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PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT 596951

XIX
We BOEHRINGER INGELHEIM INTERNATIONAL GmbH,
of D-6507, Ingelheim am Rheim,
Federal Republic of Germany

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APPLICATION ACCEPTED AND AMENDMENTS
ALLOWED 28.2.90

hereby apply for the grant of a Standard Patent for an invention entitled:

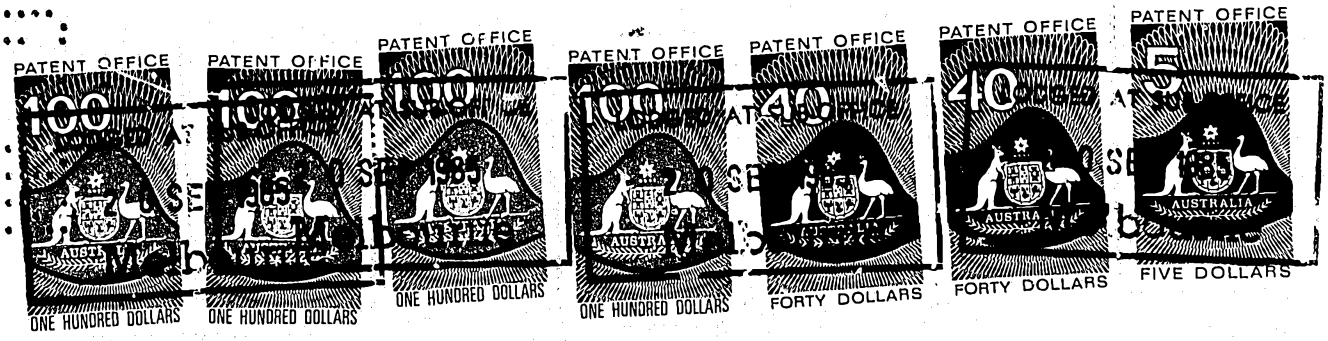
"BLOOD COAGULATION INHIBITING PROTEINS, PROCESSES FOR PREPARING THEM AND THEIR USES"

LODGED AT SUB-OFFICE
23
20 SEP 1985
Melbourne

which is described in the accompanying ~~provisional~~ complete specification.

Details of basic application(s):—

Number	Convention Country	Date
84.02904	Netherlands	21st September 1984
85.00601	Netherlands	4th March 1985



The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

Dated this 20th day of September 19 85

To: THE COMMISSIONER OF PATENTS

E. A. Rimington
.....
(a member of the firm of DAVIES & COLLISON for and on behalf of the Applicant).

Form 6 52.149-167
Form 7

Regulation 11 (1)
Regulation 11 (2)

AUSTRALIA
Patents Act 1952-1973

Declaration in Support of
(a) A Convention Application
(b) ~~An Application~~
for a Patent ~~or Patent of Addition~~

(a) Delete for Non-Convention Application.
(b) Delete for Convention Application.

In support of the ~~Application~~/Convention Application made by

(c) Insert Full Name of Applicant.

(c) Boehringer Ingelheim International GmbH

for a patent/~~patent of addition~~ for an invention entitled:

(d) Insert Title of Invention.

(d) "Blood coagulation inhibiting proteins, processes for preparing them and their uses"

(e) Insert Full Names of Declarant(s).

I/~~We~~ (e) Dr. Dieter Laudien

(f) Insert Address(es) of Declarant(s).

of (f) Carl-Orff-Str. 11, D-6500 Mainz 33, Federal Republic of Germany

do solemnly and sincerely declare as follows:—

(g) Delete when Applicant is a Company.

1. (g) ~~I am/we are the applicant(s) for the patent/patent of addition~~

~~or~~

(h) Delete when Applicant is an Individual. Fill in Name of Applicant if a Company.

(h) I am/~~we are~~ authorised by Boehringer Ingelheim International GmbH the applicant for the patent/~~patent of addition~~ to make this declaration on its behalf.

(i) For Non-Convention Application, delete. For Convention Application, fill in details of basic application.

2. (i) The basic application(s) as defined by Section 141 of the Act ~~was~~/were made in The Netherlands on the 21 day of September 1984 ~~by~~ and on 4 March 1985 by the RIJKSUNIVERSITEIT LIMBURG.

(j) Delete for Non-Convention Application by Assignee of Inventor and for Convention Application.

(k) Delete for Non-Convention Application by Inventor and for Convention Application by Assignee.

3. (j) ~~I am/we are the actual inventor(s) of the invention~~

~~or~~

(k) ~~I am/we are the actual inventor(s) of the invention referred to in the basic application.~~

~~or~~

Christian Peter Maria Reutelingsperger, of Looiersgracht 17, 6211 JK Maastricht, The Netherlands

(l) Delete for Application by Inventor. For Application by Assignee, insert name, address and occupation of Inventor

(l)

~~of~~

is/~~are~~ the actual inventor(~~s~~) of the invention and the facts upon which ~~I am/we are~~/the said Company is entitled to make the application are as follows:

(m) Insert details of Assignment, etc. Date of Assignment only is insufficient.

(m) The applicant is the assignee of the rights to the invention from the inventor Reutelingsperger. Priority is claimed from the basic applications with the consent of the basic applicant.

(4) Delete for Non-Convention Application.

4. The basic application referred to in paragraph 2 of this Declaration was the first application made in a Convention country in respect of the invention the subject of the application.

(n) Insert place and date of signature.

(n) Declared at Ingelheim this 28th day of October 19 88

SIGN X X .....

(12) PATENT ABRIDGMENT (11) Document No. AU-B-47682/85
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(54) Title
ANTICOAGULANT PROTEINS PURIFIED FROM ARTERIES

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(56) Prior Art Documents
AU 15164/83 G07G 7/00
AU 28558/71 G07G 7/00
AU 28819/71 G07G 7/00

(57) The proteins are isolated from human, bovine,murine and equine sources.

The aim of the present invention was to prepare an agent which has blood coagulation-inhibiting properties but without the disadvantageous effects on the coagulation process which accompany the anti-coagulants currently known.

Surprisingly, it has now been found possible to isolate native proteins which have blood coagulation-inhibiting properties, but do not increase the risk of bleeding. Also surprisingly, these proteins lose their inhibiting properties in the event of major bleeding, so that the normal coagulation processes can proceed without disruption and there is no danger of bleeding to death.

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CLAIM

1. Blood coagulation-inhibiting proteins, characterised in that

- they are capable of being isolated from strongly vascularised tissue,
- they do not deactivate the coagulation factors,
- they do not hydrolyse phospholipids,
- they bind reversibly to negatively charged phospholipids via the divalent cations Ca^{2+} and/or Mn^{2+},
- they are capable of displacing the factor X_a and the prothrombins from a negatively charged phospholipid surface and
- they do not inhibit the biological and amidolytic activity of the factors X_a and II_a.

C O M M O N W E A L T H O F A U S T R A L I A

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COMPLETE SPECIFICATION

(Original)

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Lodged:

Complete Specification Lodged:
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This document contains the amendments made under Section 49 and is correct for printing

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Name of Applicant: BOEHRINGER INGELHEIM INTERNATIONAL GmbH

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Actual Inventor(s): Christian Peter Maria Reutelingsperger

Address for Service: DAVIES & COLLISON, Patent Attorneys,
1 Little Collins Street, Melbourne, 3000.

Complete specification for the invention entitled:

"BLOOD COAGULATION INHIBITING PROTEINS, PROCESSES FOR PREPARING THEM AND THEIR USES"

The following statement is a full description of this invention, including the best method of performing it known to us :-

HF S008-622

Blood coagulation inhibiting proteins, processes for preparing them and their uses.

This invention relates to proteins which inhibit the coagulation of the blood, processes for preparing these proteins and their use.

Anti-coagulant proteins, which are present in most mammals, can be divided into three groups. This subdivision is based on the different mechanisms of activity of the proteins.

1. Proteins which form a complex with a coagulation factor and thereby render the coagulation factor inactive. These include the proteins:
   a) Antithrombin III (Thromb. Res. 5, 439-452 (1974))
   b) α_1-Protease Inhibitor (Ann. Rev. Biochem. 52, 655-709 (1983))
   c) α_2-Macroglobulin (Ann. Rev. Biochem. 52, 655-709 (1883))
   d) C_1-Inhibitor (Biochemistry 20, 2738-2743 (1981))
   e) Protease nexin (J. Biol. Chem. 258, 10439-10444, (1983)).

2. Proteins which proteolytically cut up a coagulating factor and thereby inactivate the coagulating factor. The only protein of this kind that has been described hitherto is protein C (J. Biol. Chem. 251, 355-363 (1976)).

3. Proteins which screen and/or hydrolyse the negatively charged phospholipids so that the phospholipid-dependent reactions of the blood coagulation mechanism are inhibited. Hitherto, only phospholipases which have

been isolated from various types of snake poison have been described (Eur. J. Biochem. 112, 25-32 (1980)).

In recent years, the step-wise coagulation system has been investigated thoroughly. It is understood to be an intensifying multi-stage system of different inter-connected proteolytic reactions in which an enzyme converts a zymogen into the active form (cf. Jackson, C.M. and Nemerson, Y., Ann. Rev. Biochem. 49, 765-811 (1980)). The speed of this reaction is decisively increased by the presence of phospholipids and other cofactors such as factor V_a and factor VIII_a. In vivo, the procoagulation reactions are regulated by a variety of inhibitory mechanisms which prevent an explosively thrombotic trauma after slight activation of the coagulation cascade.

The anti-coagulation mechanisms can be subdivided as follows (Rosenberg, R.D., Rosenberg, J.S., J. Clin. Invest. 74, 1-6 (1984)):

1. Serine-protease factor X_a and thrombin are inactivated as a result of their binding to antithrombin III or to the antithrombin/heparin complex. Both the prothrombin activation and also the formation of fibrin can be inhibited in this way. In addition to antithrombin III there are also various other plasma-protease inhibitors such as α_2-macroglobulin and antitrypsi the activity of which is dependent on time.

2. The discovery of protein C led to the discovery of another anti-coagulation mechanism. Once protein C has been activated, it acts as an anti-coagulant by selective proteolysis of the protein cofactors V_a and VIII_a, by

which prothrombinase and the enzyme which converts factor X are deactivated.

3. Plasmin cleaves monomeric fibrin 1, a product of the effect of thrombin on fibrinogen, thereby preventing the formation of an insoluble fibrin (Nossel, H.L., Nature, 291, 165-167 (1981)).

Of the above-mentioned native proteins involved in the coagulation process, at present only anti-thrombin III is clinically used. However, the increase in the tendency to bleed when this protein is administered has proved to be a serious disadvantage.

All the agents hitherto used as anticoagulants, whether native to the body or synthetic, in some way render the coagulation factors ineffective and thereby lead to side effects which may have a disadvantageous effect on the coagulation process.

The aim of the present invention was to prepare an agent which has blood coagulation-inhibiting properties but without the disadvantageous effects on the coagulation process which accompany the anti-coagulants currently known.

Surprisingly, it has now been found possible to isolate native proteins which have blood coagulation-inhibiting properties, but do not increase the risk of bleeding. Also surprisingly, these proteins lose their inhibiting properties in the event of major bleeding, so that the normal coagulation processes can proceed without disruption and there is no danger of bleeding to death.

One aspect of the invention provides anticoagulation proteins characterised in that

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- they are capable of being isolated from strongly vascularised tissue,
- they do not deactivate the coagulation factors,
- they do not hydrolyse phospholipids,
- they bind reversibly to negatively charged phospholipids via the divalent cations Ca^{2+} and/or Mn^{2+},
- they are capable of displacing the factor X_a and the prothrombins from a negatively charged phospholipid surface and
- they do not inhibit the biological and amidolytic activity of the factors X_a and II_a.

The present invention relates to the anticoagulant proteins, referred to herein as VAC (Vascular Anti Coagulant) proteins for convenience, which do not inactiviate the coagulation factors. In particular, the invention relates to VAC proteins substantially free of any animal

tissue, especially in substantially pure form. These proteins are capable of inhibiting the coagulation induced by a vascular procoagulant or by factor Xa, but cannot inhibit the coagulation induced by thrombin. They do not inhibit the biological and amidolytic activity of factors X_a and II_a.

The invention preferably relates to anticoagulant proteins which do not inactivate the coagulation factors and inhibit:

- the modified prothrombin-time experiment as herein described and/or
- the modified activated partial thromboplastin time experiment as herein described and/or
- the non-modified prothrombin-time experiment and/or
- the prothrombin activation by the coagulation factor X_a in the presence of negatively charged phospholipids and Ca^{2+} and/or
- the intrinsic X-activation by factor IX_a in the presence of negatively charged phospholipids and Ca^{2+} and/or
- the prothrombin activation of isolated stimulated blood platelets and/or
- the coagulation induced by the walls of the blood vessels and/or
- the coagulation-dependent platelet aggregation.

The invention also relates to anti-coagulant proteins which do not inactivate the coagulation factors and the inhibitory activity of which depends on the quantity of phospholipids. The proteins according to the invention induce inhibition of factor X_a -activation of prothrombin. The inhibition depends on the phospholipid concentration and is

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less at high phospholipid concentrations. The phospholipids are not hydrolysed by the proteins according to the invention.

The invention further relates to anticoagulant proteins which do not inactivate the coagulation factors and which bind, via the divalent cations Ca^{2+} and/or Mn^{2+}, to negatively charged phospholipids, which can be found, for example, in vesicles, liposomes or etherosomes and/or, via the divalent cations Ca^{2+} and/or Mn^{2+}, to negatively charged phospholipids which are coupled with Spherocil. The bonding of the proteins to the negatively charged phospholipids is reversible and can be reversed by ethylenediaminetetra acetic acid (EDTA). The proteins according to the invention are capable of displacing factor X_a and prothrombin from a negatively charged phospholipid surface.

Preferably

The invention relates particularly to anticoagulant proteins which do not inactivate the coagulation factors and have molecular weights of about 70×10^3, about 60×10^3, 34×10^3 or 32×10^3, of which the proteins with a molecular weight of 34×10^3 or 32×10^3 respectively consist of only a single polypeptide chain.

The invention preferably relates to anticoagulant proteins which do not inactivate the coagulation factors, characterised in that

- they are isolated from the walls of mammalian blood vessels and then purified,
- they are not glycoproteins,
- they are not phospholipases,
- they have an isoelectric point of pH 4.4-4.6,
- the activity of the anticoagulent proteins at 56°C is thermally unstable,
- the activity of the anticoagulating proteins in citrated plasma remains stable for some

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hours at 37°C,

- the activity of the anticoagulant proteins is not completely destroyed by trypsin and/or chymotrypsin,
- the activity of the anticoagulant proteins is not affected by collagenase and/or elastase,
- they bond, via the divalent cations Ca^{2+} and/or Mn^{2+}, to negatively charged phospholipids which can be found in vesicles, liposomes or etherosomes,
- they bond via the divalent cations Ca^{2+} and/or Mn^{2+} to negatively charged phospholipids which are coupled to Spherocil,
- the bonding of the prcteins to the negatively charged phospholipids is reversible and can be removed by ethylenediamine tetracetic acid (EDTA),
- they displace factor X_a and prothrombin from a negatively charged phospholipid surface,
- they inhibit the modified prothrombin-time experiment as herein described,
- they inhibit the modified, activated, partial thromboplastin-time experiment as herein described,
- they inhibit the non-modified prothrombin-time experiment,
- they inhibit prothrombin activation by the coagulation factor X_a in the presence of negatively charged phospholipids and Ca^{2+} in vitro,
- they do not inhibit the biological and amidolytic activity of factors X_a and II_a,
- they inhibit the intrinsic X-activation by the factor IX_a in the presence of negatively charged phospholipids and Ca^{2+} in vitro,
- they inhibit the prothrombin activation of isolated, stimulated blood platelets in vitro,
- they inhibit the coagulation induced by the walls of the blood vessels in vitro, and

- the inhibition of factor X^a -activation of prothrombin induced by the proteins is dependent on the concentration of phospholipids and is reduced at high phospholipid concentrations.

Suitable starting materials for the isolation of the VAC proteins are the blood vessel walls and greatly vascularised tissue of various mammals, e.g. cattle, rats, horses and humans, and endothelial cell cultures of these mammals. The arterial walls of cattle, rats, horses and humans and human umbilical veins and arteries are particularly suitable.

The invention also relates to a process for preparing the proteins according to the invention using isolation and purification techniques which are known per se. The following procedure is particularly suitable:

The homogenised starting material is first subjected to differential centrifugation. The supernatant liquid obtained can then be further treated as follows in any desired sequence. Undesirable contaminants can be precipitated with ammonium sulphate. The supernatant is then further purified by affinity chromatography, e.g. using hydroxyapatite, ion exchange chromatography, e.g. using DEAE-Sephacel, chromatography over a molecular sieve, such as Sephadex G-100, and immunoabsorption chromatography, e.g. with polyclonal or monoclonal antibodies. Depending on the quality of the starting material this purification plan can be modified or other purification procedures can be used, e.g. purification using phospholipid vesicles.

In addition to the classic anti-thrombosis treatment, namely coagulants taken orally, more recently biosynthetic tissue-plasminogen activator has been administered by the intravascular route for cases of manifest thrombosis (N. Engl. J. Med. 310, 609-513 (1984)).

The proteins according to the present invention are especially suitable for preventing thrombosis, e.g. during operations, particularly on account of their blood coagulation-inhibiting properties and at the same time their inhibiting effect on the coagulation-dependent aggregation of platelets.

The present invention therefore also relates to the use of the proteins according to the invention as antithrombotic agents.

The invention further relates to pharmaceutical compositions which comprise one or more proteins according to the invention in association with a pharmaceutically acceptable carrier and/or excipient.

Results which have been obtained on carrying out VAC isolation and purification from bovine arteries are shown in Table A. Determination of the level of VAC activity in the supernatant of the 100,000 xg centrifugation was erroneous owing to the presence of procoagulant activity. The components responsible for this activity were found to be precipitated with ammonium sulphate at a saturation level of 35%. It was discovered that the supernatant solution obtained after precipitation with 35% ammonium sulphate contained 100% VAC activity. In order to precipitate this activity, the solution was mixed with ammonium sulphate until 90% saturation was achieved. The resulting precipitate containing the VAC proteins was bound to a hydroxyapatite column in the presence of TBS (100 mM NaCl, 50mM Tris/HCl, pH 7.5). After washing, the VAC proteins were eluted from this column with a rising phosphate gradient. At a low ion intensity, the VAC proteins were bonded to a DEAE-Sephacel column. Elution of the VAC proteins from this column was effected with an increasing NaCl concentration gradient. In the final purification step, the proteins were separated on the basis of their molecular weight

by gel filtration on Sephadex G-100. The eluant was a highly-salted buffer for minimising interaction of VAC with the Sephadex material. VAC was eluted from this column in a volume of about 1.6 times the empty volume of the column (see Figure 1). The total yield of VAC after this final purification was 35%. By SDS-PAGE, all G-100 fractions which showed VAC activity where found to contain two polypeptides (molecular weight 34,000 and 32,000, respectively). In some cases a further fraction with a molecular weight of 60,000 showed VAC-activity.

According to SDS-PAGE, only the peak fractions 138-140 were homogeneous in relation to the two polypeptides. These fractions were used for all the other experiments concerning investigation of bovine VAC described in this specification, with the exception of the experiments for characterising the bonding of bovine VAC to phospholipid liposomes.

In the G-100 fraction 139, 3.4% of the VAC activity was found by means of a one-stage coagulation test (See Example 1) to have a specific activity of 1480 units per mg of protein. (See Table A). This fraction contained no detectable quantity of phospholipid and an extinction coefficient of

$$A^{1\%}_{1cm} = 8.5$$

was calculated for this purified VAC preparation from the absorption at 280 nm and from the protein content.

Characterisation of VAC

As is clear from Figure 2, the two polypeptides with molecular weights of 34,000 and 32,000, which are present in the purified protein material from bovine arteries and to which the VAC activity has

been ascribed, are proteins with a single chain. Using Schiff's reagent with basic fuchsin, it was established that both proteins contain few carbohydrate groups. Moreover, no γ-carboxyglutamate (Gla) residues could be found in either protein. Isoelectric focusing (see Example 1) showed that both proteins migrate in a single band corresponding to an isoelectric point of 4.4 to 4.6 (see Figure 3).

The VAC activity was obtained from the PAG plate by elution of this band from the gel. Analysis of the eluant with SDS-PAGE again showed the presence of the two proteins. It was thus possible to confirm that both proteins migrate in a single band in the pH gradient of the PAG plate. In order to check the method, human haemoglobin (Hb) was also investigated by isoelectric focusing. The value of 6.8 found agrees with the figure given in the literature (see Figure 3).

Bonding tests showed that the VAC activity can bond to negatively charged phospholipid membranes. This bonding takes place in the presence of Ca^{2+} and/or Mn^{2+}, but not in the presence of Mg^{2+} or in the absence of divalent metal ions (see Table B). This bonding of VAC activity to liposomes is reversible and can be reversed by means of the reagent EDTA.

Using SDS-PAGE, it was possible to show that both proteins can bond to liposomes in the presence of Ca^{2+} and that this bonding is disrupted when EDTA is added (see Figure 4). This is yet another indication that VAC activity can be ascribed to these two proteins.

On storage in TBS containing 10% glycerol, VAC activity is stable at -70°C for at least 3 months, at 0°C for at least 12 hours and at 37°C for at least half an hour. At 56°C, the activity disappears within two minutes.

Activity of VAC

VAC prolongs the coagulation time in a one-stage coagulation experiment (see Example 1), in which coagulation is triggered with thromboplastin from bovine brains (BTP). Replacement of BTP in this experiment with purified bovine thrombin or purified bovine factor X_a showed that VAC prolongs the coagulation time only if factor X_a is used to initiate coagulation; coagulation induced by thrombin is not affected by VAC. This indicates that VAC directly inhibits the factor X_a activity or that there is some interaction with the prothrombinase complex. For further testing, an amidolytic thrombin formation test with purified cattle factor X_a and prothrombin was carried out. Figure 5 shows that when prothrombin is activated in the presence of Ca^{2+} and phospholipid by means of factor X_a to form thrombin, VAC inhibits the prothrombin activation and the degree of inhibition is dependent on the concentration of VAC. Morover, the inhibiting effect of VAC is greater at a lower concentration of phospholipid.

Figure 6 shows the phospholipid dependency of the VAC-induced inhibition of prothrombin activation. It is noticeable that at a phospholipid concentration of zero the prothrombin activation by factor X_a is not inhibited by VAC. Control tests showed that VAC itself does not affect the system of measurement.

Incubation of 5 mcM phospholipid [1,2-dioleoyl-sn-glycero-3-phosphoserine (PS)/1,2-dioleoyl-sn-glycero-3-phospocholine (PC) 1:4 Mol/Mol] with 107 mcg/ml VAC (specific activity 1,300 units per mg) and 10 mM Ca^{2+} yielded a reduction in the procoagulant activity within 3 minutes at 37°C. This shows that VAC has no phospholipase activity.

By contrast with antithrombin III (AT-III), VAC has no effect on the amidolytic activity of purified thrombin and no lasting effect on factor X_a activity, as measured with the chromogenic substrate S 2337 (N-benzoyl-L-isoleucyl-L-glutamyl-L-pipecolyl-glycyl-L-arginine-p-nitroanilide-dihydrochloride) or S 2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide-dihydrochloride) [see Table C]. This table also shows that the inactivation of factor X_a and thrombin by AT-III is not intensified by VAC. Heparin, on the other hand, decisively increases inactivation of thrombin and factor X_a in the presence of AT-III. This shows that VAC has neither a heparin-like activity nor an AT-III-like activity.

The isolation of a novel human anticoagulant may be achieved by the same isolation procedure from, for example a homogenate of human umbilical cord arteries. In such an homogenate an anticoagulant according to the present invention has been discovered by its ability to prolong the clotting time in a prothrombin time test. The anticoagulant activity became measurable after Sephadex G-100 fractionation of the arterial homogenate [See Example IV]. From further isolation procedures, it is assumed that this activity is associated with a water-soluble substance(s), that carries an overall negative charge at pH 7.9.

Analysis of Sephadex G-75 fractions with gel electrophoresis has shown a positive correlation between the intensity of the Mw 32,000 band and the prolongation of the clotting time as measured with a modified prothrombin time test (MPTT) [See Example IV]. The connection of the 32K-band with the anticoagulant activity is demonstrated unambiguously since only from the site of the 32K-band on the polyacrylamide gel can anticoagulant activity be

eluted. In combination with the findings that the anticoagulant rapidly loses its activity upon incubation at 56°C, and proteolytic enzymes can destroy its activity, we assume the anticoagulant activity to be expressed by a single protein with an apparent molecular weight of 32,000 daltons.

Trypsin, in contrast to protease type I, is a poor inactivator of the anticoagulant. This suggests that the anticoagulant possesses only a small number of lysine- and arginine-residues that are accessible for trypsin. The nature of the anticoagulant activity has been studied by initiating coagulation in different ways. Clotting, induced by either the vascular procoagulant, HTP, (human brain thromboplastin), or factor X_a, is inhibited by the anticoagulant; thrombin induced clotting, on the other hand, is not. From these findings one can conclude that the anticoagulant interferes with thrombin formation, not with thrombin action.

To further study the anticoagulatory mechanism, prothrombinase reconstituted from purified factors and prothrombin was used [See Example IV]. Under the experimental conditions mentioned, the anticoagulant can inhibit the activation of prothrombin by complete prothrombinase (factor X_a, factor V_a, phospholipid, Ca^{2+}) and by phospholipid-bound factor X_a (factor X_a, phospholipid, Ca^{2+}) but not by free factor X_a (factor X_a, Ca^{2+}).

The time courses of prothrombin activation in the presence of the anticoagulant indicate an instantaneous inhibition of prothrombin activation, that remains constant in time. Therefore, one can conclude that the anticoagulant acts neither by a phospholipase- nor by a proteolytic activity. The fact that the activation of prothrombin by factor X_a and Ca^{2+} is not affected by the anticoagulant

at all, strongly indicates that the anticoagulatory mechanism of the vascular compound differs from that of the well known plasma protease inhibitors such as antithrombin III. Since Walker et al. ((1979) Biochima. Biophys. Acta, 571, 333-342) have demonstrated that activated protein C does not inhibit prothrombin activation by factor X_a, Ca^{2+} and phospholipid, it can also be concluded that our compound is not protein C either.

Preliminary binding studies indicate that the vascular anticoagulant probably interferes with the lipid binding of factor X_a and/or prothrombin. Whether the ability of the anticoagulant to inhibit prothrombin activation completely accounts for its prolonging effect on the prothrombin time remains to be established.

The fact that this inhibitor can be found in various types of arteries, but not in a poorly vascularised tissue indicates that a physiological modulator of haemostasis and thrombosis, active at the vascular level has been found.

On the basis of the properties and activities of VAC which have been observed, the blood coagulation mechanisim under the influence of VAC may be interpreted as follows:

VAC bonds via Ca^{2+} ions to negatively charged phospholipids which occur as a result of damage to the tissues and/or because of the stimulation of blood platelets, and thereby reduces the bonding of specific coagulation factors (vitamin K-dependent coagulation factors) to the negatively charged phospholipid surface which acts as a catalytic surface for these coagulation factors (Biochem. Biophys. Acta, 515, 163-205 (1985)), with the result that the phospholipid-dependent blood coagulation reactions are inhibited by VAC. On the basis of its mechanism of activity, VAC can be categorised in protein group 3 (see hereinbefore).

However, of critical importance is the difference between VAC and the other known proteins of this group. VAC does not hydrolyse phospholipids and therefore does not decompose any essential membrane structures.

The properties of VAC, which have not hitherto been described in any of the known anticoagulants, are also significant and advantageous, namely:

- The anticoagulation effect of VAC is dependent on the quantity of phospholipids participating in the coagulation process. This dependency means that the coagulation process, which has been initiated, for example, by slight damage to the wall of the blood vessel and/or by slight activation of blood platelets, e.g. by a thrombotic process, can surprisingly be inhibited by VAC. On the other hand, the coagulation process which is triggered by drastic damage to walls of blood vessels, wherein phospholipids occur in high concentrations, is not inhibited by VAC, precisely because of these high phospholipid concentrations. The danger of bleeding when using VAC is therefore surprisingly extremely small. VAC has these superior properties, in contrast to all the anticoagulants known hitherto, which render one or more coagulating factors ineffective and thereby increase the risk of bleeding.

- VAC surprisingly does not deactivate the coagulating factors themselves. Consequently, the coagulating factors are still in a position to perform their other functions, more and more of which are being discovered. Some active coagulating factors, for example, have the important non-haemostatic task of

chemotaxis for the inflammatory cells. These cells contribute to healing of damaged blood-vessel walls. Surprisingly, VAC does not interfere with this process.

This invention further describes for the first time a novel class of anticoagulant proteins which do not inactivate the coagulation factors. The Examples serving to illustrate the invention and the properties listed should not restrict the invention in any way. Anyone skilled in the art will be able, without any inventive effort, to obtain further proteins which have the anticoagulant properties without inactivating the coagulation factors, using the method described. These proteins also fall within the scope of protection of this invention.

The abbreviations used in this specification and their meanings are listed below:

VAC : vascular anticoagulant
PFP : platelet free plasma
TBS : 100 mM NaCl, 50 mM Tris/HCl, pH 7.5
EDTA : ethylenediamine tetraacetic acid
TBSE : TBS with 2 mM of EDTA
BTP : thromboplastin from bovine brains
: thromboplastin from human brains
TBSA : TBS with 0.5 mg/ml of human serum albumin, pH 7.9
S 2337: N-benzoyl-L-isoleucyl-L-glutamyl-L-pipecolyl-glycyl-L-arginine-p-nitroanilide-dihydrochloride
S 2238: H-D-phenylalanyl-L-pipecolyl-L-arginine-p- nitroanilide-dihydrochloride
AT-III: human antithrombin III
S.A. : specific activity
Ole_2Gro-P-Cho : 1,2-Dioleolyl-sn-glycero-3-phosphocholine
Ole_2Gro-P-Ser : 1,2-Dioleoyl-sn-glycero-3-phosphoserine

The nomenclature of the blood coagulation factors is as recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogens Intermediates.

Materials

The chemicals for analytical SDS-PAGE and hydroxyapatite HTP were obtained from Bio-rad. Sephadex G-100 and G-75, DEAE-Sephacel and the "Low Molecular Weight Calibration Kit" were obtained from Pharmacia, the chromogenic substrates S2337 and S2238 were obtained from Kabi Vitrum and the Diaflo PM-10 Ultrafiltration membrane was obtained from Amicon.

Example 1

Isolation and Purification of VAC

Bovine aortas were taken within half an hour after slaughtering the animals. Bovine blood was collected in trisodium citrate (final concentration 0.38% by weight) and centrifuged for 10 minutes at ambient temperature and 2,000 xg. The plasma containing few blood platelets was then centrifuged again (15 minutes at 10,000 xg). In this way, plasma free from blood platelets was obtained (PFP).

The aortas from the animals were throughly rinsed with TBS (100 mM NaCl, 50 mM Tris/HCl, pH 7.5) immediately after being removed. The inner coats were removed from the aortas and homogenised using a high-speed homogeniser, e.g. the Braun MX 32, in TBSE (TBS with 2 mM EDTA), containing soyabean trypsin inhibitor (16 mg/l) and benzamidine (1.57 g/l).

The material homogenised from 8 aortas and containing 20% of solids (weight/volume) was centrifuged for 60 minutes at 100,000 xg. The supernatant was saturated with solid ammonium sulphate up to 30% saturation, stirred for 30 minutes and then centrifuged for 20 minutes at 12,000 xg. The resulting supernatant was saturated with solid ammonium sulphate up to 90% saturation, stirred for 30 minutes and then centrifuged for 20 minutes at 12,000 xg.

The precipitate was suspended in a small volume of TBS and dialysed with TBS containing benzamidine (1.57 g/l). The dialysed fraction was applied to a hydroxyapatite column (1 x 20 cm) which had been equilibrated with TBS. The column was washed with 4 bed volumes of TBS. The VAC proteins were eluted from the column with 200 ml of sodium phosphate buffer (pH 7.5) with a linear gradient of 0-500 mM. The fractions containing VAC were combined and dialysed with 50 mM of NaCl and 20 mM of Tris/Hcl at pH 7.5.

The same buffer was also used to equilibrate a DEAE-Sephacel column (3 x 5 cm) on which the dialysed VAC material was chromatographed. The column was washed with 4 bed volumes of the equilibration buffer before the VAC was eluted from the column with 200 ml of NaCl solution in 20 mM of Tris/HCl, pH 7.5, with a linear gradient of 50-300 mM. The fractions containing VAC were collected, dialysed with 500 mM of NaCl and 20 mM of Tris/HCl at pH 7.5 and then concentrated in an Amicon concentration cell using a PM-10 ultrafiltration membrane. The concentrate with a volume of 2 ml was applied to a Sephadex G-100 column (3 x 80 cm) which had been equilibrated with 500 mM NaCl and 20 mM Tris/HCl, pH 7.5.

The eluate was collected in fractions of 2 ml, the active fractions were dialysed separately with 10% by volume of glycerol-containing TBS and stored at -70°C. The entire purification was carried out at 0-4°C.

Determining VAC activity

Two different methods were used to determine the VAC activity:

a) the one-stage coagulation test (modified prothrombin time test)

b) a thrombin formation test.

The one-stage coagulation test was carried out as follows:

In a dish of siliconised glass, 175 mcl of the fraction to be examined or 175 mcl of TBS as control were stirred with 50 mcl of PFP and 25 mcl of dilute BTP (900 revolutions per minute). After an incubation period of 3 minutes at 37°C, coagulation was initiated by adding 250 mcl of buffer which contained 80 mM of NaCl, 20 mM of $CaCl_2$ and 10 mM of Tris/HCl, pH 7.5. The fibrin formation was

recorded optically using a "Payton Dual Aggregation Module" (Hornstra, G., Phil. Trans. R. Soc. London B. 294, 355-371 (1981)). The coagulation time of the control sample was 65 seconds. This test was used during purification to examine the various fractions for the presence of VAC activity. In order to determine the VAC yield during purification, one unit of VAC activity was defined as the quantity of VAC which prolongs the coagulation time in the above test to 100 seconds.

In some cases, BTP was replaced by purified bovine thrombin or the purified bovine factor X_a. In this semi-purified coagulation system, the quantity of thrombin or factor X_a used were such that the coagulation time of the control sample was also 65 seconds.

The thrombin formation test was carried out as follows:

20 mcl of purified bovine factor X_a (150 nM), 30 mcl of $CaCl_2$ (100 mM), 30 mcl of dilute VAC and 30 mcl of PS/PC-phospholipid membrane (the final concentrations are given in the legend accompanying Figure 6) were placed in a plastics dish containing 181 mcl of TBSA (TBS with 0.5 mg/ml human serum albumin, pH 7.9).

The mixture was stirred for 3 minutes at 37°C with a Teflon stirrer. The thrombin formation was initiated by adding 9 mcl of purified bovine factor II (33.33 mcM). After various times, 50 mcl samples were taken from the reaction mixture and were then added to the contents of a plastics dish with a capacity of 1 ml which had been tempered to 37°C with a thermostat and which contained 900 mcl of TBSE and 50 mcl of the chromogenic substrate S 2238 (5 mM). The quantity of thrombin in the reaction mixture was calculated from the change in extinction at 405 nm, measured with a Kontron

Spectrometer Uvikon 810, using a calibration curve which had been plotted from assays with known quantities of purified bovine thrombin. The percentage inhibition caused by VAC was defined as follows:

$$\% \text{ inhibition} = \left(1 - \frac{a}{b}\right) \times 100\%,$$

wherein "a" is the speed of thrombin formation in the absence of VAC in nM II_a/min. and "b" is the speed of thrombin formation in the absence of VAC in nM II_a/min.

Proteins

The vitamin K-dependent factors prothrombin and factor X_a were obtained by purification of the citrated bovine plasma (cf. Stenflo J.; J. Biol. Chem. 251 355-363 (1976). After barium citrate absorption and elution, fractionation with ammonium sulphate and chromatography on DEAE-Sephadex, there were two protein fractions which contained a mixture of prothrombin and factor IX or factor X. Factor X was activated using the method of Fujikawa et al., Biochemistry, 11, 4882-4891 (1972) and using RVV-X (Fujikawa et al., Biochemistry, 11, 4892-4899 (1972)). Prothrombin was separated from factor IX by heparin-agarose affinity chromatography (Fujikawa et al., Biochemistry, 12, 4938-4945)). The prothrombin-containing fractions from the heparin-agarose column were combined and further purified using the method of Owens et al., J. Biol. Chem. 249, 594-605 (1974). The concentrations of prothrombin and factor X_a were determined using the method of Rosing et al., J. Biol. Chem. 255, 274-283 (1980). BTP was prepared by a conventional method described by Van Dam-Mieres et al. in "Blood coagulation enzymes, methods of enzymatic analysis" Verlag

Chemie GmbH, Weinheim. The protein concentrations were determined according to Lowry et al., J. Biol. Chem. 193, 265 (1951).

Preparation of phospholipids, phospholipid membranes and phospholipid liposomes

1,2-Dioleoyl-sn-glycero-3-phosphocholine ($18:1_{cis}/18:1_{cis}$-PC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine ($18:1_{cis}/18.:1_{cis}$-PS) were prepared as described in Rosing et al., J. Biol. Chem. 255, 274-283 (1980). Separate phospholipid membranes of PC and PS consisting of two layers were prepared using ultrasound as described by Rosing et al., J. Biol. Chem. 225, 274-283 (1980). A supply solution of phospholipid liposomes was prepared by dissolving the required quantity of phospholipid in chloroform. The chloroform was evaporated using nitrogen. The residual phospholipid was suspended in TBS containing 5% glycerol carefully mixed with a few glass beads for 3 minutes and then centrifuged for 10 minutes at 10,000 xg. The above solution was discarded and the residue was carefully re-suspended in TBS containing 5% glycerol; in this way the phospholipid-liposome supply solution was obtained. These liposomes were stored at ambient temperature. The phospholipid concentrations were determined by phosphate analysis according to Bottcher et al., Anal. Chim. Acta. 24, 203-207 (1961).

SDS-PAGE

Gel electrophoresis on plates in the presence of SDS was carried out according to the method described by Laemmli, Nature, 227, 680-685 (1970) using a gel which contained 10% by weight of acrylamide, 0.27% by weight of N,N^3-methylene-bisacrylamide

and 0.1% by weight of SDS. 5% by weight of β-mercaptoethanol was present in gel samples with reduced disulfide bridges. The gels were stained as follows:

1.) with 0.25% by weight of Coomassie Blue R-250 in 50% by weight of ethanol and 15% by weight of acetic acid and decolorised in 10% by weight of ethanol and 10% by weight of acetic acid.

2.) with Schiff's reagent prepared from basic fuchsin (Merck) using the method of Segrest et al., described in "Methods in Enzymology", Vol. 28, 54-63 (1972) or

3.) with silver as described by Merril et al. in Electrophoresis, 3, 17-23 (1982).

Electrofocussing

The isoelectric pH measurements of proteins were carried out with the readymade thin layer polyacrylamide gels which contain amfoline carrier ampholyte (PAG plates, LKB) at a pH in the range from 3.5 to 9.5 in accordance with the manufacturer's instructions. The pH gradient in the gel was determined immediately after electrofocusing by cutting off a strip of the gel along a line between the anode and the cathode. The electrolytes were eluted from each strip using distilled water and the pH value of the water was measured with a combined glass electrode.

Determination of glutamic acid

The Gla determination was carried out by HPLC on a "Nucleosil 5SB" column (CHROMPACK) using the method of Kuwada et al., Anal. Biochem. 131, 173-179 (1983).

Example II

Coupling of phosholipids to Spherocil

The required phospholipids were dissolved in chloroform and added to the column material Spherocil (Messrs. Rhone-Poulenc) in a ratio of 5 mg of phospholipid per gram of Spherocil. The chloroform was evaporated with N_2 gas and the dry Spherocil phospholipid was then washed with the buffer in which VAC had been suspended. VAC bonds to Spherocil-coupled phospholipid in the presence of Ca^{++} and/or Mn^{++} when some of the phospholipids are negatively charged.

Example III

50 mcl of citrated/platelet-free plasma were mixed with 200 mcl of buffer (25 mM Tris/HCl, pH 7.5, 100 mM NaCl), in which kaolin (artificial surface which catalyses the essential coagulation, in this case ground glass), inositin (phospholipid source) and VAC were present. This mixture was incubated for 3 minutes at 37°C, after which 250 mcl of Ca^{++} buffer (200 mM Tris/HCl pH 7.5, 80 mM NaCl, 20 mM $CaCl_2$) were added. The coagulation time was measured as in Example I.

Example IV

Human blood was collected by venepuncture in trisodium citrate (final concentration about 13 mM citrate) and centrifuged at 2000xg for 10 minutes at room temperature. The resulting plasma was recentrifuged at 10,000xg for 15 minutes in order to obtain platelet free plasma (PFP). A standard pool of PFP was prepared by mixing plasmas obtained from several healthy donors.

Human umbilical cords were obtained within 15 minutes after delivery. The arteries were immediately perfused with ice-cold TBS-buffer, subsequently

prepared free from the Jelly of Warton and homogenized in TBS using a whirl mixer, Braun MX32. A 10% homogenate (w/v) was then fractionated.

Fractionation of the supernatant of a 10,000xg spin of the homogenate on Sephadex G-100 results in a reproducible specific profile (see Figure 7). The fractions affecting the coagulation system as measured with the MPTT are indicated in Fig. 7. A procoagulant activity eluted with the void volume. This activity can only be detected in the presence of factor VII in the MPTT, as indicated by experiments in which human congenital factor VII-deficient plasma was used. Therefore this procoagulant must be considered to be tissue thromboplastin.

Certain fractions expressed a distinct anticoagulant activity. These fractions were pooled and further purified with DEAE-Sephacel chromatography (see Figure 8A). The anticoagulant appeared to bind to the DEAE-Sephacel at 50 mM NaCl and 50 mM Tris/HCl pH 7.9. Elution of the activity with a linear gradient of NaCl at pH 7.9 was achieved at 150-160 mM NaCl. The DEAE-fractions expressing anticoagulant activity were pooled and submitted to Sephadex C-75 gel filtration (Figure 8B). The column (1.5 x 50 cm) was equilibrated with TBS. The activity appeared in the fractions which correspond to molecular weights of about 30,000-60,000 daltons.

The MPTT was used as a quantitative assay for the determination of the amount of anticoagulant activity (see Figure 9). One unit of anticoagulant activity was defined as the quantity which prolongs the clotting time in the MPTT, with HTP (final concentration 95 ug protein/ml) as initiator of coagulation, from its control value of 65 s to 100 s. With this assay, it was calculated that from 10 g wet arterial tissue 2 mg protein with approximately 1200 units anticoagulant activity can be isolated.

Modified Prothrombin Time Test (MPTT)

The modified prothrombin time test (MPTT) was carried out as follows:
In a siliconized glass cuvette 50 mcl PFP was stirred at 37°C with 150 mcl TBS, 25 mcl of a standard HTP-dilution, and 25 mcl TBS (control) or 25 mcl of a fraction of the arterial homogenate. After incubation for 3 minutes, coagulation was started at time zero with the addition of 250 mcl Ca^{2+} buffer (80 mM NaCl, 20 mM $CaCl_2$ and 10 mM Tris/Hcl pH 7.9). Fibrin formation was monitored optically with a "Payton Dual Aggregation Module". When factor X_a was utilized to initiate coagulation in the MPTT, HTP was omitted and 25 mcl purified factor X_a was added together with the 250 mcl Ca^{2+}-buffer to the diluted PFP.

Modified Thrombin Time Test (MTT)

This assay was carried out similar to the X_a-initiated MPTT described above, with the only exception that the X_a-preparation was replaced by 25 ml of purified thrombin.

Proteins

Protease type I and trypsin (EC 3.4.2.1.4) were obtained from Sigma. HTP was prepared from human brain as described by van Dam Mieras et al (1984) Methods of Enzymatic Analysis 5 pp. 352-365. Factor X_a, prothrombin and thrombin were purified from citrated bovine blood as described by Rosing et al. (1980) J. Biol. Chem. 255, 274-283. Factor V was purified from bovine blood as described by Lindhout et al (1982) Biochemistry 21, 4594-5502. Factor V_a was obtained by incubating factor V with thrombin. Prothrombin concentrations were calculated from Mw 72,000 and $A_{280}^{1\%}$-15.5 (Owen et al. (1974) J. Biol. Chem. 249, 594-605) and

factor V concentration was calculated from M_W=330,000 and $A_{280}^{1\%}$=9.6 (Nesheim et al. (1979) J. Biol. Chem. 254, 508-517). Factor X_a and thrombin concentrations were determined by active site titration (Rosing et al. (1980) J. Biol. Chem. 253, 274-283). Other protein concentrations were determined as described by Lowry et al. (1951) J. Biol Chem. 193,265.

Preparation of phospholipid and phospholipid vesicles

Ole_2Gro-P-Cho(1,2-Dioleoyl-sn-glycero-3-phosphocholine) and Ole_2Gro-P-Ser(1,2-Dioleoyl-sn-glycero-3-phosphoserine) were prepared as described by Rosing et al. (1980). Single bilayer vesicles composed of Ole_2Gro-P-Ser/Ole_2Gro-P-Cho (molar ratio 20:80) were prepared by sonication. Phospholipid concentrations were determined by phosphate analysis according to the method of Böttcher et al. (1961) Anal. Chim. Acta. 24, 203-207.

Measurement of prothrombin activation

The time course of prothrombin activation was examined at different concentrations of the anticoagulant. Mixtures of (X_a, Ca^{2+}), (X_a, phospholipid, Ca^{2+}) or (X_a, V_a, phospholipid, Ca^{2+}) were stirred with different amounts of the anticoagulant at 37°C in 50 mM Tris/HCl, 175 mM NaCl, 0.5 mg/ml human serum albumin at pH 7.9. After 3 minutes, prothrombin activation was started by the addition of prothrombin. At different time intervals, a 25 mcl sample was transferred from the reaction mixture into a cuvette (thermostated at 37°C), containing TBS, 2 mM EDTA and 0.23 mM S2238 (final volume: 1 ml). From the absorption change at 405 nm, measured with a Kontron Spectrophotometer Uvikon 810, and a calibration curve, plotted on the basis of assays with known amounts of purified thrombin, the amount of thrombin formed was calculated at different concentrations of the anticoagulant.

Phospholipid was added as vesicles composed of Ole_2Gro-P-Ser and Ole_2Gro-P-Cho with a molar ratio of 20:80.

Characterisation

Several fractions of the G-75 chromatography were tested in a MPTT and analysed with SDS-PAGE. The results (Figure 10) suggested that the anticoagulant has a molecular weight of approximately 32,000 daltons. The association between the anticoagulant activity and the 32K-band was confirmed by slicing the polyacryl-amide gel and subsequent elution of the proteins out of the slices with TBS, containing 0.5 mg/ml bovine serum albumin. An anticoagulant activity was found only in the eluant from the slice corresponding to the 32K-band. Furthermore this activity was found to be thermolabile at 56°C and demonstrated a similar dose response relationship in the MPTT as the starting material.

The G-75 fractions containing the peak anticoagulant activities were pooled and used for further characterization of the anticoagulant. Incubation of the anticoagulant at 56°C rapidly decreases its activity until after 2 minutes no activity can be measured. The anticoagulant loses its activity completely within 2 hours upon incubation at 37°C with protease type I, whereas trypsin hardly inactivates the anticoagulant after an incubation period of 3 hours (Figure 11). The protease type I and the trypsin concentration used in these experiments, completely inactivates 2.5 nM thrombin in 15 minutes. The amounts of protease type I and trypsin, carried over from the reaction mixtures to the MPTT, have no effect on the control clotting time.

Mode of action

The MPTT is prolonged in the presence of the anticoagulant (Figure 12) both when triggered with HTP and when started with factor X_a. Thrombin-induced coagulation however is not inhibited.

Because of these findings, we investigated the effect of the anticoagulant on the conversion of prothrombin to thrombin by factor X_a, factor V_a, phospholipid and C^{a2+}. Under the experimental conditions mentioned, thrombin formation is inhibited by the anticoagulant in a dose-dependent way (Figure 13A). The activation of prothrombin by factor X_x, phospholipid and Ca^{2+} in the absence of factor V_a can be inhibited also by the anticoagulant (Figure 13B). However this inhibition is not observed if the activation takes place in the absence of phospholipid (Figure 13C).

Example V

Polyclonal Antibodies against VAC

Polyclonal actibodies against bovine VAC were raised in rabbit. Bovine VAC, purified according to the method as described in Example I, was mixed with equal amounts of complete Freund's adjuvant. The mixture was injected subcutaneously into a rabbit. After a period of 4 weeks, the rabbit was boostered with a subcutaneous injection of purified bovine VAC. The booster was repeated twice with a two-weeks interval. 10 days after the last booster, the rabbit was bled and the collected blood was allowed to clot in order to obtain serum.

Immunoglobulins (Ig) were isolated from the serum according to the following method.

a) The serum was heated for 30 min at 56°C

b) Subsequently, the serum was applied to DEAE- Sephacel, which was equilibrated with 50 mM Tris, 100 mM NaCl, pH 8.2

c) The non-bound protein was precipitated with $(NH_4)_2SO_4$ at 50% saturation.

d) The precipitated proteins were pelleted by centrifugation and the pellet was resuspended in 50 mM Tris, 100 mM NaCl pH 7.9 and dialyzed extensively against the same buffer

e) The resulting protein mixture contained anti-VAC Ig.

From bovine aorta, bovine lung, rat and horse aorta, and human umbilical cord arteries protein fractions which express VAC-activity were isolated following the procedure as described.

The proteins were separated by electrophoresis on a polyacrylamide gel in the presence of dodecyl sulfate and under non-reduced conditions. After completion of the electrophoresis, the proteins were transferred from the gel to nitrocellulose sheets as described by Towbin et al. (1979) Proc. Natl. Acad. Sci. USA 76 4350-4354. The sheets were incubated with the anti-VAC Ig and after a throrough wash the sheets were incubated with goat anti-rabbit Ig coupled to horse radish peroxidase. The latter was visualized with diamine bezidine tetrahydrochloride, which is a substrate for the peroxidase.

A brown band on the nitrocellulose sheet, after completion of the described procedure, indicated the present of goat anti-rabbit Ig. Furthermore, on this spot were present anti-VAC Ig and proteins to which the anti-VAC Ig was bound.

Immunoblots of proteins with VAC-activity, isolated from bovine aorta, bovine lung, rat and horse aorta, and human umbilical cord arteries are presented in Figure 14.

From these results one can conclude the following: Using essentially the isolation procedure as described, a protein fraction with VAC-activity can be obtained from bovine aorta, bovine lung, rat and horse aorta, and human umbilical cord arteries. Moreover, the

isolated protein fractions with VAC-activity contain proteins, with Mw 10 of about 32,000 about 34,000 and about 70,000, that react with anti-VAC Ig raised against purified bovine VAC in rabbits.

Example VI

Purification Step for VAC, using Large Volume Phospholipid Vesicles

Large volume phospholipid vesicles (LVV), composed of 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC), were prepared by the known method of P. van de Waart et al. Biochemistry (1983) 22, 2427-2432

For the purification step, LVV containing PS/PC (molar ration 20:80) was used. However, other molar ratios are useful too, with the restriction that negatively charged phospholipids must be present. The chain length of the fatty acids in the phospholipids can be varied too.

LVV, $\pm$ 1 mM phospholipids in 50 mM Tris/HCl, 100 mM NaCl, pH 7.9, were mixed with an equal volume of a protein fraction, containing VAC-activity. The proteins were in 50 mM Tris/HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.9. The mixture was allowed to stand for 5 min at ambient temperature. Subsequently, the mixture was centrifuged for 30 min at 20,000xg. The pellet was resuspended in 50 mM Tris/HCl, 100 mM NaCl, 10 mM $CaCl_2$, pH 7.9, and recentrifuged. The resulting pellet was then resuspended in 50 mM Tris/HCl, 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.9, and recentrifuged. The resulting supernatant contained the VAC-activity.

The above described procedure is an efficient purification step in the procedure to obtain purified VAC.

Table A

Summary of the purification of VAC from inner coats of bovine aortas

Purification Step	Protein[a] mg	VAC[b] Units	Specific activities units/mg	Yield %	Degree of purification
Supernatant liquid with 35% $(NH_4)_2 SO_4$	630	19.000	31.0	100	1.0
Precipitate with 90% $(NH_4)_2SO_4$	470	19.000	40.4	97	1.3
Hydroxyapatite fraction	206	17.300	84.0	89	2.7
DEAE fraction	35.8	13.900	388	71	12.5
Sephadex G-100 fraction 139	0.45	0.666	1480	3.4	47.7

a) The quantity of protein was determined using the method of Lowry et al. (Lowry, O.H., Rosebrough N.V., Farr A.L. and Randall R.J. J.Biol. Chem. 193 (1951) 265).

b) The VAC units were determined using the one-stage coagulation test described in Example 1 by a series of test dilutions. The coagulation time of the control samples was 65 seconds. One unit of VAC activity was defined as the quantity of VAC which prolongs the coagulation time to 100 seconds.

Table B

The metal-dependent bonding of VAC to negatively charged phospholipid liposomes

Cation (10 mm)	t_c, seconds[a] supernatant liquid	EDTA[c]
Control (no liposomes)	180	N.D.[d]
Control (no cation)	174	64.8
$CaCl_2$	64.2	134
$MgCl_2$	165	N.D.
$MnCl_2$	65.1	N.D.

a) The coagulation time (t_c) was determined using the one-stage coagulation test described in Example I.

b) 50 mcl of the supply solution of phospholipid liposomes (PS/PC; 50/50 mol/mp: 1 mm), 50 mcl VAC (250 mcg/ml, Specific activity = 700 units per mg) and 100 mcl of TBS which contained 5% glycerol and the added cation, pH 7.5, were mixed together at ambient temperature and centrifuged for 15 minutes at 15,000 xg 25 mcl of the supernatant liquid obtained was diluted with TBS to a final volume of 175 mcl and then tested by the one-stage coagulation test. The remainder of the supernatant liquid was analysed with SDS-PAGE (Figure 4).

c) The liposome precipitate obtained was again suspended in 150 mcl of TBS containing 5% glycerol and 10 mm EDTA, pH 7.5. The suspension was centrifuged for 15 minutes at 15,000 xg. The VAC activity of the supernatant liquid was analysed as described in b).

d) N.D. = not determined.

Table C

The effect of VAC on the amidolytic activity of factor X_a and factor II_a

	A_{405}/Min.10^3 (a) VAC	
	-	+
X_a	110.5	110.5
X_a, AT-III	80.0	81.5
X_a, Heparin	110.5	109.0
X_a, Heparin, AT-III	47.5	N.D.[b]
II_a	7.5	7.5
II_a, AT-III	5.4	5.6
II_a, Heparin	7.5	7.1
II_a, Heparin, AT-III	0.56	N.D.

a) The amidolytic activity was measured as follows: Factor X_a or Factor II_a was diluted with the above-mentioned agents in TBSA. The reaction mixture was stirred with a Teflon-coated stirrer in a plastics dish which had been tempered to 37°C using a thermostat. After 10 minutes, a sample of 100 mcl (X_a) or 50 mcl (II_a) was taken from the dish. This sample was placed in another plastics dish which had been tempered to 37°C with a thermostat and which contained 800 mcl of TBSE and 100 mcl of TBSA and 100 mcl of S 2337 (2 mM) or 900 mcl of S 2238 (5 mM). The change in absorption at 405 nM was measured with a Kontron Spectrophotometer Uvikon 810 which had been tempered at 37°C by means of a thermostat.

The final concentrations of the various agents in the reaction mixtures were as follows: 18.7 nM of Factor X_a; 1.5 nM of Factor II_a; 18.7 nM human AT-III; 1 unit per ml of heparin and 10.7 mcg/ml of VAC (Specific activity: 1300 units per mg).

b) N.D. = not determined.

Description of the figures

Figure 1 Gel filtration of VAC on Sephadex G-100.

The column (3 x 80 cm) was prepared with a pressure height of 60 cm and equilibrated with 500 mM NaCl and 20 mM Tris/HCl, pH 7.5. The VAC-containing fraction obtained after DEAE chromatography was concentrated down to 2 ml and then passed over the Sephadex G-100. The pressure height of 60 cm was maintained. The empty volume was 245 ml (fraction 70). Then fractions of 2 ml were collected. The fractions were dialysed with TBS containing 10% glycerol, after which they were tested for VAC activity by the one-stage coagulation test as described in Example I. The coagulation times were obtained at dilutions of 1:10 of the G-100 fractions with TBS. The coagulation time in the absence of VAC was 65 seconds.

Figure 2 Analytical SDS-PAGE of VAC

SDS-PAGE with gels containing 10% by weight of acrylamide, 0.27% by weight of N,N^3-methylene-bisacrylamide and 0.1% by weight of SDS was carried out according to Laemmli (Laemmli, U.K. (1970) Nature 227, 680-685). The significance of the figures on the x axis is as follows:

1) Reference Proteins of known molecular weight in which any disulphide bonds are reduced.

2) 25 mcg of reduced VAC,

3) 25 mcg of non-reduced VAC.

The gel was stained with Coomassie Blue and decolorised in the manner described in Example I.

Figure 3 Determination of the isoelectric pH of VAC.

Electrofocussing was carried out with PAG plates in a pH range of from 3.5-9.5 (see Example 1). 200 mcg of human H_b^1 and 20 mcg of VAC were applied to the gel after the pH gradient had formed in the gel. Human H_b was used as a reference (known isoelectric point: pH 6.8). Before the gel was subjected to staining with Coomassie Blue, the gel was fixed for 30 minutes with 0.7 M trichloroacetic acid.

Figure 4 Analysis of the bonding of VAC to negatively charged phospholipid liposomes with SDS-PAGE

SDS-PAGE was carried out according to Laemmli (Laemmli, U.K. (1970) Nature 227, 680-685) on the same plates as described in Example 1. The samples analysed were obtained from the bonding experiments as mentioned in the explanation to Table B. The significance of the figures on the x axis is as follows:

1) Reference Proteins of known molecular weight in which any disulphide bonds are reduced.

2) Supernatant liquid obtained after centrifuging a VAC preparation in the absence of liposomes.

3) Supernatant liquid obtained after centrifuging VAC in the presence of liposomes.

4) Supernatant liquid obtained after centrifuging VAC in the presence of liposomes and Ca^{++}.

5) Supernatant liquid obtained after centrifuging the liposome precipitate of 4) which had been resuspended in TBS containing 10 mM EDTA.

Figure 5 The effect of the concentration of VAC on the inhibition (%) of thrombin formation

The concentrations of VAC mentioned are the final concentrations present in the test systems. The thrombin formation was measured with 1 mcM prothrombin, 10 nM factor X_a and 0.5 M (▲–▲) or 5 M (● —●) phospholipid membrane (PC/PS; 4:1, mol/mol) in 10 mM of TBSA containing $CaCl_2$. The reaction mixture was stirred with the specified quantities of VAC (Specific activity: 1300 units/mg) for 3 minutes at 37°C without prothrombin. By adding prothrombin to the mixture, as in Example I, the thrombin formation was initiated and the speed was measured. The speed of thrombin formation in the absence of VAC was 3.3 nM II_a/min. (▲-▲) or 10.9 nM II_a/min. (● —●).

Figure 6 The effect of the phospholipid concentration on the inhibition (%) of thrombin formation by VAC.

Thrombin formation was measured at 1 mcM prothrombin, 10 nM factor X_a, 10.7 mcg/ml of VAC

(Specific activity: 1300 units/mg) and at various concentrations of phospholipid membrane (PC/PS; 4:1, mol/mol) in TBSA. Factor X_a, VAC and phospholipid were stirred in TBSA for 3 minutes at 37°C. The thrombin formation was initiated by adding prothrombin to the reaction mixture. The speed of thrombin formation was measured as described in Example I. The percentage inhibition of thrombin formation (•—•) was measured for each phospholipid concentration with the corresponding speed of thrombin formation in the absence of VAC (▲–▲).

Figure 7. Gel filtration of the 10,000 x g supernatant of an umbilical cord artery homogenate on Sephadex G-100.

2 ml of the 10,000 x g supernatant was loaded on a Sephadex G-100 column (1.5 x 80 cm), which was pre-equilibrated with TBS. The column was eluted with TBS. Aliquots of the resulting fractions were tested in the MPTT. Certain fractions (▬) express a procoagulant activity and initiate coagulation in the MPTT without the addition of HTP, factor X_a or thrombin. Other distinct fractions (▭) prolong the clotting time in the MPTT, using HTP to initiate coagulation. These fractions were pooled and further fractionated.

Figure 8. Chromatography of the anticoagulant on DEAE-Sephacel (A) and Sephadex G-75 (B).

The pool, containing the anticoagulant, from the Sephadex G-100 column was applied to DEAE-Sephacel. Elution was performed with a 200 ml linear gradient of 50 mM - 300 mM NaCl (- - -). Fractions (4 ml) were collected. A_{280} was determined for each fraction (—) and anticoagulant activity was assayed

in the MPTT, using HTP (final concentration 95 mcg protein/ml) as initiator of coagulation (●- -). The fractions with anticoagulant activity were pooled, concentrated and subsequently applied to Sephadex G-75 (B). Fractions (2 ml) were collected. A_{280} was determined for each fraction (-) and also anticoagulant activity (●). V_o represents the void volume of the column.

Figure 9. Dose response of the anticoagulant in the MPTT.

Varying amounts of the anticoagulant were added to the MPTT. Coagulation was initiated with HTP (final concentration 95 mcg protein/ml) The control clotting time was 65 s.

Figure 10. Gel electrophoresis of several fractions of the G-75 eluant.

A fixed amount of several fractions of the G-75 eluant were analysed by SDS-PAGE. The gels were silver-stained according to Merril et al. (1982) Electrophoresis J. 3, 17-23. Lane 1: reduced low molecular weight standards; lanes 2-6 unreduced aliquots of the G-75 fractions numbers 35,39,41,43 and 50 respectively.

Figure 11. The effect of proteolytic enzymes on the activity of the anticoagulant.

The anticoagulant was incubated at 37°C with protease type I (■, final concentration 0.11 units/ml), trypsin (▲ , final concentratin 88 BAEE units/ml) and without proteolytic enzymes (●). At the time points indicated, 5 mcl containing 6 mcg protein of the anticoagulant, was removed from the reaction

mixture and added to a MPTT. Clotting was initiated with HTP (final concentration 18 protein mcg/ml). Control clotting time was 110 s. The units given in this legend for the proteolytic enzymes are calculated from the values supplied by the manufacturer.

Figure 12. The effect of the vascular anticoagulant on the clotting times, induced in the M(P)TT by either HTP, factor X_a or thrombin.

The concentrations of the initiators of coagulation (HTP 16 mcg protein/ml, 1.5 nM factor X_a or 0.4 nM thrombin) were chosen so as to give control clotting times of about 110 seconds (open bars). When factor X_a was used, phospholipid vesicles (final concentration 10 mcM), composed of Ole_2Gro-P-Ser/Ole_2Gro-P-Cho (molar ratio, 20:80) were added to the reaction mixture. Clotting times induced by the indicated agents in the presence of 3.5 mcg protein of the anticoagulant are represented by the shaded bars.

Figure 13. The effect of the anticoagulant on prothrombin activation by (X_a, V_a, phospholipid, Ca^{2+}), (X_a, phospholipid, Ca^{2+}) and (X_a, Ca^{2+}).

The reaction mixtures contained: (A), 1 mcM prothrombin, 0.3 nM X_a, 0.6 nM V_a, 0.5 mcM phospholipid and 10 mM $CaCl_2$ with 12.0 mcg/ml anticoagulant (■), 4.8 mcg/ml anticoagulant (▲) and without anticoagulant (●). (B) 1 mcM prothrombin, 10 nM X_a, 0.5 mcM phospholipid and 10 mM $CaCl_2$ with 2.4 mcg/ml anticoagulant (■), 0.48 mcg/ml anticoagulant (▲) and without anticoagulant (●). (C) 1 mcM prothrombin, 75 nM X_a and 10 mM $CaCl_2$ with 120 mcg/ml anticoagulant (▲) and without anticoagulant (■). At the times indicated, samples were removed and thrombin was determined.

Figure 14. Immunoblots.

The blots were obtained by procedure described in Example V. Lane 1: protein fraction with VAC-activity isolated from bovine aorta. Lane 2: protein fraction with VAC-activity isolated from bovine aorta. Lane 3: protein fraction with VAC-activity isolated from bovine lung. Lane 4: protein fraction with VAC-activity isolated from human umbilical cord arteries. Lane 5: protein fraction with VAC-activity isolated from rat aorta. Lane 6: protein fraction with VAC-activity isolated from horse aorta.

Figure 15. Gel electrophoresis (A) and the anticoagulant activity (B) of the various fractions of the eluate from G-75.

Specific quantities of the various fractions of the eluate from G-75 were subjected to gel electrophoresis as described hereinbefore. The bands were stained with silver using the method described by Merril C.R., Goldman D., and Van Keuren M.L. (1982) Electrophoresis 3, 17-23. Electrophoresis lane 1: standard material with a low molecular weight; electrophoresis lanes 2-6: non-reduced equal quantities of the G-75 fractions, with increasing elution volumes. Specific quantities of the G-75 fractions which had been analysed by gel electrophoresis were tested in the MPTT (see the preceeding description) using HTP to initiate coagulation. The control coagulation time is represented by the open bar. The figures under the shaded bars correspond to the numbers of the electrophoresis lanes in Figure 15A.

Figure 16. The heat deactivation of the vascular anticoagulation agent.

The anticoagulation agent was incubated at 56°C and, after the various incubation periods, samples of 5 mcl containing 3.6 mcg of protein were taken, immediately cooled with ice and then tested in the MPTT using HPT as coagulation initiator. The coagulation time of the control sample was 110 seconds.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Blood coagulation-inhibiting proteins, characterised in that

- they are capable of being isolated from strongly vascularised tissue,
- they do not deactivate the coagulation factors,
- they do not hydrolyse phospholipids,
- they bind reversibly to negatively charged phospholipids via the divalent cations Ca^{2+} and/or Mn^{2+},
- they are capable of displacing the factor X_a and the prothrombins from a negatively charged phospholipid surface and
- they do not inhibit the biological and amidolytic activity of the factors X_a and II_a.

2. Anticoagulant proteins as claimed in claim 1, characterised in that they inhibit the coagulation induced by a vascular coagulant or by factor X_a but they do not inhibit coagulation induced by thrombin.

3. Anticoagulant proteins as claimed in claim 1 or claim 2 characterised in that they inhibit

- they modified prothrombin-time experiment, as hereinbefore described, and/or
- the modified, activated, partial thromboplastin-time experiment, as hereinbefore described, and/or
- the non-modified prothrombin-time experiment and/or
- the prothrombin activation by the coagulation factor X_a in the presence of negatively charged phospholipids and Ca^{2+} and/or
- the intrinsic X-activation by the factor IX_a in the presence of negatively charged phospholipids and Ca^{2+} and/or
- the prothrombin activation of isolated,

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stimulated blood platelets and/or
- the coagulation induced by the blood vessel walls and/or
- the coagulation-dependent aggregation of platelets.

4. Anticoagulant proteins as claimed in any one of claims 1 to 3, characterised in that
- their inhibiting effect is dependent on the quantity of phospholipids, and
- the inhibition of factor X_a-activation of prothrombin induced by the proteins is dependent on the phospholipid concentration.

5. Anticoagulant proteins as claimed in any one of the preceding claims characterised in that
- they reversibly bond, via the divalent cations Ca^{2+} and/or Mn^{2+} to negatively charged phospholipids which are coupled with Spherocil.

6. Anticoagulant proteins as claimed in any one of the preceding claims, characterised in that they have molecular weights of about 70×10^3, about 60×10^3, about 34×10^3 or about 32×10^3.

7. Anticoagulant proteins as claimed in any one of the preceding claims characterised in that they can be isolated from arteries.

8. Anticoagulant proteins as claimed in any one of the preceding claims characterised in that
- they are isolated from the walls of mammalian blood vessels and then purified,
- they are not glycoproteins,
- they are not phospholipases,
- they have an isoelectric point at pH 4.4-4.6,
- the activity of the anticoagulant proteins at

56°C is thermally unstable,

- the activity of the anticoagulating proteins in citrated plasma remains stable for some hours at 37°C,
- the activity of the anticoagulant proteins is not completely destroyed by tripsin and/or chymotrypsin,
- the activity of the anticoagulant proteins is not affected by collagenase and/or elastase,
- they bond, via the divalent cations Ca^{2+} and/or Mn^{2+}, to negatively charged phospholipids which can be found in vesicles, liposomes or etherosomes,
- they bond via the divalent cations Ca^{2+} and/or Mn^{2+} to negatively charged phospholipids which are coupled to Spherocil,
- the bonding of the proteins to the negatively charged phospholipids is reversible and can be reversed by ethylenediamine tetracetic acid (EDTA),
- they displace factor X_a and prothrombin from a negatively charged phospholipid surface,
- they inhibit the modified prothrombin-time experiment as hereinbefore described,
- they inhibit the modified, activated, partial thromboplastin-time as hereinbefore described,
- they inhibit the non-modified prothrombin-time experiment,
- they inhibit the prothrombin activation by the coagulation factor X_a in the presence of negatively charged phospholipids and Ca^{2+} in vitro,
- they do not inhibit the biological and amidolytic activity of factors X_a and II_a,
- they inhibit the intrinsic X-activation by the factor IX_a in the presence of negatively charged phospholipids and Ca^{2+} in vitro,
- they inhibit the prothrombin activation of

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isolated, stimulated blood platelets in vitro,
- they inhibit the coagulation induced by the walls of the blood vessel in vitro, and
- the inhibition of factor X_a-activation of prothrombin induced by the proteins is dependent on the concentration of phospholipids and is reduced at high phospholipid concentrations.

9. Human anticoagulant proteins as claimed in any one of the preceding claims.

10. Bovine anticoagulant proteins as claimed in any one of the claims 1 to 8.

11. Murine anticoagulant proteins as claimed in any one of claims 1 to 8.

12. Equine anticoagulant proteins as claimed in one of claims 1 to 8.

13. An anticoagulant protein as claimed in any one of the preceding claims having a molecular weight of 32 x 10^3.

14. A process for preparing anticoagulant proteins as claimed in claim 1 which comprises homogenisation of mammalian blood vessel walls, strongly vascularised tissue or endothelial cell cultures followed by differential centrifugation and subsequently one or more of the following purification treatments in any desired sequence:

(b) precipitation with salt
(c) affinity chromatography
(d) ion exchange chromatography and
(e) chromatography using a molecular sieve, if desired dialysis of the products being carried out between stages (b) and (c), (c) and (d) and (d) and (e).

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15. A process as claimed in claim 14, which further comprises a step wherein immunoadsorption chromatography is employed.

16. A process as claimed in claim 14 or 15, wherein the anticoagulant proteins are purified using phospholipid vesicles.

17. A process as claimed in any one of claims 14 to 16, wherein ammonium sulphate is used for precipitation in step (b), hydroxyapatite is used for chromatography in step (d) and Sephadex G-100 or G-75 is used for chromatography in step (e).

18. A process as claimed in any one of claims 14 to 17, wherein blood vessels derived from cattle, rats, horses or humans are employed.

19. A process as claimed in claim 18 wherein human umbilical veins and/or arteries are employed.

20. A process as claimed in claim 18 wherein bovine aortas are employed.

21. An anticoagulant protein prepared by a process as claimed in any one of claims 14 to 20.

22. A pharmaceutical composition which comprises one or more proteins as claimed in claim 1 in association with a pharmaceutically acceptable carrier and/or excipient.

23. An anticoagulant protein as claimed in claim 1 for use in therapeutic treatment.

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24. Anticoagulant proteins as claimed in claim 1 substantially as hereinbefore described with reference to Examples I, IV and V.

25. A process as claimed in claim 14 substantially as hereinbefore described with reference to the Examples I, IV and VI.

Dated this 20th day of February, 1990.

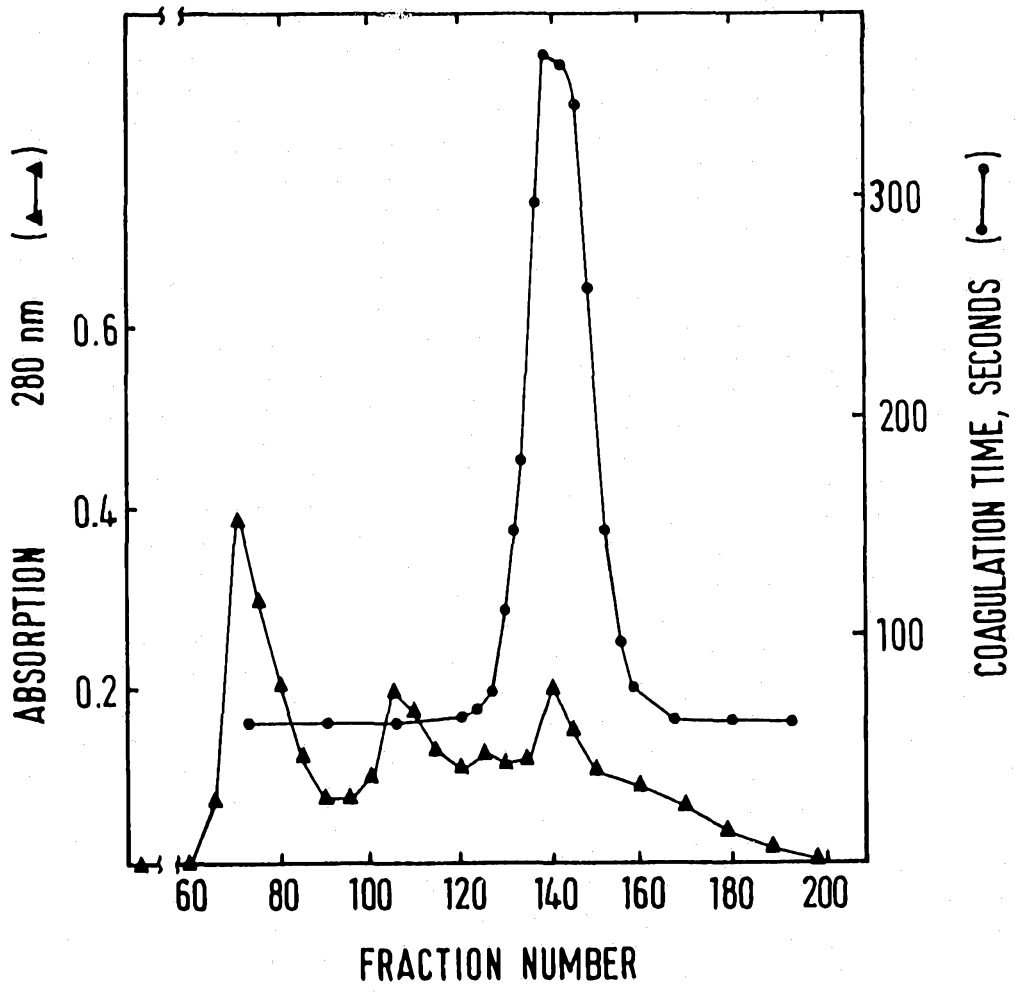
BOEHRINGER INGELHEIM INTERNATIONAL GmbH,

By its Patent Attorneys,

DAVIES & COLLISON.



FIG. 1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0.4
0.2
300
200
100
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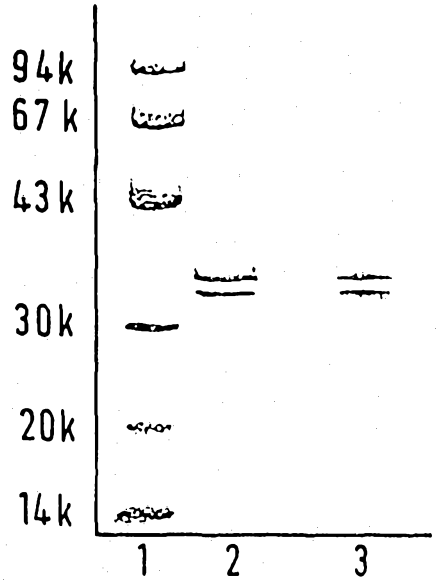
80
100
120
140
160
180
200
FRACTION NUMBER


94k
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30k
20k
14k
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2
3

FIG. 2

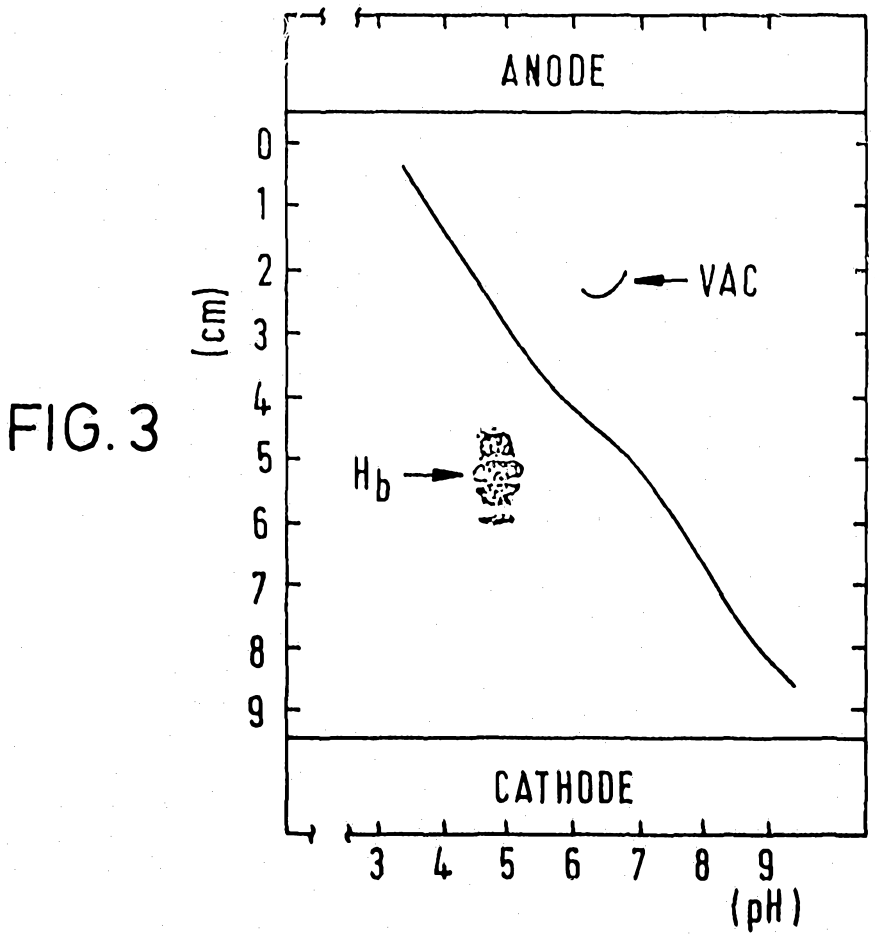
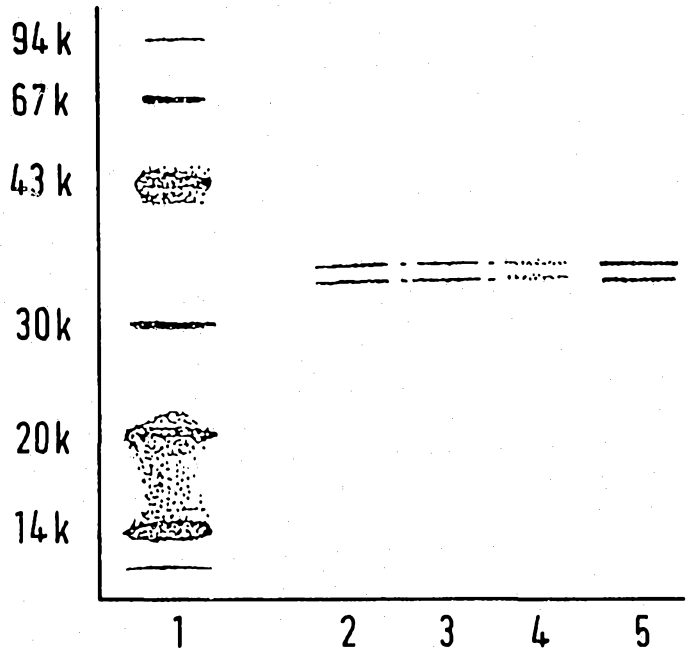

ANODE
CATHODE
VAC
Hb
(cm)
0
1
2
3
4
5
6
7
8
9
3
4
5
6
7
8
9
(pH)

FIG. 3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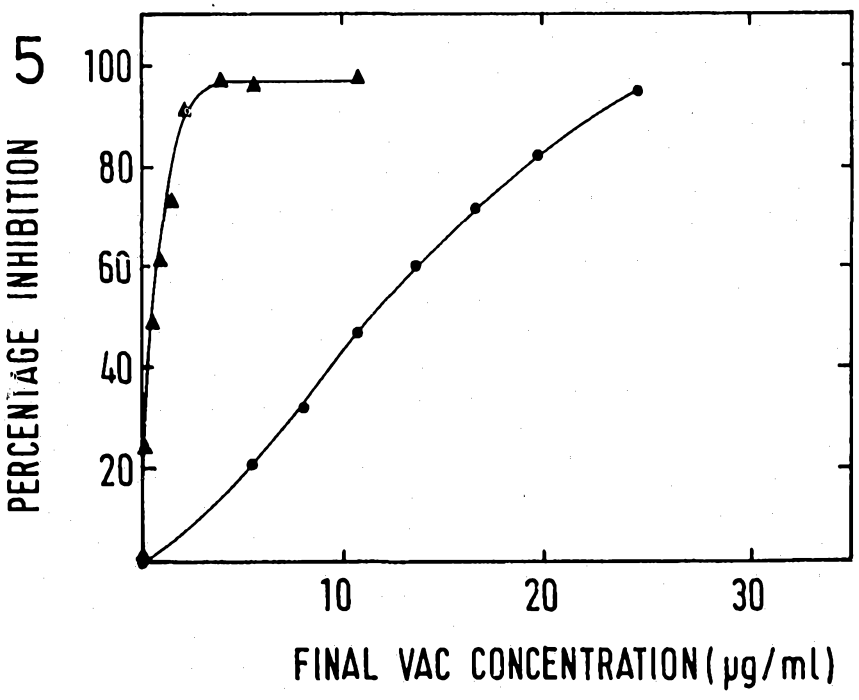
30k
20k
14k
1
2
3
4
5

FIG. 4

FIG. 5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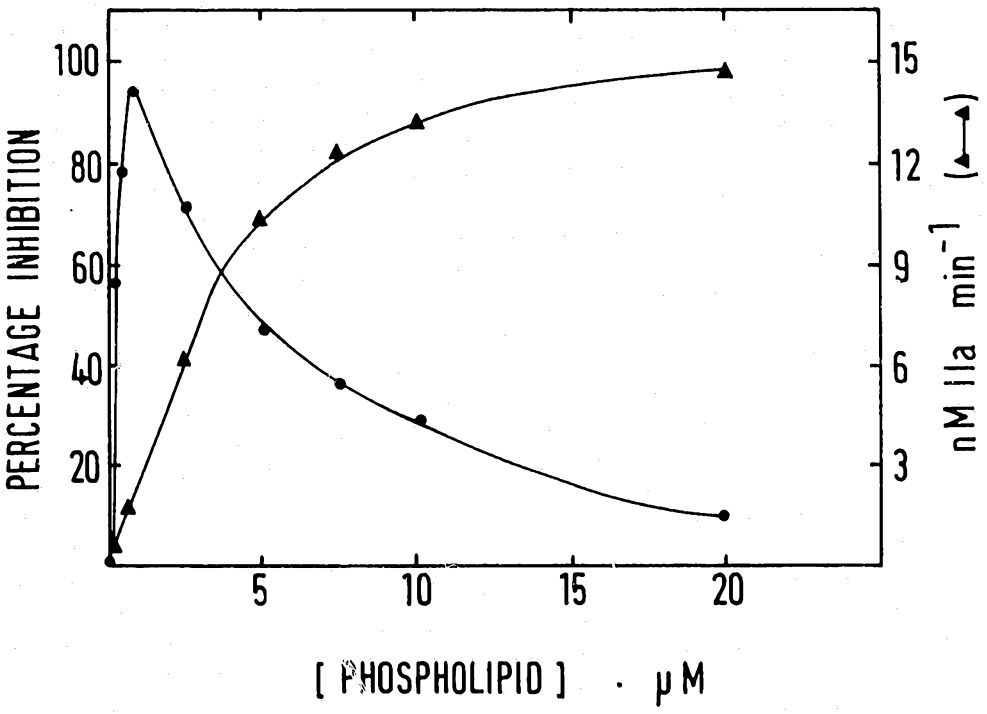
80
60
40
20
10
20
30
FINAL VAC CONCENTRATION (µg/ml)

FIG. 6


PERCENTAGE INHIBITION
100
80
60
40
20
5
10
15
20
15
12
9
6
3
nM IIa min^{-1} (▲—▲)
[PHOSPHOLIPID] · μM

0 1285 47642

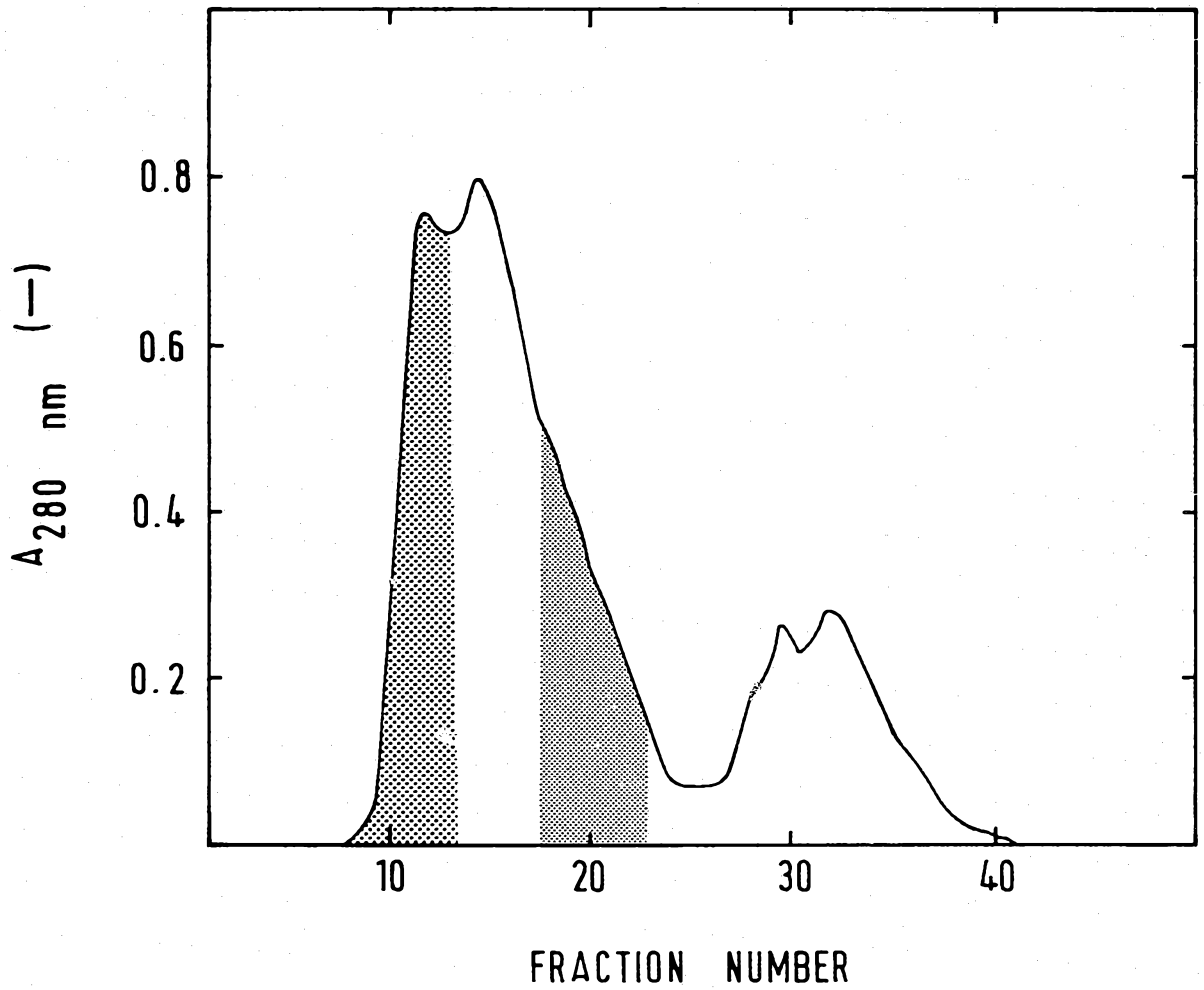


FIG. 7

6 12 85 47682

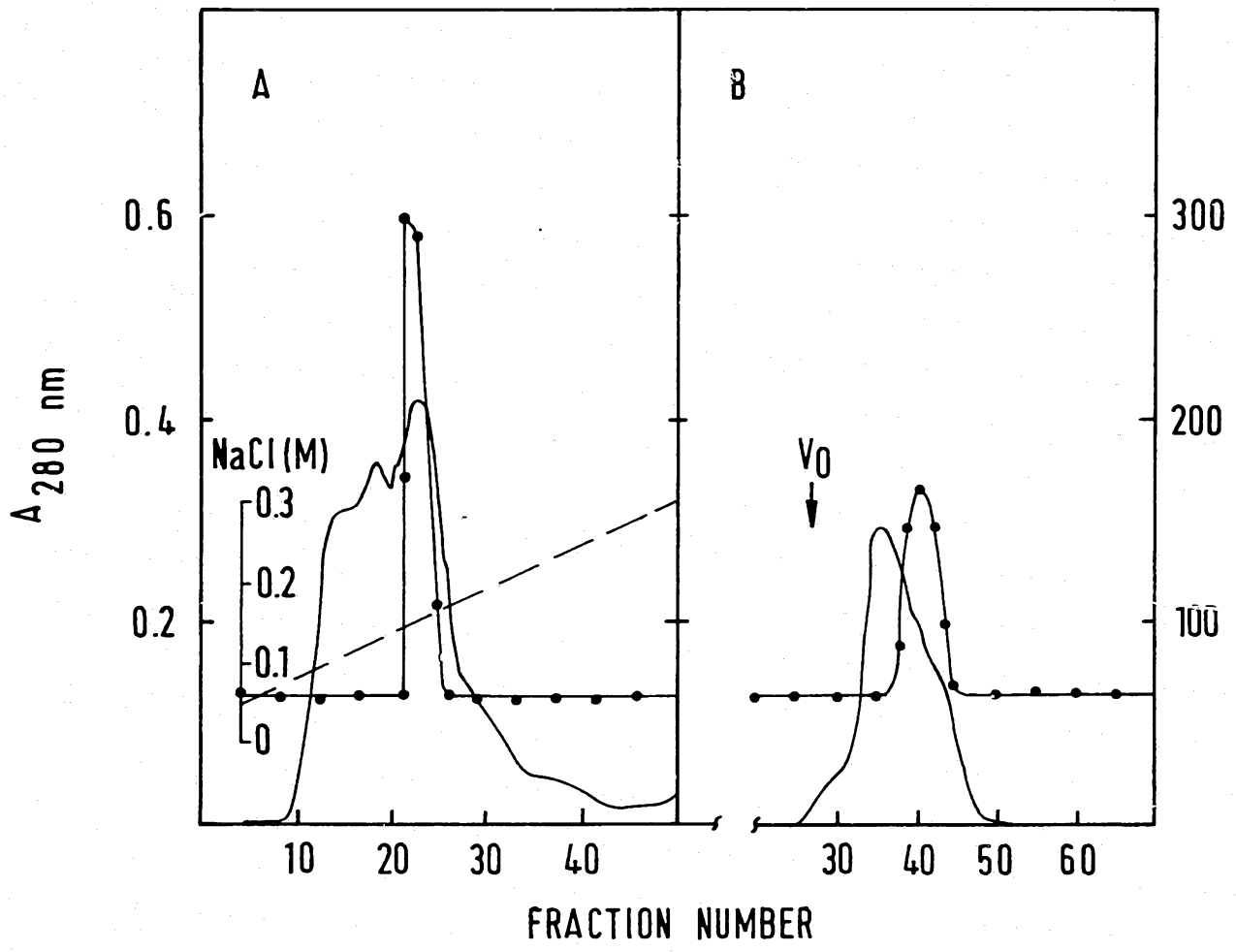


FIG. 8

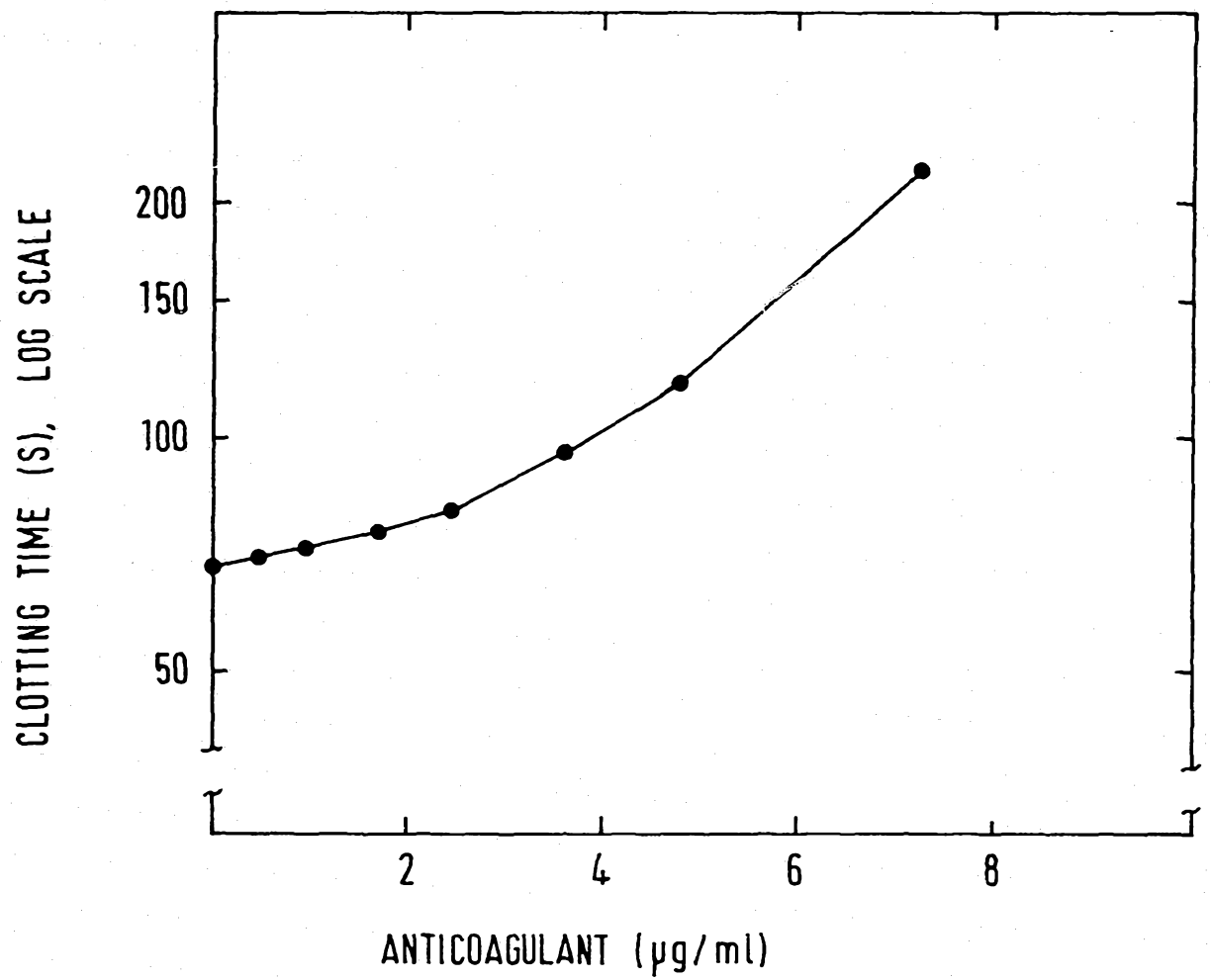
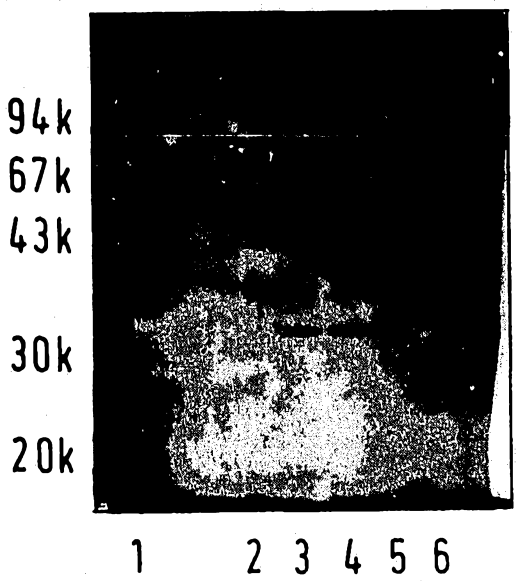

CLOTTING TIME (S), LOG SCALE
200
150
100
50
2
4
6
8
ANTICOAGULANT (µg/ml)

FIG.9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43k
30k
20k
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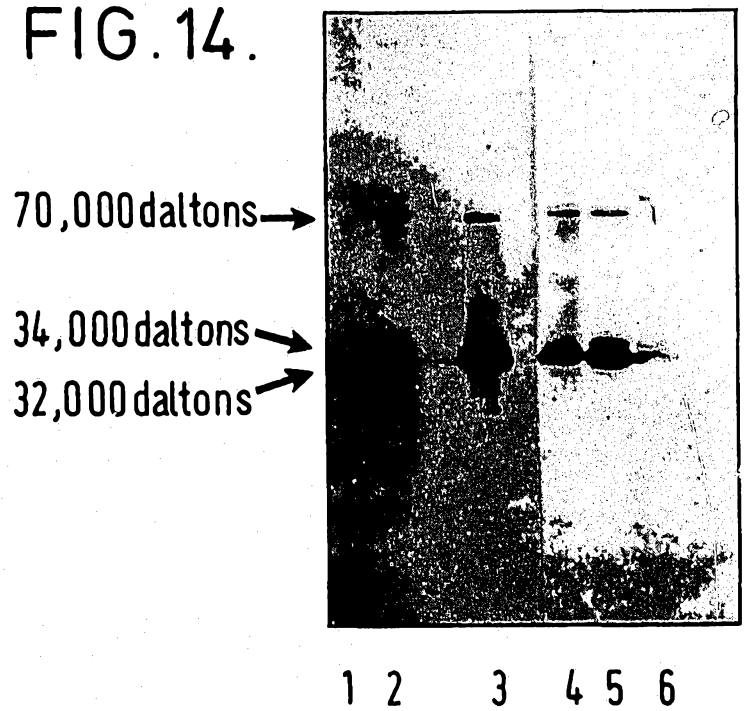
FIG. 14.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34,000daltons
32,000daltons
1 2 3 4 5 6

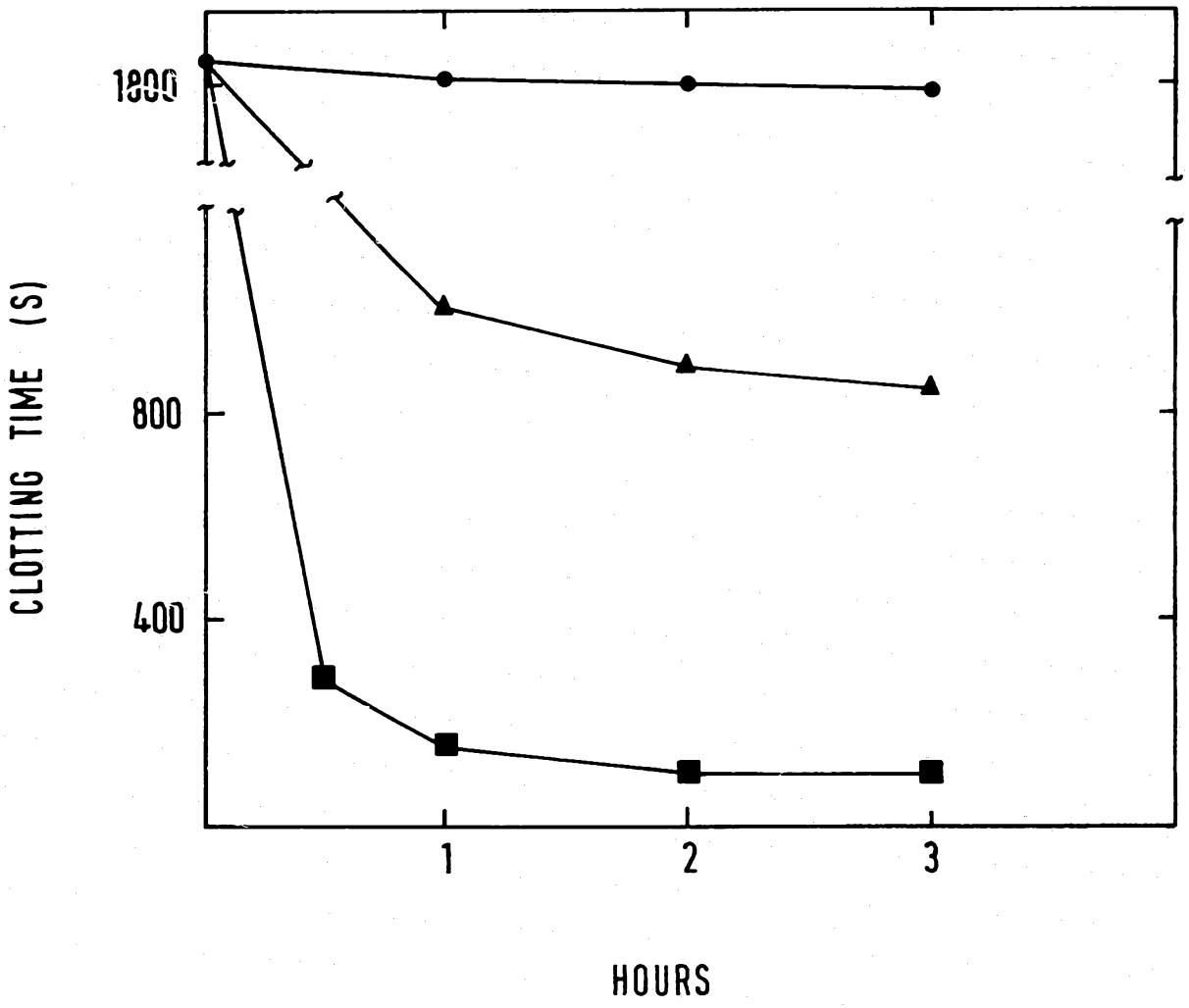

FIG. 11

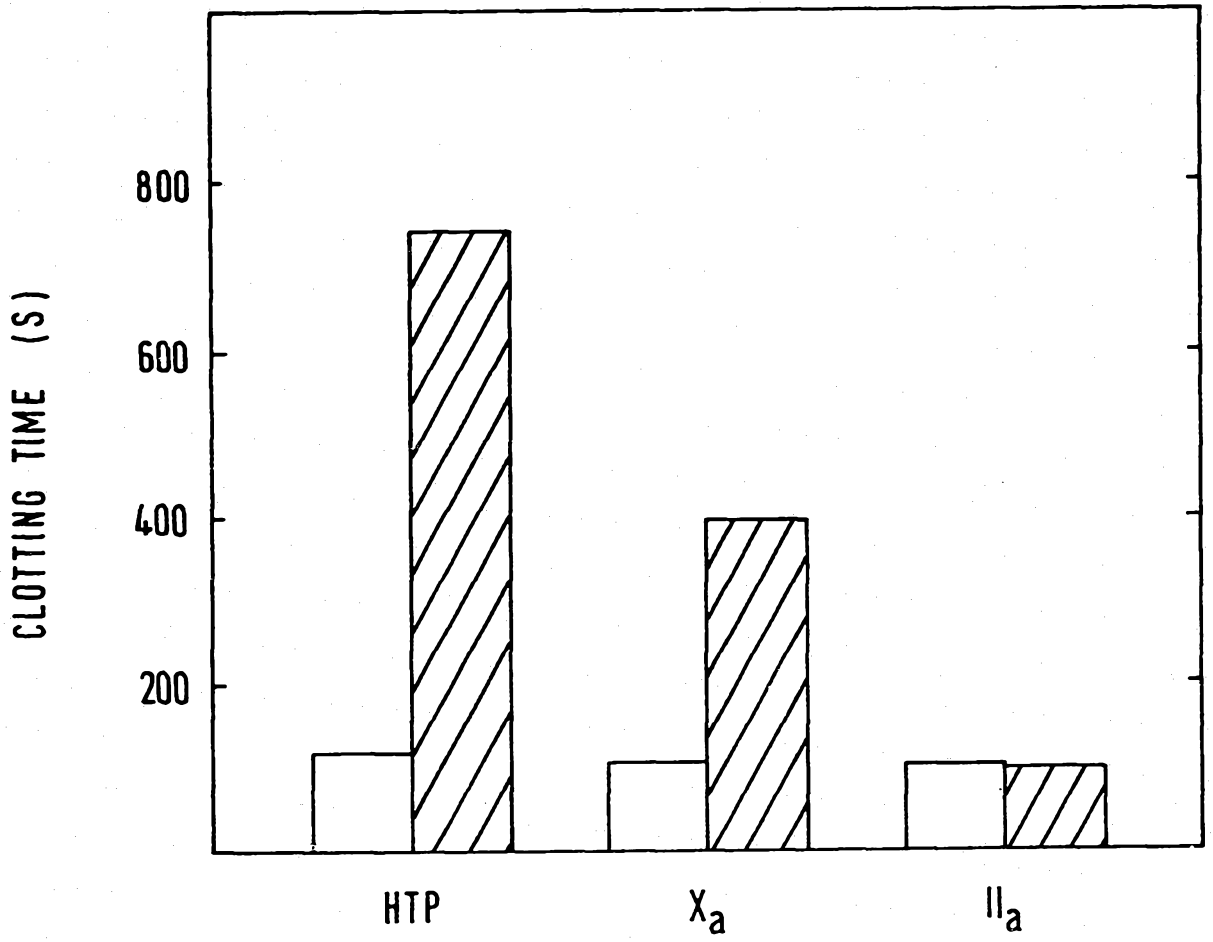

FIG. 12

6 12 85 4682

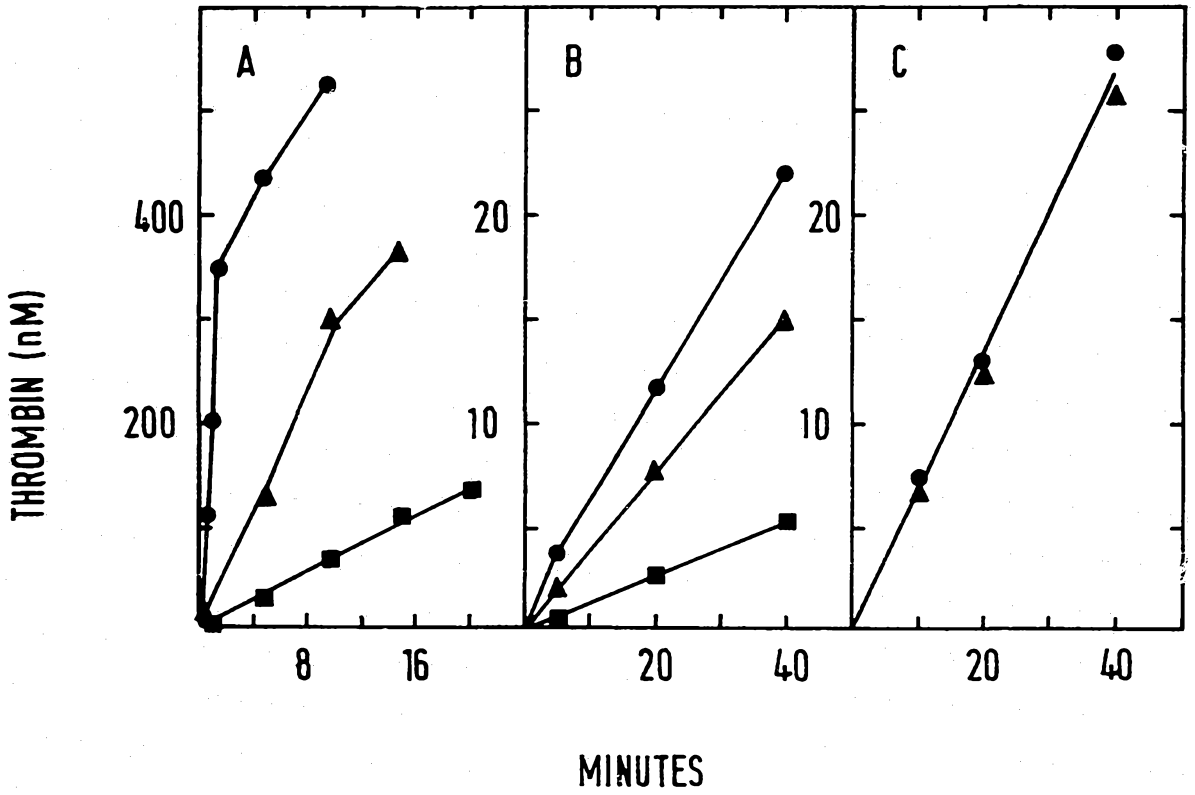

FIG. 13

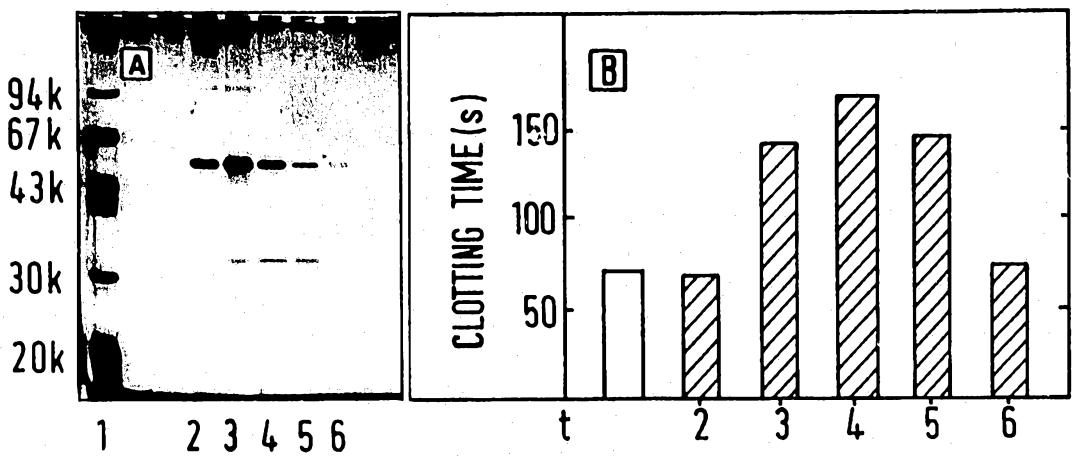
A
94k
67k
43k
30k
20k
1 2 3 4 5 6
B
CLOTTING TIME (s)
150
100
50
t 2 3 4 5 6

FIG. 15

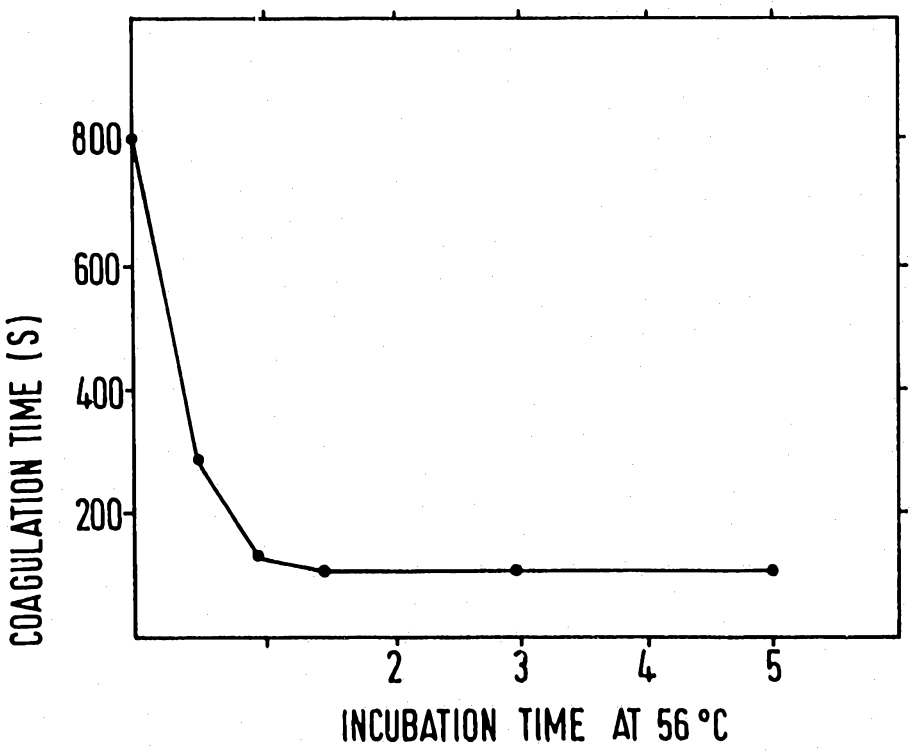
800
600
400
200
COAGULATION TIME (S)
2 3 4 5
INCUBATION TIME AT 56 °C

FIG. 16