

*For m' Watt* *0035*

Application for a Research Grant in Support of Work Destined to Improve the  
Stability and Yield of Factor VIII (Antihæmophilic Factor) Isolated from  
Human Plasma

by *for your file*

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Health Department on 15th June 1978.

Synopsis:

The current supplies of Factor VIII concentrates from NHS sources do not meet the demands of adequate therapy for hæmophiliacs in the U.K. Importation from commercial sources with the attendant problems of hepatitis and increased costs, have been necessary. Although the availability of raw material (fresh frozen plasma) for the production of Factor VIII concentrate is restricted, the overall problem is greatly aggravated by poor yields of biologically active Factor VIII, such that more than 70% of the Factor VIII obtained from the blood donor is lost during subsequent handling and/or separation procedures. This situation is partly due to the instability of Factor VIII coagulant activity and it is the object of this work to identify the factor(s) responsible for this phenomenon, with particular reference to the role of vascular plasminogen activator and the development of soluble fibrin monomer complexes. It is hoped that from these studies will emerge fundamental data which will contribute to future improvements in production methods for therapeutic factor VIII concentrates.

### III Introduction

Early investigations into the stability of F VIII coagulant activity aimed primarily at improving the clinical efficacy of blood products for haemophilia A therapy have shown quite clearly that the essential biological activity of anti-haemophilic factor (AHF) is very labile and in whole blood stored at 4°C, decays rapidly for the first 1-2 days and then more slowly over the ensuing 3 weeks (Penick & Brinkhous 1956; Kekwick & Walton 1962 and 1965; Goldstein et al 1964; Weiss 1965; Britten & Grove-Rasmussen 1966; Preston 1967; Stibbe et al 1972). These studies highlighted the need for care in the choice of anticoagulants, mixing and storage of blood, rapid separation and freezing of plasma at low temperature. It was also evident that the decay of factor VIII coagulant activity was an enzymic process (Kekwick & Walton 1962; Weiss 1965; Stibbe et al 1975; Vermeer et al 1976a, 1976b), but this could be influenced to some extent by the Ca<sup>++</sup> concentration, pH and ionic strength of extraction buffers.

In order to administer reasonable volumes of blood product it is necessary to concentrate the AHF, and cryoprecipitation has played a major role in this respect because of its inherent ease and safety of operation in blood banks. However, the product (known as cryoprecipitate) is subject to considerable variation in yield and although much has been investigated, few clear variables have been identified as requiring strict control (Pool 1967 and 1975; Kasper et al 1975; Slichter et al 1976; Vermeer et al 1976a and 1976b; Rock & Tittley 1977). Several procedures have been developed to further purify and concentrate AHF on a large scale, and these are inevitably accompanied by further losses (Newman et al 1971; Wickerhauser 1976).

In parallel with the work described above, considerable effort has been applied to achieve a more fundamental understanding of the biosynthesis, structure, mode of action and decay of F VIII and its various associated activities. Immunological techniques using human and animal antisera clearly show that F VIII is a high molecular weight complex of two proteins, one carrying the so called F VIII related antigen is stable and non-covalently complexed to a second, smaller molecule which carries the AHF activity and is very unstable (Bennett et al 1972; Poon et al 1975; Ratnoff et al 1976; Poon & Ratnoff 1977; Koutts et al 1977; Poon et al 1977; Yang & Kuzur 1977). Under certain circumstances (high salt concentrations in vitro, exercise or transfusion of von Willebrand's patients in vivo) a functional separation of these two moieties appears to occur (Bennett & Ratnoff 1972; Poon & Ratnoff 1976; Harris et al 1977; Ratnoff 1977; Stibbe 1977; Weiss et al 1977).

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Although it is far from clear whether they play a physiological role *in vivo*, it has been known for some time that serine proteases can destroy F VIII coagulant activity. Plasmin rapidly inactivates F VIII coagulant activity (Pasquini & Hershtgold 1973; Kirby et al 1974; Vermylen & Bottechia 1976; Hedner et al 1978) whilst thrombin first activates and then inactivates F VIII coagulant activity (Rick & Hoyer 1974 and 1977; Vehar and Davie 1977). An observation by one group (Furlan et al 1977a and 1977b) of a non-proteolytic reversible dissociation of the F VIII complex by Rhizopus lipase has not yet been confirmed. The same group has also observed the presence of an unidentified endogenous contaminating protease in preparations of F VIII which may explain some of its variable behaviour (Beck et al 1976; Furlan & Beck 1977).

Despite the general inability to improve the yield of F VIII coagulant activity by existing methods, it has been shown that careful pH control will improve stability on freeze drying (Godfrey et al 1975), that some amino acids, notably lysine, will stabilise the low MW form of AHF (MacDonald et al 1977) and that useful yields of AHF can be fractionated from plasma which is between 6 and 24 hours old (Avoy et al 1976; Whitman et al 1976). With the exception of one group, little attention has been paid to the way in which *in vitro* assays of F VIII correlate with the recovery and half-life in *in vivo* therapy (Nilsson & Hedner 1977).

During the last twelve months, work carried out in this department under the author's supervision (see appendix) has indicated that the vascular plasminogen activator from endothelial cells (VPA) may be one of the primary causes of F VIII decay. This enzyme has been isolated and characterized by Mr. Rodger Allen (who has been supported by a grant from the Scottish Home and Health Department to the author). We are in a unique position to extend this work by applying the theoretical knowledge of fibrinolytic activation to the practical problem of protecting F VIII from subsequent proteolysis. We believe this enzyme is latent in plasma because (a) soluble inhibitors are present in plasma and (b) the reactants, VPA and plasminogen, do not bind to fibrinogen. When, however, a solid (or polymeric) phase of fibrinogen is present, adsorption and activation take place rapidly and cannot be inhibited. Thus cryoprecipitate which contains insoluble fibrinogen polymers is treated as if it were a fibrin clot and proteolysis is automatically triggered. We have come to this conclusion after noting that VPA is specifically concentrated in cryoprecipitate, that venous occlusion (which raises the levels of F VIII and VPA in the occluded circulation) enhances the rate of decay when cryoprecipitate is subsequently prepared; and that artificially lowering or raising the level of VPA in resuspended cryoprecipitate

gives a corresponding increase or decrease in the stability of F VIII coagulant activity, (see appendix).

#### Aims

1. To evaluate the F VIII antigen/F VIII coagulant activity ratio (Hargreaves et al 1977; Sas et al 1974) as a means of diagnosing the degree of enzymatic decay which may have occurred in cryoprecipitate or fractionated F VIII concentrates. It may also provide a quality control parameter for routine clinical products.
2. To evaluate the possibility of inhibiting VPA enzymatic activity in plasma or intermediate products by the addition of specific inhibitors, or the removal by adsorption of either component onto a specific solid phase. The synthesis or procurement of appropriate inhibitors/adsorbents and where possible the development of isotopically labelled inhibitors to act as radio-tracers of VPA during the isolation of F VIII.
3. To evaluate the effect of fast freezing and thawing of plasma on the yield and purity of factor VIII. Both Newman et al (1971) and Vermeer et al (1976a) have shown that the rate of freezing and duration of storage can have a profound effect on the amount of denatured (insoluble) fibrin present in the final product. This could prove useful in reducing the amount of VPA co-precipitated and could thus be a practical solution applicable to all blood banks.
4. To evaluate the rate of thermal decay of F VIII coagulant activity in solution at 37°C as a diagnostic indicator of the VPA: inhibitor balance. As discussed in the Appendix, we have reason to believe that the deleterious effects of VPA are kept in check by the fortuitous presence of an unidentified plasma inhibitor (Gallimore 1975). We would therefore expect that high yields of F VIII in the final product would correlate with a slower rate of decay in this test. It is a test which can be applied retrospectively to production batches and therefore is of value in evaluating processing variables.
5. To evaluate methods of separating fibrinogen/fibrin soluble polymers from cryoprecipitated factor VIII. Since we have shown (Appendix) that the enzyme VPA is absorbed onto these materials, one way to avoid its damaging effects is to differentially remove them from the product. This could be achieved either by differential adsorption of fibrin/fibrinogen onto a solid

matrix (or F VIII) or by differential solvent extraction. Both Kekwick & Walton (1965) and Newman et al (1971) have used differential solubility to improve purity, but neither group examined the stability of the final product or its VPA content.

6. To evaluate synthetic chromogenic substrates and radioimmunoassays as direct methods of measuring VPA in plasma and intermediate purity F VIII concentrates. If a correlation is found between VPA content and F VIII yield and/or stability, then a direct measurement of the enzyme would be more precise and less labour intensive than the indirect thermal decay test. Since commercial substrates (Kabi AB type 2444) already exist for the similar enzyme urokinase, the same substrate may be useful for quantiting VPA in a simple spectrophotometric assay. If this substrate proves to be insensitive or non specific, an alternative assay based on the immunological properties of the activator might be considered. Such a radioimmunoassay is currently under development by the author and Mr. Rödger Allen of this department for clinical applications.
7. To identify and isolate the natural anti-activator(s) in plasma and evaluate them as possible stabilising agents in clinical concentrates of F VIII. Both Hedner (1973) and Gallimore (1975) have described unidentified anti-activators in plasma. Should this activity prove to reside in one of the existing by-products of the plasma fractionation centre, it may be feasible to consider it as a natural, non-toxic preservative for F VIII.
8. To explore various methods of producing artificial F VIII deficient substrate plasma. A continuing problem with large scale research of this nature is the supply of substrate plasma which is voluntarily donated by haemophiliacs and is necessarily of variable nature and supply. It should be possible, by affinity chromatography on insolubilised F VIII antigen and/or human antibodies (to F VIII coagulant protein) to remove the biologically active portion of normal plasma without affecting any other component (especially factor V) and thus produce artificial haemophilia plasma.

#### Plan of Investigation

(a) Methods to be used:

We plan to investigate the antigen/activity ratio and thermal decay of F VIII concentrates including cryoprecipitate produced in the Blood Transfusion Service, Edinburgh, by conventional and fast freeze/thaw methods. Since the procoagulant activity is sensitive to proteolysis and

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the F VIII antigen insensitive (or even increases, Atichartakarn et al 1978), this antigen:activity ratio may represent a way of monitoring expressed enzymic activity both within process samples and in the final clinical concentrate. It also has the advantage of being applicable retrospectively which would make it a particularly valuable quality control parameter for the evaluation of process variables. F VIII antigen will be assayed by Laurell rocket immunoassay and F VIII coagulant activity by an automated one stage activated partial thromboplastin time. It is also hoped to use the newly developed immunoradiometric assay for F VIII coagulant antigen (Peake & Bloom 1978).

One way in which enzymic activity can be limited is to reduce both the time and temperature at which F VIII is present in the cryo-precipitation phase. These requirements tend to be mutually exclusive. Nevertheless, the 'thaw-siphon' technique recently developed by Mason & co-workers in Brisbane B.T.S. (Personal communication) and which incorporates some of these possibilities has been evaluated by us in a limited number of samples and it seems capable of doubling the yield of F VIII coagulant activity from 75 u/pack to 150 u/pack. If this result holds for larger scale routine operation, then it will be a valuable method immediately applicable to all Regional Blood Transfusion Centres. Subject to inherent engineering difficulties it may also find application in the Plasma Fractionation Centres. It has yet to be demonstrated whether the improved yields of F VIII seen in single unit cryoprecipitate are a result solely of reduced enzymic activity, or of reduced solubility of F VIII coagulant activity or a combination of both. The antigen:activity ratio will be of significant value in exploring this problem.

Should it be demonstrated that enzymatic activity is a major cause of factor VIII coagulant loss during processing, two basic theoretical approaches can be contemplated (a) the addition of inhibitors or (b) the removal of VPA or the F VIII complex by specific adsorption. At the present time, inhibitors which are effective include the serine protease inhibitors DFP, PMSF and TLCK and the as yet ill-characterised plasma anti-activator. The former are toxic and active only at high concentrations, however they are capable of improvement by synthesising specific peptide sequences which correspond to the 'target sequence' of the active site of VPA and this is already known from the work carried out in Sweden by AB Kabi who have synthesised GLU-GLY-ARG pNA as a chromogenic substrate for plasminogen activators. When the p-nitroanilide group is replaced by a chloromethylketone group, the inhibitor should be active at

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a concentration of one million fold less than DFP or PMSF (Kettner & Shaw 1977). Even if still toxic in vivo, these compounds, when radioactive or fluorescent, would be valuable tools in the labelling of 'tracer' VPA for following the purification of F VIII and the immunoassay of VPA therein. An alternative inhibitor (which is of interest because it may reside in one of the by-products of the existing PFC fractionation scheme) is the natural plasma anti-activator (Hedner 1973). This inhibitor has the advantage of not requiring toxicity testing in the same way as synthetic inhibitors.

It has now been established by Mr. Rodger Allen (working in this department) that vascular activator binds strongly to soluble fibrin polymers and to cryoprecipitate as well as to insoluble fibrin (Gurewitsch et al 1975) and that the enzyme circulates in the plasma as an inactive reversible complex with one or more carrier proteins. We believe it may be possible to design techniques which would extract VPA (together with its binding proteins) whilst leaving F VIII in the solid phase (or vice versa). Such a development may be of value in large scale fractionation procedures.

V No patients, normal volunteers or animals will be required in this work.

VI The department is fully equipped with all necessary preparative apparatus (centrifuges, chromatography systems, ultrafiltration cells) and analytical apparatus (electrophoresis, immunoassay, physico-chemical measurements). Supervision of the work will be carried out by the grant applicant (DSP) within the existing laboratory suite (2 x 300 square feet). Ancillary services (blood donation, component processing, coagulation assay, autoclaving and wash-up) are all provided within the department and this project falls naturally within the interests of the Blood Transfusion Service and the community, namely to maximise the usefulness of any given blood donation.

VII No other funding is currently available for this project.

VIII The work requires one technician with appropriate experience in blood banking/coagulation/component production/protein chemistry. It is hoped that this person will be funded for not less than three years with an option of a fourth year to allow full co-operation with the fractionation service, where batch processing and stringent regulations necessarily allow only a slow rate of change in process variables.

## IX Curriculum Vitae:

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1960 - 1963 University College London, Department of Chemistry, B.Sc.  
 1963 - 1964 S.W. Essex Technical College, Lecturer.  
 1964 - 1967 University College Hospital Medical School, London,  
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 1967 - 1969 Research Associate, American National Red Cross, Washington D.C.  
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 1969 - S.E. Scotland Regional Blood Transfusion Service, Edinburgh.  
 Senior Scientific Officer 1969 - 1974. Principal Scientific  
 Officer 1974 - present.

## Recent Relevant Publications:

Pepper, D.S. & Allen, R. (1978) "Isolation and Characterization of Human  
 Cadaver Vascular Endothelial Activator" in "Progress in Chemical Fibrinolysis  
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 Activator, Cryoprecipitation and the Decay of F VIII Coagulant Activity".  
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 Medical School, 13th January, 1978 and Brit. J. Haematol. (Abstract) in press.

Note: Since much of the impetus for this proposed work arises from developments  
 taking place in this laboratory which have not yet been published, an Appendix  
 is added to summarise the experimental basis of the ideas.



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XI Financial Support:

(a) The grant would be administered by the Common Services Agency, Edinburgh, Treasurer, Mr. J. Morrison.

(b) The duration of the grant shall be not less than 3 years with an option to extend for a fourth year.

(c) (i) Salary of a state registered technician (a suitable candidate is available with 6 years post-registration experience).

1st year	(7th point)	£3,679
2nd year	(8th point)	£3,789
3rd year	(9th point)	£3,903
4th year	(10th point)	£4,016

(ii) Employers share of National Insurance & Superannuation is 17%

(iii) Equipment: a Warner "Coag-a-pet" automatic coagulometer (£6,000) is required to perform the large number of bioassays for F VIII coagulant activity. Because of the labour-intensive nature of the assay, it is not possible to perform thermal decay experiments manually and an automatic coagulometer is essential for this aspect of the work.

(iv) Reagents, antisera, disposable plastic ware etc. £500/annum

(v) Travel to meetings within the U.K., equivalent to two visits to London per year. £100.

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## Appendix

The following experiments have led us to the conclusion that the enzyme vascular plasminogen activator is responsible (at least during in vitro processing) for the large losses of F VIII procoagulant activity.

Cryoprecipitate was prepared from normal fresh frozen plasma in the blood bank. The fibrinolytic activity of the starting plasma, the cryoprecipitate and the cryo-supernatant plasma were assayed directly on fibrin plates and after euglobulin precipitation, on fibrin plates. In every case cryoprecipitate showed spontaneous fibrinolytic activity without the need for euglobulin precipitation. When the activity in each fraction was expressed as specific activity (square:mm lysis per mg-protein) it was found that cryoprecipitate contained a 10 fold higher concentration of plasminogen activator than in the starting plasma. This implies that (a) activator is bound to the precipitated proteins and (b) that the intrinsic plasma inhibitors of fibrinolysis are not bound.

Venous occlusion was performed on one arm of a volunteer for 20' and blood was then removed for the production of cryoprecipitate. A control sample was prepared at the same time from the other arm. The amount of F VIII procoagulant activity and fibrinolytic activity were measured in both samples and the F VIII procoagulant activity was assayed at hourly intervals for a further 5 hours incubation at 37°C. When the amounts of F VIII procoagulant activity present at zero time were normalized to 100%, it was seen that venous occlusion gave a cryoprecipitate with enhanced levels of fibrinolytic activity and a significantly greater rate of decay than the control cryoprecipitate. It was noted also that cryoprecipitate per se lost procoagulant activity at a much faster rate than plasma or cryo-supernatant plasma; this effect could be considerably reduced if cryoprecipitate was resuspended in plasma rather than buffered saline. (see Fig 1).

Based on the assumption that this enhanced thermal decay was due to expressed enzymic activity consequent on adsorption to fibrin(ogen) and removal from the milieu of plasma inhibitors, we tried different inhibitors of serine proteases. Only PMSF (phenylmethyl-sulphonylfluoride) and DFP (diisopropylphosphofluoridate) were able to prevent the decay of F VIII procoagulant activity. Significantly, known inhibitors of fibrinolysis (eACA and Trasylol) do not prevent loss of F VIII procoagulant activity (fig 2). We interpret this to be due to the direct action of the serine protease VPA upon the low molecular weight protein which probably forms the procoagulant portion of the F VIII complex. Plasminogen does not seem to be involved in the reaction since eACA and Trasylol are ineffective inhibitors. Purified VPA enzyme itself is only inhibited by PMSF and DFP.

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To confirm that VPA enzyme could indeed produce the changes we observed, we added partially purified material (isolated by Mr. Rodger Allen of this department) from cadaver limbs, at concentrations which were similar to those seen in cryoprecipitate. It was found (Fig 3) that increasing amounts of VPA enzyme (1 to 32 u/ml) caused accelerated rates of decay of F VIII procoagulant activity. Of particular note was the finding that even at the highest enzyme doses used, 20% of the F VIII procoagulant activity remained; this is in direct contrast to plasmin which will rapidly destroy 100% of procoagulant activity. This may be taken as additional indirect evidence that VPA is a serine protease which acts directly on the F VIII coag. protein with only a limited amount of cleavage.



Figure I

Decay of F VIII Procoagulant Activity at 37°C

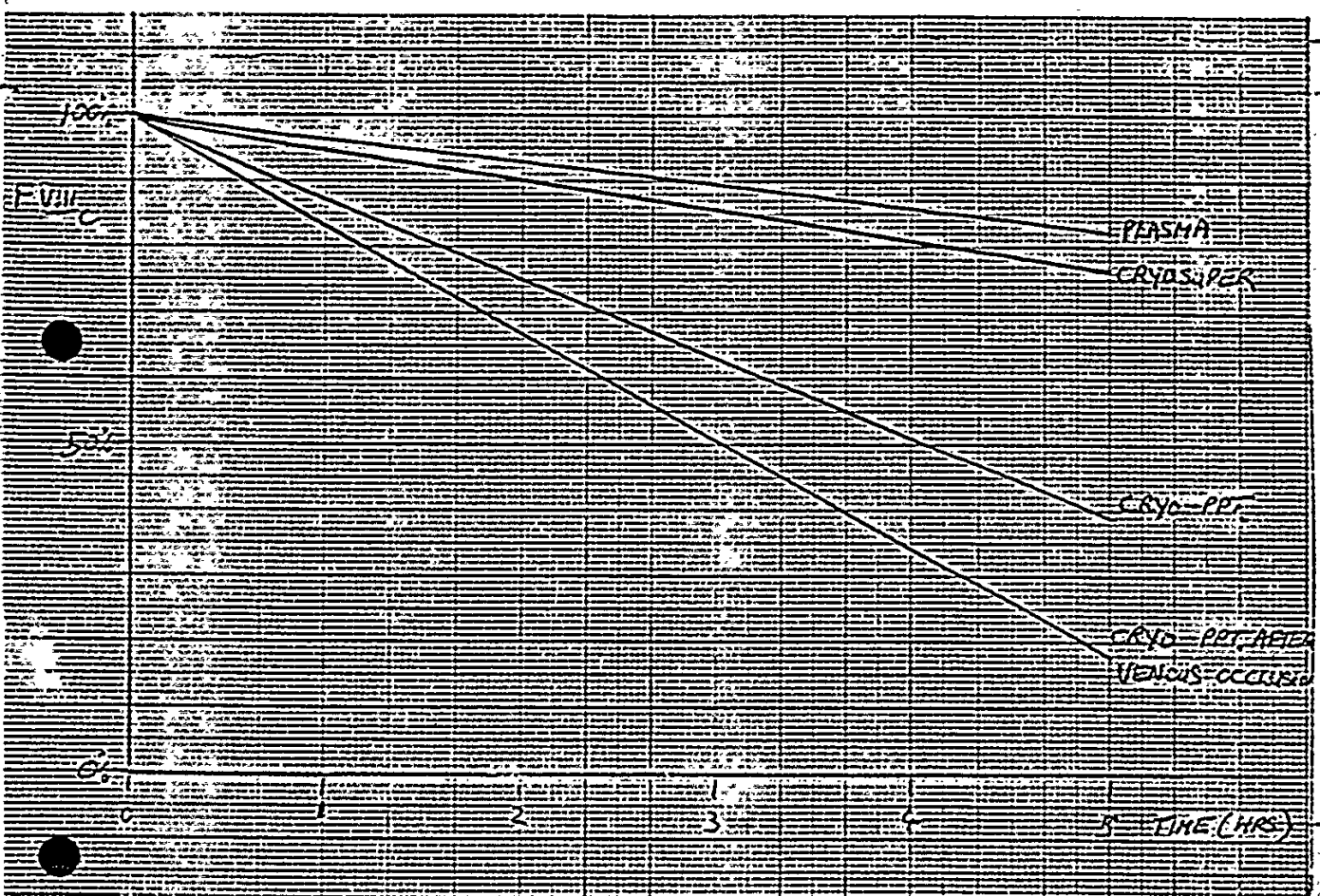


Figure 2

Inhibition of the Thermal Decay of F VIII Procoagulant Activity  
by Various Serine Protease Inhibitors.

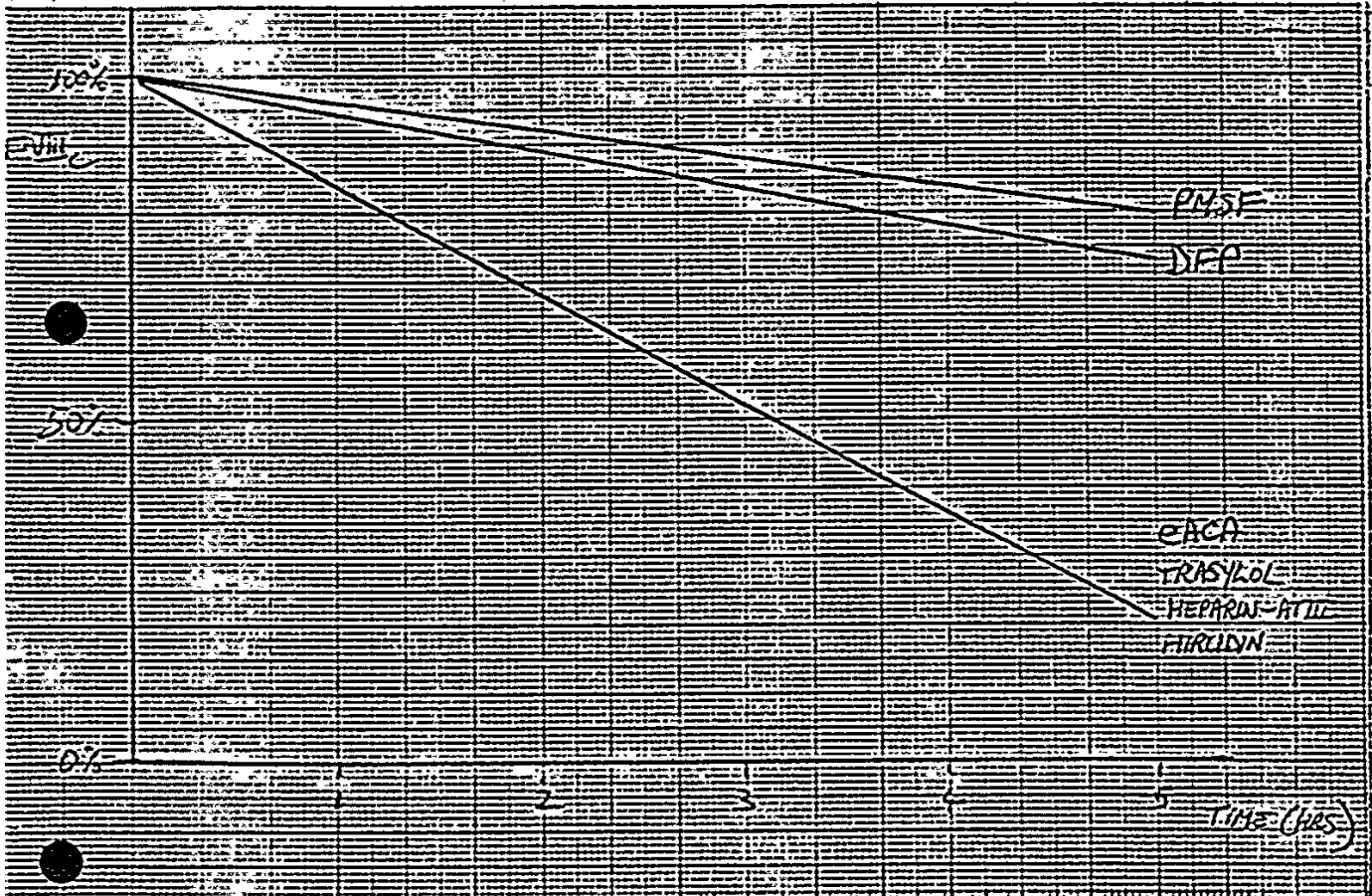


Figure 3

Enhanced Thermal Decay of F VIII Procoagulant Activity  
After Addition of Partially Purified Vascular Plasminogen  
Activator Enzyme.

