01 M in  $5 \times 10^{-2}$  M Tris buffer, pH 7.4, containing  $1 \times 10^{-3}$  M CaCl<sub>2</sub> unless otherwise indicated. 2  $\mu$ g of factor D were incubated with 1  $\mu$ mol of synthetic ester in a final vol of 120  $\mu$ l for 1 h at 37°C. The amount of hydrolyzed ester was determined by the colorimetric method of Roberts (16). In addition, the esterase activity of various enzymes and factor D was quantitated with *P*-tosyl-L-arginine methyl ester HCl (TAME) using a spectrophotometric procedure (17). The rate of increase of adsorbance of 247 nm was proportional to

TABLE I  
Synthetic Substrates

L-Lysyl methyl ester	(LME)
N-Acetyl-L-arginine methyl ester HCl	(AAME)
N- $\alpha$ -Acetyl glycy-L-lysine methyl ester	(AGLME)
N-Acetyl-L-tyrosine methyl ester, hemihydrate	(ATME)*
N- $\alpha$ -Benzoyl-L-arginine methyl ester HCl	(BAME)
N- $\alpha$ -Benzoyl-L-lysine methyl ester HCl	(BLME)
p-Tosyl-L-arginine methyl ester HCl	(TAME)
N- $\alpha$ -Tosyl-L-lysine methyl ester HCl	(TLME)
N- $\alpha$ -CBZ-L-lysine methyl ester HCl	(CBZ LME)
N-CBZ-Glycyl-L-tyrosine methyl ester	(CBZ GTME)*

All chemicals were purchased from Cyclochemical Co., Los Angeles, Calif., with the exception of TAME which was obtained from Calbiochem.

\* Solubilized by addition of 5% ethanol.

enzyme concentration allowing measurement of TAME units (1  $\mu$ mol of TAME hydrolyzed/min) per mg of enzyme.

*Assay for Proteolytic Activity.* A mixture of 100  $\mu$ g of B chain of oxidized bovine insulin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 35  $\mu$ g of factor D (or 1  $\mu$ g of trypsin which was used as positive control) in a final vol of 75  $\mu$ l of VB<sup>++</sup> was incubated for 2 h at 37°C. Degradation of B chain of insulin was then examined by thin-layer chromatography on cellulose sheet (no. 13255, Eastman Kodak Co.) in 1-butanol-acetic acid-water (200:30:75). After 7 h, the chromatogram was developed with ninhydrin spray (18).

*Factor D Depletion from Human Serum.* Factor D depleted sera were obtained by three methods: first, gel filtration on a Sephadex G75 column (1.5  $\times$  30 cm) of 2 ml of human serum containing 0.01 M EDTA. After elution with VBE, the high molecular weight proteins contained in 0.6 column vol were pooled and concentrated to the original serum volume. This procedure was repeated twice and the serum finally dialyzed against VB. This technique was also used for GPS and the pool of low molecular weight proteins eluted after 0.6 column vol was used as source of guinea pig factor D. Second, 10 ml of serum containing 0.01 M EDTA and adjusted to pH 7 was passed over a Bio-Rex 70 column (1.5  $\times$  15 cm) equilibrated with VBE, pH 7. The breakthrough fraction was collected and dialyzed against VB. Third, immune adsorption was used as published (19).

*Electrophoresis.* Electrophoresis in 1.2% agarose was performed in barbital buffer, pH 8.6, 0.05 mS, 0.01 M EDTA, for 2 h at 4.6 vol/cm. 7% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Weber et al. (20). Isoelectrofocusing electrophoresis in Pevikon blocks was performed according to Reisfeld et al. (21) with 2% ampholine (pH 5-9).

*Sucrose Density Gradient Ultracentrifugation.* It was performed in 5 ml linear 5-20% sucrose density gradients in half isotonic VB<sup>++</sup> at pH 6.5, 7.3, or 8.3 at 130,000 g for 5.5 h at 4°C in a Beckman L5-65 ultracentrifuge using an SW 50 L rotor.

## Results

*Purified Factor D: Correlation of Protein, Hemolytic Activity, and Radioactivity.* Purified factor D was radiolabeled with <sup>125</sup>I according to Bolton and Hunter (22), because the techniques using chloramine T (23) or lactoperoxidase (24) did not achieve iodine incorporation. Iodine uptake was approximately 15% and the specific radioactivity of factor D was 0.4  $\mu$ Ci/ $\mu$ g. The position of the radioactivity in SDS polyacrylamide gels after electrophoresis was identical in presence or absence of dithiothreitol (DTT) and corresponded to the single protein band observed upon staining with Coomassie Blue (Fig. 1). Factor D hemolytic activity and radioactivity were also superimposed and found in the  $\gamma$ 1 region upon agarose electrophoresis (Fig. 2).

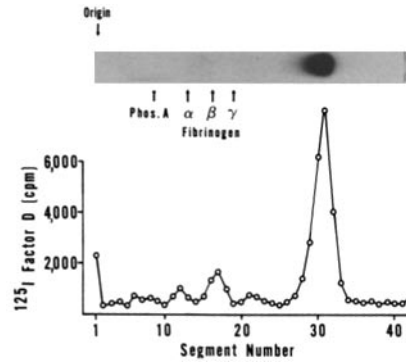


FIG. 1. Isolated factor D: identical distribution of factor D protein and protein bound radioactivity after 7% SDS polyacrylamide gel electrophoresis. Upper panel: gel containing 40  $\mu\text{g}$  of factor D stained with Coomassie Blue. Lower panel: radioactivity distribution of gel containing 0.5  $\mu\text{g}$   $^{125}\text{I}$  factor D subjected to electrophoresis under identical conditions. Arrows indicate position of reference proteins (phosphorylase A and reduced fibrinogen) contained in a third gel.

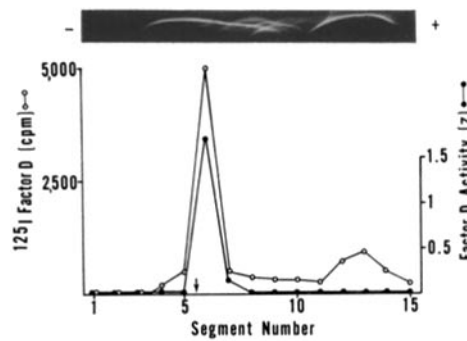


FIG. 2. Isolated factor D: identical distribution of hemolytic activity (●) and radioactivity (○) after agarose gel electrophoresis at pH 8.6 (lower panel). For comparison the immunoelectrophoretic pattern of whole human serum is shown as developed in the same gel (upper panel).

*Absolute Factor D Requirement for the Activation of Proenzyme C3/C5 Convertase.* To investigate the possibility that uncleaved factor B bound to EC3b,P has C3/C5 convertase activity, EC3b,P,B was incubated for 5 min at 37°C in absence or in presence of excess factor D. The two resulting intermediate complexes will be referred to, respectively, as proenzyme C3/C5 convertase (EC3b,P,B) and as C3/C5 convertase (EC3b,P,Bb). We found that the proenzyme bearing cells were lysed in GPS-EDTA, but to a lesser extent than the cells bearing the activated enzyme. Proenzyme-dependent lysis was not due to contamination of factor B with factor D since the proenzyme complex remained fully stable over a 45-min period at 37°C in GVB<sup>++</sup> (Fig. 3). Proenzyme-dependent lysis was due to activation of the enzyme by factor D supplied by addition of GPS-EDTA, as evidenced by the fact that factor D-depleted GPS-EDTA did not cause lysis. The activity of the depleted GPS was restored by either guinea pig or human factor D (Table II). The results show that factor D is absolutely required for activation of the proenzyme and that although proenzyme formation is Mg<sup>++</sup> dependent, its activation by factor D proceeds in presence of EDTA.

*Absolute Requirement of Factor D for Activation of the Alternative Pathway in Serum or*

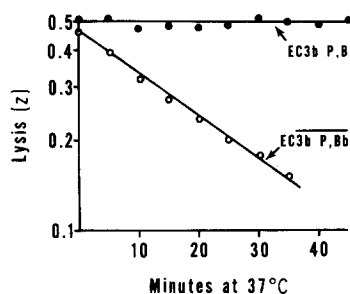


FIG. 3. Stability of proenzyme C3/C5 convertase at 37°C.  $2 \times 10^8$  EC3b/ml was incubated with 3 µg/ml P, 5 µg/ml (O) or 25 µg/ml (●) factor B. After 5 min at 37°C the cells were washed in GVB<sup>++</sup> and 200 ng/ml factor D was added to the cells prepared with the low amount of factor B. 50-µl samples were then withdrawn at 5-min intervals and added to 1 ml GPS-EDTA. Lysis was quantitated after 10 min at 37°C.

TABLE II  
*Lack of Hemolytic Activity of Proenzyme C3/C5 Convertase in Absence of Factor D*

Reagent	Lysis (z) of:	
	EC3b,P,B (-factor D)	EC3b,P,Bb (+ factor D)
GPS-EDTA	0.28	1
D-depleted GPS-EDTA	0	1
D-depleted GPS-EDTA + D <sup>GP</sup>	0.30	1
D-depleted GPS-EDTA + D <sup>Hu</sup>	0.17	1

Reaction mixture contained per ml  $2 \times 10^8$  EC3b, 10 µg P, 30 µg factor B in GVB<sup>++</sup>. After 5 min at 37°C the cells were washed in GVB<sup>++</sup> and 50-µl aliquots of cell suspension were further incubated for 5 min at 37°C in presence or absence of factor D (100 ng/ml). Lysis was measured after incubation for 10 min at 37°C in 1 ml of reagent.

**Plasma.** Treatment of human plasma prepared from Mg-EGTA blood with 20 mM DFP<sup>2</sup> for 30 min at 37°C completely abolished the alternative pathway-dependent lysis of rabbit erythrocytes (E<sub>R</sub>). The activity of the pathway in the treated plasma was fully restored by addition of physiological amounts of isolated factor D (Fig. 4). Increasing the concentration of factor D in untreated and DFP-treated plasma to approximately nine times the physiological value, lysis increased 9-fold, namely from  $z = 0.18$  to  $z = 1.6$ . At approximately 18 µg/ml plasma, factor D was no longer limiting.

Depletion of factor D from serum by methods based either on molecular size (Sephadex G75), charge (Bio-Rex 70) or antigenicity (anti-factor D immune absorbent) gave the same results. The data indicate that under physiological conditions no other protease in serum or plasma is capable of replacing factor D.

**Lack of Incorporation of Factor D into the Multimolecular Enzymes.** First, no depletion of factor D in the fluid phase could be detected after incubation with increasing concentrations of cellular intermediates. A limiting amount of factor D (0.2 ng) was incubated at 4°C or 37°C in GVB<sup>++</sup> or DGVB<sup>++</sup> for 10 s or 5 min with  $10^7$  to  $2 \times$

<sup>2</sup> Treatment for 30 min at 37°C in VB with 20 mM DFP was required also for complete inactivation of isolated factor D; 5 mM resulted in only 70% activation.

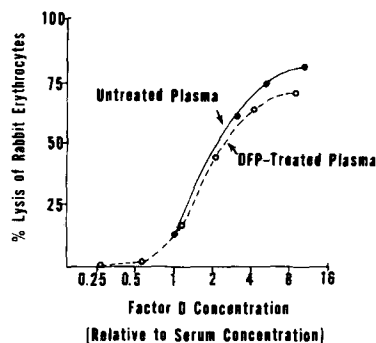


FIG. 4. Absolute requirement of factor D for alternative pathway-dependent lysis of  $E_R$  in plasma. 25  $\mu$ l of Mg-EGTA plasma (either treated by DFP [O] or nontreated [●]) was incubated in presence of increasing amounts of factor D with  $10^7 E_R$  in a final 150  $\mu$ l vol of Mg-GVB. After 5 min at 37°C, the lysis was measured by addition of 1 ml VBE.

$10^8$  EC3b. EC3b was either used as such or after preincubation with P (10  $\mu$ g/ml), P and factor B (40  $\mu$ g/ml), or nephritic factor (NF) (5  $\mu$ g/ml) and factor B. Even in presence of a 1,000-fold molar excess of cell bound factor B over factor D in the fluid phase, there was no detectable depletion of factor D hemolytic activity. Second, after incubation of the same cellular intermediates with an excess of radiolabeled factor D, radioactivity was never found associated with the cell pellets after separation from the fluid phase by centrifugation through 200- $\mu$ l cushions of 20% sucrose DGVB<sup>++</sup>. Nor was factor D hemolytic activity found associated with the cell pellets after three washings, transfer, and decay. Third, we studied the elution of  $^{125}$ I factor D (0.5  $\mu$ g) at 4°C from columns of Sepharose (S)-C3b, S-C3b,P, and S-C3b,P,B. While about 50  $\mu$ g of P and 25  $\mu$ g of factor B were bound, more than 95% of factor D was recovered in 1.5 column vol. Fourth, the interaction between  $^{125}$ I factor D with either factor B, C3, or C3b was investigated by sucrose density gradient ultracentrifugation at conditions described in Materials and Methods. No interaction could be detected with 100 M excess of factor B, C3, or C3b. Neither was it possible to detect incorporation of radioactive factor D into the NF-dependent fluid phase C3 convertase (20  $\mu$ g of NF, 10  $\mu$ g of C3, 2  $\mu$ g of factor B, 50 ng of  $^{125}$ I factor D).

*Serum Concentration of Factor D as Measured by RIA and Hemolytic Activity.* Plasma and serum samples were obtained from eight healthy donors. A portion of each serum sample was treated with zymosan (5 mg/ml, 30 min, 37°C) which resulted in more than 90% C3 consumption. The concentration of factor D was determined on all samples by the two methods. As shown in Table III, all mean values are approximately 2  $\mu$ g/ml. No statistically significant difference was found between the values for plasma and serum, and no statistically significant difference was found between the values obtained by either RIA or hemolytic assay. Further, serum and zymosan-activated serum had identical factor D concentrations.

*Identical Distribution of Factor D Antigen and Hemolytic Activity in Plasma or Serum After Fractionation by Chromatography or Electrophoresis.* After fractionation of fresh serum on QAE Sephadex-A50, only one symmetrical peak of factor D antigen was detected, the distribution of which corresponded to that of factor D hemolytic activity (Fig. 5). The same result was obtained with fresh plasma after isoelectrofocusing electrophoresis in a Pevikon block (not shown).

TABLE III  
Factor D: Plasma and Serum Concentration Measured by RIA and Hemolytic Activity

	Factor D concentration*	
	RIA	Hemolytic activity $\mu\text{g/ml} \pm \text{SD}$
Plasma	$1.9 \pm 0.7$	$2.4 \pm 0.9$
Serum	$2.0 \pm 0.6$	$2.3 \pm 0.9$
Zymosan-activated serum	ND	$2.3 \pm 0.9$

\* Expressed as average of eight donors  $\pm$  SD. The protein concentration of purified factor D was determined by Folin analysis with bovine serum albumin as reference protein.

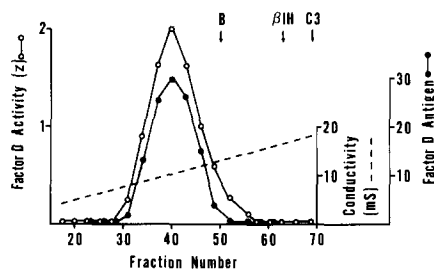


FIG. 5. Identical distribution of factor D antigen and hemolytic activity in serum after anion exchange chromatography. 20 ml of fresh serum in EDTA (0.01 M) was dialyzed against Tris buffer, 0.01 M, pH 8.5, adjusted to 4 mS with NaCl. The sample was applied to a  $5 \times 30$  cm column of QAE Sephadex A50. The column was washed with starting buffer and the material eluted with a linear salt gradient (600 ml of the starting buffer and 600 ml of this buffer adjusted to 25 mS). 10 ml fractions were collected and assayed for factor D hemolytic activity (○) and for factor D antigen (●) by RIA (expressed in percent of inhibition of specific binding). Elution volumes of factor B,  $\beta$ 1H, and C3 are indicated by arrows.

*Comparison of Factor D with Other Serine Esterases: Lack of Relationship with  $\alpha$ -Thrombin.* The specific factor D hemolytic activity expressed in terms of  $\text{z}/\mu\text{g}$  is shown for different serine esterases, including factor D, in Table IV.  $\alpha$ -thrombin had no detectable hemolytic activity. Streptokinase activated human plasminogen and bovine trypsin were, respectively, 4,200 and 35 times less active than factor D. For comparison, the ability of these serine esterases to cleave TAME is shown. Factor D has no detectable esterolytic activity<sup>3</sup> and the esterolytic activities of  $\alpha$ -thrombin, plasmin and bovine trypsin are in agreement with those found in the literature (25, 26, 17). The absence of factor D hemolytic activity from  $\alpha$ -thrombin and the absence of esterolytic activity from factor D demonstrates that these two molecules are functionally distinct. Further, as shown in Fig. 6, the binding of factor D in the RIA was inhibited by unlabeled factor D, but not by  $\alpha$ -thrombin. This result led to the conclusion that  $\alpha$ -thrombin and factor D do not share antigenic determinants.

<sup>3</sup> No esterolytic activity of factor D was found with respect to nine additional synthetic substrates, and no cleavage of oxidized B chain of bovine insulin was observed (Materials and Methods).

TABLE IV  
Enzymatic Activity of Factor D and other Serine Esterases

	Esterolytic activity (TAME cleavage)	Factor D hemolytic activity (factor B activation)
	$\mu\text{mol}/\text{min}/\text{mg}$	$\text{z}/\mu\text{g}$
Factor D	<0.006	21,000
$\alpha$ -Thrombin	21	<0.01*
Plasmin	7	5*
Bovine trypsin	204‡	560* ‡

\* Pretreatment of EC3b cells with enzymes in a control experiment did not result in impairment of bound C3b function.

‡ Completely abolished by treatment of enzyme (30 min at 37°C in VB) with 1 mM DFP or 1 mM PMSF.

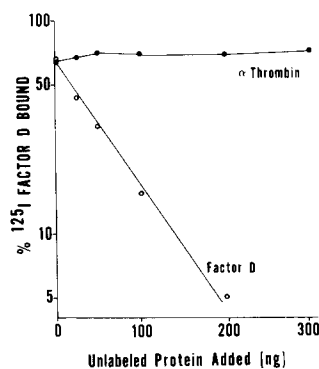


FIG. 6. Lack of immunochemical cross reaction between factor D and  $\alpha$ -thrombin. A constant amount of  $^{125}\text{I}$  factor D (40 ng) was mixed with increasing amounts of unlabeled factor D (○) or  $\alpha$ -thrombin (●) and assayed by RIA (Materials and Methods).

### Discussion

C3 proactivator convertase was postulated to be an enzyme of the C3 activator system in 1971 (5), described as such in 1972 (2), shown, as factor D, to be required for formation of cobra factor-dependent C3 convertase in 1973 (27) and obtained in isolated form in 1974 (10, 28).

The present study was undertaken to delineate the mechanism of action of factor D in molecular terms. In view of the outcome of the study, the following observations reported in the literature require reinterpretation: (a) occurrence in plasma of zymogen factor D, in addition to the activated enzyme,  $\bar{D}$  (29); (b) activation of the zymogen by properdin or trypsin (30); (c) physical incorporation of factor D activity and antigen into the C3/C5 convertase (31); (d) lack of factor D requirement for the formation of the properdin or nephritic factor dependent C3 convertase (30, 32, 33); (e) expression of esterolytic activity of factor D with respect to synthetic esters (34, 35); and (f) existence of an antigenic and functional relationship to  $\alpha$ -thrombin (36).

Our study yielded the following novel results. Human plasma or serum contain only enzymatically active factor D. This conclusion is based on the following evidence: the distribution of factor D hemolytic activity paralleled that of factor D antigen when plasma or serum were subjected to various separation procedures. An antigenically related, inactive proenzyme with charge or size differing from those of active



factor D could not be found. During purification, or upon alternative pathway activation in serum, an increase in specific factor D activity (hemolytic activity in  $z/\mu\text{g}$  of factor D protein) was not observed. Isolated factor D and factor D in whole serum possessed the same specific activity. In addition, there was no consumption of factor D as a result of alternative pathway activation in serum or upon interaction of isolated factor D with cell-bound proenzyme C3/C5 convertase. These results indicate that factor D requires no activation, can recycle and is not inhibited by the various protease inhibitors of serum. These results also are in accord with the finding that factor D is not incorporated into the C3/C5 convertase as an essential subunit. In fact, it was not possible to demonstrate any association of factor D with the complex enzyme or with its subunits, either by measurement of factor D radioactivity or hemolytic activity.

Although factor D is not a part of the C3/C5 convertase subunit structure, it is absolutely necessary for the formation of the enzyme. This absolute requirement is in agreement with the results reported by others (3, 4, 19, 37). The proenzyme C3b,P,B expresses no convertase activity and, unlike the activated enzyme, is completely stable at 37°C. Formation of the proenzyme is  $\text{Mg}^{++}$ -dependent, but its activation by factor D is not.

To some extent tryptic enzymes can substitute for factor D function. Pronase, trypsin, and plasmin have been shown to be active in this respect (38–40). However,  $\alpha$ -thrombin is incapable of mimicking factor D. An antigenic and functional relationship between the two enzymes, proposed by others (36), could not be verified.

Comparing further the functional properties of factor D with those of trypsin, it is noted that in contradistinction to trypsin, factor D does not act on factor B in absence of C3b and  $\text{Mg}^{++}$ , has no detectable esterolytic activity toward synthetic substrates, no proteolytic activity toward the B chain of insulin, and is relatively insensitive to inactivation by DFP (6, 29). Complete inactivation of factor D necessitates more than 20 times the DFP concentration needed for trypsin inactivation. However, the specific hemolytic activity of factor D, measured in terms of C3/C5 convertase activation, is 35 times greater than that of trypsin.

Although some tryptic enzymes exert a factor D-like effect, no other protease in normal serum or plasma is able to replace factor D. Selective removal of factor D activity by a variety of methods, including DFP treatment, totally impaired the alternative pathway. However, pathway activity could be restored in a dose-dependent fashion upon addition of isolated factor D to the depleted serum. These experiments also revealed that factor D is present in human serum in limiting concentration. Factor D became nonlimiting only at nine times physiological serum concentration.

The mechanism of action of factor D may be formulated in terms of the cryptic site hypothesis. Factor D occurs in fresh plasma not as proenzyme, but in enzymatically active form. Factor D has no discernible substrate in unactivated plasma and, unlike most plasma enzymes, it is not controlled by plasma enzyme inhibitors. The substrate is formed upon activation of the alternative pathway and consists of the C3b- $\text{Mg}^{++}$  factor B complex. It is within this association product that factor B becomes susceptible to cleavage and activation by factor D. It is unlikely that association with C3b is necessary to reveal the susceptible bond in factor B because trypsin, which also activates factor B, can act on it directly, i.e., in absence of C3b and  $\text{Mg}^{++}$ . It is proposed therefore that C3b induces factor B to fit into the substrate binding site of

factor D. This binding site is envisaged to be cryptic because factor D, an active trypsin-like enzyme, neither reacts with plasma enzyme inhibitors nor with its natural substrate unless factor B is induced to fit by its helper molecule, C3b.

That factor D is not only essential for amplification of the alternative pathway, but also for its initiation has been established (41). The question arises therefore how the initial C3 convertase may be formed in absence of apparent C3b. It is conceivable that a reversible association product of native C3 and factor B occurs in unactivated plasma in very low concentration and that this complex constitutes a surrogate substrate for the always active factor D. The very small numbers of resulting C3b molecules deposited on the  $\beta$ 1H control restricted surface of activator particles (15, 42) could then initiate the chain reaction of factor D catalyzed C3 convertase formation.

### Summary

Factor D (C3 proactivator convertase) of human serum has been shown to be absolutely necessary for alternative pathway function, for activation of the C3/C5 convertase of that pathway and not to be a subunit of this enzyme. Factor D was found to be present in human plasma in active form only, at a concentration of 2  $\mu$ g/ml, and not to be controlled by plasma protease inhibitors or by spontaneous decay. Unlike trypsin, factor D cleaves and activates factor B only when it is in  $Mg^{++}$ -dependent complex with C3b, has no esterolytic activity, and is unable to cleave the B chain of insulin. The alleged functional and antigenic relationship of factor D to  $\alpha$ -thrombin could not be verified. The results of this study led to the description of the mechanism of action of factor D in terms of the cryptic site hypothesis.

We wish to thank Ms. Lorraine Wood for skillful technical assistance.

*Received for publication 18 August 1978*

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