IMMUNOLOGICAL AND TOXICOLOGICAL STUDIES ON DRUGS AND BLOOD COAGULATION FACTORS

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ABSTRACT

1) Monoclonal antibodies directed against both Factor FVIII procoagulant protein (FVIII C) and fibrinogen were produced and characterised

2) Polyclonal antisera directed against a BSA-diazepam conjugate was produced These were found to be directed against the conjugate rather than diazepam

3) In vitro studies on the effects of both coumarin and 7-hydroxy-coumarin on the growth of cells indicated that neither drug was cytotoxic, but they both slowed the growth of cells in vitro 7-hydroxycoumarin was found to have more potent cytostatic effects than coumarin

4) The results from a clinical study on the effects of coumarin on liver function indicated that 0 36% of patients experienced an idiosyncratic hepatotoxicity to coumarin

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The following are a list of commonly used abreviations

A Aminopterin

ALT Alanine aminotransferase

AP Alkaline Phosphatase

APTT Activated partial thromboplastin time

AST Aspartate aminotransferase

BSA Bovine Serum Albumin

C Coumarin

DCM Dichloromethane

DMEM Dulbecco's Modification of Eagle's medium

DMF Dimethylformamide

DMSO Dimethylsulphoxide

ELISA Enzyme-linked immunoabsorbent assay

EtSH 2-Mercaptoethanol

F Fungizone/Amphotericin B

FCS Foetal Calf Serum

FVIII Blood coagulation factor VIII

GOT Glutamate-Oxaloacetate Transaminase (AST)

GPT Glutamate-Pyruvate Transaminase (ALT)

H Hypoxanthine

HAM Ham's F-12 medium

LATC Landschutz Ascites Tumour Cells

LFT Liver Function Test

LG L-Glutamine

MoAB Monoclonal Antibody

MP Melting point

7-OHC / 7HC 7-Hydroxycoumarin

ONPG o-Nitrophenyl-β-D-galactopyranoside

OPD o-Phenylenediamine

PAGE Polyacrylamide Gel Electrophoresis

PBS Phosphate buffered saline

PEG Polyetheleneglycol 4,000

PRISTANE 2,6,10,14-Tetramethylpentadecane

PS Penicillin - Streptomycin

SDS Sodium Dodecyl Sulphate

SP2 SP2 0 Ag1-4 myeloma cell line

T Thymidine

THF Tetrahydrofuran

vWF von Willebrands factor

SECTION I

INTRODUCTION

I INTRODUCTION

While inoculation had been used by the Chinese many centuries ago, immunology as a modern science began with the introduction of vaccination by Jenner in 1798 His simple technique of innoculating people with a minor infection like cowpox, to protect them from a more serious infection like smallpox, revolutionised medical therapy Preventative medicine based on scientific principles was born, and immunology looked as if it was going to become the most important discipline in medical science It was thought that these advances would lead to the eradication of disease and thus, to a disease free society Yet, 150 years later very little progress had been made in immunology, with many of the major advances in immunology only occurring in the last 30-40 years

There are two reasons for this lack of progress in immunology Firstly, immunology, like all branches of science, cannot develop in isolation Thus, immunology had to await developments in other branches of science before it could advance further In particular, improvements in electrophoretic techniques, advances in tissue culture techniques, the development of new protein chemistry techniques, and the major advances in genetics, especially cloning techniques, played key roles in the advances in immunology

The second reason for the slow progress in immunology was the failure to realise the importance of immunology While it was known that antibody production by B-cells was important in the prevention of disease, this was only part of the picture Both T-cells and macrophages have important functional and regulatory roles in the immune system The importance of the regulatory mechanisms in the immune system have only recently been realised (Sinlair and Panoskaltsis, 1988) The role of autoimmunity in diseases such as rheumatoid arthritis (Cohen, 1988), as well as the problem of graft rejection in organ transplant surgery, led to the development of immunosuppressive drugs The severe consequences of immune suppression as seen in HIV infection, has produced a search for new, and more powerful drugs for stimulating the immune system (Merigan, 1988) The use of these drugs in the treatment of cancer is also currently under investigation (Hadden,

1982)

One of the most important advances in immunology was the somatic cell hybridization technique of Kohler and Milstein (1975) It was not long before the commercial, and scientific value of this technique was realised The resultant availability of large quantities of pure, highly characterized antibodies, as well as the advances in immunoassays such as Enzymes Linked ImmunoSorbent Assay (ELISA), Enzyme Multiplied Immunoassay Technique (EMIT), and RadioImmunoAssay (RIA) brought immunoassay techniques to the forefront of the lucrative assay market (Montgomery et al , 1986) At present immunoassays are probably the most widely used assays for biologically active substances

While monoclonal antibodies quickly became the cornerstone of the newly developing biotechnology industry, they did not eliminate the need for conventional polyclonal sera The main reason for this is their cost Production costs are very high due to the specialist techniques, and equipment that are needed This is reflected in their retail cost, which is much higher than polyclonal antisera Thus, for much work polyclonal antisera are still used .

It is in the areas of research and therapy that monoclonal antibodies have been of real benefit Monoclonal antibodies have been very successfully used in the isolation and localization of many biologically active compounds, eg blood coagulation factors (Fass et al , 1982) This, coupled with gene cloning techniques, has been responsible for many of the major advances in biology, and medicine in recent years Monoclonal antibodies are particularly useful for in vivo use because of their pure and highly defined nature Clinically they are being used for treating drug overdoses (Smith et al , 1982), for tumour localization, and for treating cancer (Hersh et al , 1981, Schlom, 1986)

The work described in this thesis involves three major areas of research

(i) The production of monoclonal antibodies to blood coagulation factors (Section III)

(ii) The production of antibodies to drugs (Section IV)

(iii) Studies on the potential use of coumarin as a therapeutic

agent This included studies on the effects of coumarin on in vitro growth of tumour cells (Section V), and a study on clinical toxicity of coumarin (Section VI)

This work touches on a number of different areas of immunology including the production and uses of monoclonal antibodies, the role of the immune system in toxic effects of drugs, and the therapeutic roles for immunostimulants

Replacement therapy in blood factor deficient states has a number of problems Firstly, there is the risk of infection from the donor plasma, and secondly, there is the problem of production of antibodies by the recipient to the administered blood factors These problems can be reduced by using purified blood factors FVIII was chosen for this work because of the prevalence of haemophilia in the world, and in particular, to the high risk of HIV infection among haemophiliacs treated with crude FVIII concentrates (Ward et al , 1988) Immobilised monoclonal antibodies can be used to isolate pure FVIII (Fass et al , 1982) This enables the FVIII to be isolated from plasma in a pure form, free from other blood factors, and infectious particles Thus, patients can receive a highly purified FVIII preparation, which has a low risk of infection, especially if it is heat-treated as well (Hink et al , 1957) The low level of contaminants in it will reduce the risk of antibody formation to other blood factors, thus preventing the patient from developing new acquired disorders Other uses for monoclonal antibodies to blood coagulation factors are in the study of the coagulation process and its disorders, and in immunoassays for plasma levels of these factors A more detailed discussion on the uses of monoclonal antibodies to FVIII and to fibrinogen can be found in section III 3 10

Both anti-FVIII and anti-fibrinogen antibodies have a use in the study of different cancers vWF is a marker for endothelial cells (Jaffe et al , 1973) and can be used in the detection/classification of endothelial tumours Similarly FVIII CAg is a marker for hepatocytes, and spleen cells (Wion et al , 1985), and anti-FVIII CAg antibodies may have a role in the detection and classification of tumours in these tissues Fibrinogen is involved in the growth and proliferation of tumours, and thus, has a role in the study and detection of certain tumours (Brown et al , 1988)

The production of antibodies to drugs has many important uses They can be used in immunoassays (Budd and Walkin, 1980, Dixon, 1982) and they have been successfully used in the treatment of drug overdoses (Butler et al , 1973) The work presented here highlights the difficulties in the production of both monoclonal and polyclonal antibodies to drugs, in this case diazepam

In recent years coumarin has become increasingly popular as a therapeutic agent due to its properties as an immunostimulant (Piller, 1976b, Thornes, 1983b, Zanker et al , 1984) Initially it was used in the treatment of high protein lymphoedema (Clodius and Piller, 1978), but currently it is undergoing extensive trials for use in various forms of cancer (Zanker et al , 1984, Marshall et al , 1987) Two major questions have prevented more wide spread use of coumarin in clinical medicine The first involves the mechanism of action of coumarin Are its actions solely on the immune system, and is coumarin, or a metabolite, the active agent? A more serious doubt about coumarin is its reputation as a hepatotoxin from animal studies (Hazelton et al , 1956) In section V the effects of coumarin, and its major metabolite in humans, on the in vitro growth of tumour cells is investigated

The clinical study presented in section VI reports a form of hepatotoxicity that appears to have an immunological basis, in a small number of patients treated with coumarin Thus, stimulation of the immune system may have a side effect of producing disorders of the autoimmune type This could be due to coumarin only, or it could be a problem with other types of immunostimulatory therapy (Shoham, 1985)

Since the research described here covers a number of distinct but related topics, the thesis has been divided into 8 sections which, it is hoped, will help clarify the results There is a common Materials and Methods section (Section II), and Reference section (Section VIII) Sections III, IV, V and VI each have their own introduction, results, and discussion sections Section I (this section) provides a general introduction to the research, while section VII provides an overview of the results

SECTION II

MATERIALS AND METHODS

II 1 1 MATERIALS

All materials were purchased from Sigma Chemicals, Poole, Dorset, UK, unless otherwise indicated All solvents, and acids were purchased from Riedel de Haen, Aktiengesellschaft, Wunstorfer Str 40, D-3016 Seelze 1/Hannover, W Germany All tissue culture media, and reagents were purchased from Flow laboratories, Ayrshire, Scotland Tissue culture flasks, and plates were also purchased from Flow laboratories Other disposable tissue culture plastics were purchased from Sterilin Laboratory, 43/45 Broad Street, Teddington, Middlesex, UK Tris(hydroxymethyl) methylamine (Tris), glycine, and SDS were obtained from BDH, Poole, Dorset, UK

II 1 2 BUFFERS

The following buffers were used

Phosphate buffered saline (PBS) Phosphate buffered saline, 145 mM NaCl, 2 5 mM NaH_2PO_4, 7 5 mM Na_2HPO_4, pH 7 2

PBS-TWEEN PBS with 0 1%(v/v) Tween 20

PBS-BSA PBS containing 1%(w/v) BSA

Perox substrate buffer 0 05 M citric acid-0 25 M Na_2PO_4, pH 5 0

Carbonate Buffer 0 025 M carbonate/bicarbonate buffer, pH 9 5

II 2 1 BLOOD FACTOR ASSAYS

There are two main types of blood factor assays The first type depends on binding of a specific antiserum to a blood factor (antigen) This can be in the form of an ELISA assay, or quantitative immunoelectrophoresis (Giddings and Peake, 1981) The second type assays the biological function of the blood factor This is usually a coagulation assay (Barrowcliffe and Kirkwood, 1981), but there are also chromogenic assays of biological activity (Blomback, 1981) The results obtained from both these assays do not always correspond This is due, in part, to the presence of inactive protein which is quantified in the immunoassay and not in the coagulation assay Thus, in certain types of haemophilia an immunoassay can detect normal levels of FVIII, while the coagulation assay detects very low levels of activity

The standard used for FVIII assays is normal plasma Normal plasma is pooled plasma from a large number of donors and it is assumed to have 1 IU of FVIII procoagulant activity per ml Similarly the level of vWF in normal plasma is 1 IU/ml Fibrin(ogen) is measured in terms of mg/ml and is supplied in a relatively pure form for use as a standard

II 2 2 ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)

This assay uses a FVIII deficient plasma which allows the quantity of FVIII to be assayed specifically The assay is based on the principle that coagulation cannot proceed beyond FVIII without calcium Thus, when a sample containing FVIII is mixed with FVIII deficient plasma and an activator, which contains rabbit brain cephalin, and kaolin (APTT reagent), the coagulation pathway is activated, but coagulation cannot occur due to the absence of calcium When calcium is then added the rate limiting step will be the quantity of functional FVIII present To ensure that that there is no calcium present in the test, all plasmas were collected using sodium citrate as an anticoagulant Reagents for coagulation assays were obtained from Warner Lambert and from Sigma (see also III 1 13)

All of the reagents were reconstituted according to the manufacturers instructions A 100% standard was prepared by diluting normal plasma 1/10 in 0 85% saline A standard curve was prepared by diluting the 100% standard in saline to give 100%, 50%, 25%, 12 5% and 6 25% solutions All samples were assayed in duplicate, and the reagents were kept at 37°C throughout the assay Borosilicate glass tubes (Corning) were used for the assay, since they are less likely to bind protein non-specifically Great care must be taken with the reconstituted reagents due to the risk of hepatitis from plasma FVIII-deficient plasma, which is obtained from haemophiliacs, poses an additional risk due to the high level of HIV infection in these patients Many commercially available FVIII deficient plasmas, especially those sold before routine screening was established, are anti-HIV positive, and could pose a serious risk to the operator

0 1 ml of FVIII-deficient plasma was mixed with 0 1 ml of sample, or standard, and incubated for 2 min 0 1 ml of APTT reagent was then added to the bottom of the tube, and allowed to incubate for 5 min 0 1 ml of calcium chloride (0 02 M) was then added, and the time taken for a clot to form was measured The tilt tube method was used, and the time was taken when the first fibrin strands became visible The results were plotted on log-log graph paper

II 2 3 FACTOR VIII ELISA

This assay uses polyclonal antisera to detect the presence of FVIII This assay only detects von Willebrands Factor (FVIIIR Ag)

Plates were coated with 5 mg/l sheep anti-human FVIII antibodies (Serotec) in carbonate buffer (see II 8 1) Plates were washed 3 times in PBS-Tween before use Serial dilutions of normal plasma, and test sample were made in PBS-Tween 100 µl of each dilution were added in duplicate to the plates, and incubated at 37°C for 2 hrs The plates were then washed 3 times in PBS-Tween, and 100 µl of the peroxidase-labelled sheep anti-human FVIII antibody (Serotec), diluted in PBS-Tween, was added to each well The plates were incubated for 1-2 hrs at 37°C The plates were washed 3 times in PBS/Tween, and 100 µl of perox substrate buffer, containing 40 mg OPD and 20 µl of H_2O_2, were added After 15 min at room temperature the reaction was stopped by the addition of 25

µl of sulphuric acid (20% v/v) The absorbance was read at 490 nm on an ELISA plate reader (Biotek; EL307)

II 2 4 LAURELL ROCKET IMMUNOELECTROPHORESIS

Quantitative immunoelectrophoresis, based on the method of Laurell (1966), was also used to assay the quantity of FVIIIR Ag present in samples This technique involved electrophoresing samples of FVIII in an agarose gel containing anti-human FVIII antiserum The resulting precipitin lines formed peaks The height of each peak was proportional to the FVIII concentration in the sample (Zimmerman et al , 1975)

A 1%(w/v) solution of electrophoresis grade agarose (Gibco) in barbitone buffer (25mM diethylbarbituric acid (BDH), pH 8 6, containing 75mM Tris and 0 5 mM calcium lactate (Riedel de Haen)) was prepared by boiling the suspension until all of the agarose was dissolved This was then stored in 12 ml aliquots at 4°C These aliquots were then boiled in a water bath until completely melted, and allowed to cool to 50°C when 10 µl of goat anti-human FVIII antisera (Miles) was then added A 12 ml aliquot was then poured onto glass plates (8 5x9 5 cm) and allowed to cool Some plates were stored overnight, in a humidity chamber before use Eight wells were then cut in the agarose using the LKB mould and punch

Normal plasma was diluted in 0 85% saline to produce a range of concentrations for a standard curve 12 µl of the standards, and suitably diluted samples, were applied to the wells in the agarose The plates were then placed on the Multiphor 2117 electrophoresis tank (LKB) with wicks soaked in barbitone buffer at each end of the plates The protein was electrophoresed from cathode to anode overnight with a current of 10 mA

After electrophoresis the gels were covered with filter paper, and a weight was placed on top This was repeated 3 times to dry the gel The gels were then washed twice in 0 1 M saline (at least 15 min each), dried with filter paper, and then dried to a clear film with hot air They were then stained for 5 min in Coomassie blue (see II 10 6) rinsed in distilled water, soaked in destain solution for 10 min (or longer if necessary) until clear, and again dried under hot air

The heights of the standards were measured, and a standard curve was plotted This was used to calculate the concentration of FVIIIR Ag in the unknown samples

II 2 5 FIBRINOGEN ASSAYS

The assay used for fibrinogen was a coagulation assay based on the method of Clauss (1957) This uses thrombin to convert fibrinogen into fibrin, which then forms a clot With diluted plasma, the rate limiting step in the presence of excess thrombin, is the fibrinogen concentration This is reflected in the time taken for clot formation The assay was performed using a kit from Sigma (see also III 1 6)

Reagents were reconstituted according to the manufacturers instructions Test plasma, and the standard fibrinogen preparation were diluted in diluent supplied with the kit 0 2 ml was incubated in borosilicate glass tubes for 1-5 min, at 37°C 0 1 ml of thrombin was then added and incubated at 37°C The time taken to clot formation for the standard was measured, and plotted on log-log graph paper The clotting time for the samples could then be converted to concentration using this standard curve

II 3 FACTOR VIII ISOLATION AND PURIFICATION

II 3 1 REAGENTS

Three sources of factor VIII (FVIII) were used The first was prepared from fresh frozen plasma (a gift from The Blood Transfusion Service Board, Dublin) by ethanol cryoprecipitation The second was isolated crude cryoprecipitate (kindly donated by The Blood Transfusion Board, Dublin) The third source was Koate, a commercially available, high purity FVIII (Cutter Laboratories)

II 3 2 ISOLATION OF FVIII BY CRYOPRECIPITATION

The method used was similar to the ethanol cryoprecipitation method of Cohn et al , (1946) as modified by Newman et al (1971)

Frozen plasma, which had not been stored for more than 3 months, was softened by immersion in a water bath at 37°C for 5 min It was then crushed in the bag and transfered to a stainless steel container in an ethanol bath at 0°C Ice cold ethanol (50%v/v) was added, with stirring, to a final concentration of 3%(v/v) The plasma was then melted under a stream of air but its temperature was not allowed to rise above 2°C It was then centrifuged at 5,000 g for 20 min The resulting precipitate was washed with 8%(v/v) ethanol at -5°C After washing, the precipitate was dissolved (by stirring at 20°C for 30 min) in 125 ml 0 02 M Tris, pH 7 0, per litre of starting plasma The solution was then passed through glass wool to remove any residual precipitate The Tris solution containing FVIII was then adsorbed with Aluminium hydroxide The Aluminium hydroxide was prepared by diluting Aluminium hydroxide gel F500 (Reheis) to a concentration of 1 6% (w/w) Al_2O_3 (as determined by titration against lead nitrate according to the British Pharmacopeia assay) and heated to 95°C for 3hr to inactivate it The Tris-Aluminium hydroxide mixture was centrifuged at 5,000g for 20 min, and filtered through an AP20 glass fibre prefilter (Millipore) to remove any remaining particles Trisodium citrate (0 5 M) was then added to a final concentration of 0 02 M

II 3 3 PURIFICATION OF FVIII WITH POLYETHYLENE GLYCOL

The contaminating fibrinogen was then removed by precipitation with PEG By increasing the PEG concentration the FVIII was then precipitated

Cryoprecipitate prepared from plasma (see above) or lyophilised FVIII concentrate (Blood Transfusion Service Board, Dublin) was acidified to pH 6 1 with 0 02 M citric acid, and PEG 4000 (Riedel de Haen) was added to a final concentration of 4-5 g/100 ml This precipitated most of the fibrinogen, which was removed by centrifugation at 5,000g for 5 min PEG 4000 was then added to the supernatant, to a final concentration of 12 g/100 ml, to precipitate the FVIII The solution was centrifuged at 6,000g for 10 min The resulting pellet was then gently washed (without dislodging it) with 8% (v/v) ethanol in 0 02 M Tris, pH 7 0 at -5 °C The pellet was dissolved in 0 02 M Tris-0 02 M citrate buffer, pH 7 0, and diluted to approximately 1/200th of its original plasma volume It was stored at -20°C until further use

II 3 4 FURTHER PURIFICATION OF FVIII BY CHROMATOGRAPHY

Both PEG precipitated FVIII and Koate were further purified by gel chromatography according to the method of Fay et al , (1982) A 76x2 5 cm column of Sepharose CL 6B (Pharmacia) was used The void volume and total volume were estimated with dextran blue, and phenol red, respectively The elution buffer was 5 mM sodium citrate containing 1 mM $CaCl_2$, 135 mM NaCl, 5% (w/v) Dextrose, 0 02% (w/v) sodium azide, and pH 7 35 One bottle of Koate (220 IU), or the PEG precipitate, was dissolved in 5 ml of elution buffer The 5 ml of Koate or 2 ml of the PEG precipitate were eluted from the column with elution buffer and a flow rate of 1 63 ml/min Sixty 10 ml fractions were collected Each fraction was assayed for protein (OD at 280 nm), and for FVIIIR Ag (ELISA) The fractions from the same protein peak were pooled, and were stored at -20°C These were later used for screening hybridomas for antibody production

II 4 PRODUCTION AND PURIFICATION OF FIBRINOGEN FRAGMENTS

The action of plasmin on fibrinogen produces 4 fragments viz X,Y,D and E The X and Y fragments are produced first, and have a larger molecular weight than the D, and E fragments The method used for the production of the early fragments was based on that of Nieuwenhuizen and Gravessen (1981), while the method for producing the late degradation products was based on that of Haverkate et al , (1979)

II 4 1 PRODUCTION OF EARLY FIBRINOGEN DEGRADATION PRODUCTS

20 mg of fibrinogen purified from Koate by column chromatography (see II 3 4) was added to 20 ml of 0 04 M sodium barbitone buffer, pH 7 75, containing 0 1 M NaCl, and 10 mM EGTA Digestion was started by adding 10 CU of plasmin (Kabivitrum), and it was allowed to proceed for 30 min at 37°C Digestion was stopped by adding 0 2 ml of 0 1 M phenylmethylsulphonylfluoride (PMSF)

Saturated ammonium sulphate, pH 7 0, was then added to a final concentration of 20% (w/v) After centrifugation at 15,000 rpm for 15 min the supernatant was collected and its ammonium sulphate concentration was increased to 50% (w/v) The precipitate was collected after centrifugation at 15,000 rpm for 15 min

The pellet was dissolved in the minimum volume of 0 15 M potassium phosphate buffer (mixed), pH 7 5, and applied to a Sepharose CL 6B (Pharmacia) column (76 x 2 5 cm) This was then eluted in the same buffer overnight with a flow rate of 0 5 ml/min 10 ml fractions were collected and protein peaks, as determined by absorbance at 280 nm, were pooled, and concentrated by the addition of solid ammonium sulphate to a concentration of 50% (w/v) saturation The precipitates were collected after centrifugation at 15,000 rpm for 20 min The pellet was dissolved in the minimum volume of PBS, and dialyzed overnight

II 4 2 PRODUCTION OF LATE FIBRINOGEN DEGRADATION PRODUCTS

10 mg of fibrinogen purified from Koate was added to 5 ml of 0 04 M sodium barbitone, pH 7 4, containing 0 1 M NaCl, 2 mM $CaCl_2$, and 2 CU of plasmin This was then incubated for 24 hrs at 37°C 1,000 KIU of aprotinin was then added, to stop the action of plasmin and the preparation was lyophilised

The sample was then dissolved in the minimum volume of 0 01M sodium bicarbonate containing 10 KIU/ml of aprotinin, and dialysed against this buffer The sample was then applied to a Sepharose CL 6B column (76 x 2 5 cm) equilibrated in the same buffer

10 ml fractions were collected, and protein peaks, determined by absorbance at 280 nm, were pooled, and dialysed against distilled water They were then lyophilised, redissolved in PBS, and stored at -20°C

II 4 3 PURIFICATION OF FIBRINOGEN CHAINS

Fibrinogen consists of 3 chains - Aα, Bβ, and γ These were prepared by the method of Doolittle et al , (1977)

100 mg of fibrinogen were dissolved in 10 ml of 6M guanidinium chloride - 0 2M Tris, pH 8 2, containing 0 01 M dithioerythritol (DTE) After 30 min at room temperature 10 ml of 6M guanidinium chloride containing 0 03M iodoacetic acid was added This was left for 30 min in the dark, and was then dialysed against 6M guanidinium chloride for 60min, also in the dark The mixture was then dialysed against a number of changes of distilled water, at 4°C

After dialyses the chains were lyophilised, and redissolved in 8 M urea-5 mM sodium acetate, pH 5 2 A sample of this was then applied to a CM-cellulose column (20 ml), and eluted, using a linear gradient, with 8 M urea - 0 125M sodium acetate, pH 5 2 as the limit buffer 5 ml fractions were collected, and those containing protein, as determined by absorbance at 280 nm were pooled, dialysed against water, and lyophilised

Since diazepam is too small to be antigenic, it has to be conjugated to a carrier protein This conjugate becomes the antigen If there is sufficient epitopes of diazepam present then most of the antibodies will be directed to the diazepam The conjugation method used was based on that of Peskar (1973) This involved conjugating the p-aminoacetanilide derivative of diazepam (Ro 20-9748) to BSA Initially this derivative was not available, so it was synthesised from Ro 7-3551

II 5 1 REAGENTS

Diazepam (Ro 5-2807) 7-Chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

Ro 7-3351 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-1-methyl-2H-1,4-benzodiazepin-2-one

Ro 20-9748 5-[3-(4-Aminophenylazo)-4-hydroxyphenyl]-7-chloro-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one

Ro 20-9749 5-[3-(Acetamidophenylazo)-4-hydroxyphenyl]-7-chloro-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one

Ro 7-3551, Ro 20-9748 and diazepam were gifts from Hoffman La-Roche

II 5 2 SYNTHESIS OF Ro 20-9749

The method of Earley et al (1979) was used A solution of 0 45g of sodium nitrite (BDH) in 5 ml of water was added to a mixture of 1 2 ml of conc HCl, 5 g ice and 0 9 g p-aminoacetanilide (Riedel de Haen) This mixture was then added to 0 5 g Ro 7-3351 in 25 ml of Tetrahydrofuran (THF) containing 1ml 1N NaOH, and 20 g ice The reaction was incubated for 2 5 days at 4°C The crystals were recrystallised from methylene chloride - methanol (50 50) mixture, and recrystallised from a methylene chloride-petroleum ether (50 50) mixture The expected mp was 276-278°C, and the expected IR should have a broad peak at 1700 cm^{-1}

II 5 3 SYNTHESIS OF Ro 20-9748

0 6 g of Ro 20-9749 were added to 50 ml methanol, containing 10 ml concentrated HCl, and heated for 10 min on a steam bath The mixture was left for 2 hr at room temperature and the methanol was evaporated The solution was then made basic with ammonium hydroxide, and extracted with 50 ml methylene chloride The

organic layer was collected and evaporated The residue was crystallised from methanol, and recrystallised from methylene chloride-methanol (50 50) Brown prisms with a melting point of 262-268°C, and IR peaks at 3450, 3350, and 1682/cm were expected

II 5 4 SYNTHESIS OF Ro 20-9748 (REVISED)

The revised synthesis of Ro 20-9748 by Fryer (personal communication) is based on a combination of the 2 reactions above It uses Ro 7-3351 as a starting compound to form Ro 20-9748 without the intermediate step of isolating Ro 20-9749

A solution of 8 6 g of sodium nitrite in 100 ml of water was added to a mixture of 100 g of ice, 24 ml conc HCl and 18 g p-aminoacetanilide, with stirring This was then added, with stirring, to 100 ml of THF containing 10 g Ro 7-3551, 240 ml 1N NaOH, and 400 g ice 4 g of sodium acetate was added, and the the solution was adjusted to pH 8-9 with conc HCl After 3 hrs at 4°C the resulting crystals were collected

This crude material was dissolved in 1400 ml methanol containing 280 ml conc HCl After standing for 18hrs the solution was adjusted to pH 7-8 with conc ammonium hydroxide and the methanol was removed under vacuum Water was added, and the solid collected by filtration This was then air dried and dissolved in THF An insoluble tar was removed by filtration, and the filtrate was concentrated The residue was dissolved in dichloromethane (DCM), and THF (50 50) mixture and filtered through 200 g of Florisil (Sigma) The product was eluted with DCM followed by ether The solvents were evaporated, and the residue was recrystallised twice from THF/ethanol to give reddish brown prisms, MP=265-268°C

II 5 5 SYNTHESIS OF Ro 20-9748-PROTEIN CONJUGATE

(i) Method of Peskar et al , (1973) 20 mg of Ro 20-9748 were dissolved in DMF and the pH was adjusted to about pH 1 5 with 1ml of 1N HCl All procedures were done on ice A solution of 100 mg of sodium nitrite in 1ml water was added slowly to this 50 mg of ammonium sulfamate in 1 ml water was then added dropwise This mixture was added to 40 mg of BSA in 5 ml of 0 1 M borate buffer (0 05 M boric acid containing 0 05 M potassium chloride), pH 9 The mixture was maintained above pH 8 by the addition of borate buffer A bright orange colour developed immediately The

reaction mixture was adjusted to pH 9 and stirred overnight in the dark at 4°C This was followed by dialyses against several changes of water The solution was then lyophilised

(ii) Method of Dixon (1982) 0 06 ml of 1 M sodium nitrite was added to 2 ml DMF containing 20 mg of Ro 20-9748, 0 6 ml water, and 0 2 ml of 1 M HCl, and the mixture was stirred for 30 min 0 05ml of 1 M ammonium sulfamate was then added This solution was added to 200 mg BSA in 5 ml 0 16 M borate buffer, pH 9 The solution became dark blue but turned to brown after stirring for 1 hr, and was kept at pH 9 by the addition of 1 N NaOH It was incubated overnight at 4°C, and then dialysed against several changes of 0 05 M sodium bicarbonate This was followed by dialyses against several changes of distilled water

II 6 IMMUNISATION PROTOCOLS

Two different immunisation protocols were used One was for immunising mice for the production of monoclonal antibodies The second was for the production of antibody rich ascitic fluid In the former instance both FVIII and diazepam conjugate were used In the latter only the diazepam conjugate was used

II 6 1 IMMUNISATION FOR FUSIONS

Antigen was prepared by diluting stock solutions to a concentration of 1 mg protein/ml (or 10 IU FVIII/ml) in PBS 0 1 ml of this preparation was emulsified with 0 3 ml of either complete (CFA), or incomplete (IFA) Freunds adjuvant (Gibco) It was then injected ip into 8 week old Balb/C mice as follows

Day 0 100 µg (1IU) in CFA
Day 21 100 µg (1IU) in IFA
Day 42 100 µg (1IU) in IFA
Day 63 100 µg (1IU) in PBS

On day 66 the mouse was killed, and its spleen was removed asceptically. A fusion was then performed using the isolated spleen cells, or else the spleen cells were frozen down in liquid nitrogen for later use

II 6 2 PRODUCTION OF ASCITIC FLUID RICH IN POLYCLONAL ANTIBODIES

Antigen was prepared by emulsifying protein with CFA in the ratio 9 1 (CFA protein) 0 2 ml of this was injected ip into 12 week old Balb/C mice as follows

Day 0, 14, 45, 52, 59, and 81
100-500 µg protein in 0 2 ml CFA
Day 86 0 5 ml of pristane
Day 91 100-500 µg protein in 0 2 ml CFA

Ascites fluid was tapped from mice when necessary by inserting a sterile 21G needle into the peritoneal cavity, and allowing the fluid to flow out It was necessary to set up 6-10 mice at a time as some died, and many failed to produce ascites (Tung, 1983)

II 6 3 TREATMENT OF ASCITIC FLUID

Ascitic fluid was obtained by 2 methods The first was from the growth of a hybridoma in the peritoneal cavity (see II 7 8), and as an antibody rich fluid (see above) Ascitic fluid was centrifuged at 2,000 rpm for 10 min, and the supernatant stored at -20°C until it was processed further If the ascitic fluid contained a growing hybridoma, the pellet, which contains the cells, was either injected into other mice or frozen

Ascitic fluid produced by the same clone was pooled Similarly, ascites produced from immunisation with the same antigen was pooled The fatty layer was removed with a pasteur pipette, and the fluid was centrifuged at 15,000 rpm at 4°C for 20 min All subsequent steps were performed at 4°C The fluid was then filtered through glass wool, and the pH lowered to 4 9-5 1 with HCl After 1 hr it was centrifuged at 15,000 rpm for 20 min to remove the precipitated fibrinogen

The pH was raised to 7 0-7 2 with NaOH, an equal volume of saturated ammonium sulphate was added, and left for 1 hr The ascites was centrifuged at 15,000 rpm for 20 min The pellet was then washed with 50% saturated ammonium sulphate, and centrifuged at 15,000 rpm for 20 min The pellet was then dissolved in the minimum of distilled water, dialysed against 0 1 M Tris-HCl (pH 8 0), and was then stored at -20°C

II 7 TISSUE CULTURE AND FUSION METHODS

A number of different media were used for the different tissue culture procedures Unless otherwise indicated "media" refers to DMEM containing 2mM 1-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 5 µg/ml fungizone, and 10% (v/v) FCS

II 7 1 FUSION METHOD

Sp2 myeloma cells were cultured in antibiotic free media prior to the fusion Cells in the mid-log phase were centrifuged at 2,000 rpm for 10 min The pellet was washed 3 times in serum free media Immunised mice were killed by cervical dislocation, soaked in alcohol, and their spleen removed asceptically The spleen was cut into 4 pieces, and a total of 10 ml of serum free medium was injected into the pieces The cell suspension was then centrifuged at 2,000 rpm for 10 min and the pellet was then resuspended in 2 ml of haemolysis buffer (0 017 M Tris, pH 7 2, containing 0 144 M NH_4Cl) for 5 min to lyse the red blood cells This was then underlayed with 5 ml of FCS, and centrifuged at 2,000 rpm for 10 min, which separates unlysed cells from membranes Haemolysis was repeated a second time if the pellet was still heavily contaminated with red blood cells The pellet was then washed 3 times in serum free medium This yields a pellet which is free from red blood cells, and also free from FCS, which interferes with the fusion process

After counting, viable spleen, and Sp2 cells were were mixed in a 10 1 ratio The cells were then centrifuged at 2,000 rpm for 10 min The pellet was resuspended in serum free medium, and pelleted at 4,000rpm for 5 min All the supernatant was removed, and 1 ml 50% (w/v) PEG (Riedel de Haen), dissolved in serum free medium, and filter sterilised, was added with stirring over 1 min Cells were stirred at 37°C for 1 5-2 min 3 ml of serum free medium were then added over 3 min, followed by 6 ml over 3 min The cells were stirred continuously throughout this procedure The cells were then pelleted at 2,000 rpm for 10 min, and resuspended in HAT medium (media containig 13 611 µ g/ml hypoxanthine, 0 176 µg/ml aminopterin, and 3 876 µg/ml thymidine) at a concentration of 1 10^6 viable spleen cells per ml 100 µl of this was added to each well of a 96 well plate which had been precoated with a feeder layer, usually consisting of macrophages (see II 7 2)

The plates were sealed with parafilm, and incubated for at least 7 days at 37°C in 5%CO_2 prior to examination

After 1 week cells were fed by removing 100 µl of media, and adding in fresh HAT media Subsequently cells were fed, when necessary, in HT media (HAT media without the aminopterin) until they were expanded After expansion they were grown in supplement free media When the cells were almost confluent in the wells, the supernatants were harvested, screened by ELISA, and the cells in the positive wells were expanded into 24 well plates Cells that grew in 24 well plates were rescreened, and the positives were expanded into a 6 well plate, or into additional 24 well plates These were then transfered into flasks, and then were subsequently frozen down in liquid nitrogen Some of the hybridomas were then selected for cloning, and characterisation

II 7 2 FEEDER LAYERS

Schofield mice were killed by cervical dislocation, and their bodies were soaked in alcohol All procedures were then performed asceptically The fur was removed from the abdomen, and 10 ml of media (HAT media was used if the feeder layer was for a fusion rather than for cloning) were injected intraperitoneally The macrophage-rich media was then drawn out with a syringe and was allowed to stand for a few minutes so that any contaminating faecal matter could be seen, indicating puncture of the intestine The cells were then counted, resuspended to a concentration of 1 5 10^5 cells per ml, and 100 µl were plated into each well of a 96 well plate These were incubated overnight at 37°C, and 5% CO_2, and checked for contamination before use Since some mice produce more macrophages than others, cells from more than one mouse were pooled Macrophage supernatant was also used as a feeder Human endothelial cell supernatant (HECS) was also very effective as a feeder layer (Astaldi, 1980) It can be prepared from culture of endothelial cell from a human umbilical cord, or can be obtained from Costar

II 7 3 CLONING CELLS BY LIMITING DILUTION

Cells were washed down, centrifuged at 2,000 rpm for 10min, resuspended in HAM media (Ham's F12, containing LG, PS, F and 10% FCS) and counted For the first cloning they were plated out at a concentration of 1, 5, and 10 cells per well For the second cloning they were plated out at 0 5, and 1 cell per well A

feeder layer in media was also used The cells were plated out into two 96 well plates Supernatants from wells containing growing cells were then screened by ELISA In the first cloning cells from positive wells with the fewest clones (checked by eye) were expanded, and aliquots were frozen down A number of clones were also cloned a second time During the second cloning positive wells with only one clone per well (checked by microscope at an early stage) were expanded Aliquots of these were frozen down, and were also grown as ascitic tumours

II 7 4 FREEZING AND THAWING CELLS

Cells from confluent flasks (or 24 well plates) were washed down, and pelleted at 2,000 rpm for 10 min All supernatant was removed, and the pellet was resuspended in 1 ml of FCS containing 5-10% (v/v) Dimethylsulphoxide (DMSO), or in 0 5 ml media to which 0 5 ml FCS containing 10%(v/v) DMSO was added Cells were then added to sterile freezing vials, lowered slowly (1 5-2 hrs) into the vapour phase of liquid nitrogen, and eventually immersed in it When thawing, cells were rapidly thawed at 37°C, added to 10 ml of cold media and centrifuged for 10 min at 2,000 rpm The pellet was then resuspended in media, and grown in a flask (or 24 well plate) at 37°C in 5% CO_2

II 7 5 CELL COUNTS AND VIABILITY TESTING

A cell suspension was added to an equal volume of acridine orange - ethidium bromide (0 1mg/100ml of each in PBS), and an aliquot was counted in a haemocytometer (Neubauer) Viable cells stained green under UV, while the dead cells stained orange

II 7 6 MYCOPLASMA SCREENING

A vial of Normal rat kidney (NRK) cells (Duc-Nguyen et al , 1966) was thawed out, and grown in antibiotic free media at 37°C, in 5% CO_2 for at least 3 passages The media was poured off, and 5ml of trypsin 2 5% (w/v) in Hank's Balanced Salt solution, was added for 5 min at 37°C Cells were pelleted at 2,000 rpm for 10 min, and resuspended at 1 10^4 cells per ml of antibiotic free media 1 ml of cells was added to a sterile coverslip in a sterile 35x10mm petri dish (Lux), and incubated overnight at 37°C in 5% CO_2 1-2 ml of supernatant from the cells to be tested were then added to the petri dishes, and incubated for 3 days at 37°C in 5% CO_2

The coverslips were then removed from the petri dishes, and washed 3 times in PBS They were then fixed for 6 min in a 1 1 mixture of methanol, and acetone at -20°C The cells were then washed twice in PBS, and stained in Hoechst 33528 (0 05 µg/ml in balanced salt solution) for 10 min The coverslips were then washed twice in PBS, dried, and mounted on slides The cells were examined under UV light with a 100x oil immersion lens (Nikon optiphot microscope fitted with a B2 combination filter) Uncontaminated cells had a strong fluorescence in the nucleus only, while the contaminated cells also had specks of fluorescence throughout the cytoplasm

II 7 7 CONTAMINATION

All types of contamination occured during the fusions Bacterial contamination was probably due to operator error The use of antibiotics such as penicillin and streptomycin helped control it, but there were often resistant strains of bacteria, and their use can often encourage mycoplasma contamination Thus, the use of antibiotics was restricted to prevention of contamination in fusion plates Bacteria could be detected by cloudy media with a low pH (ie yellow), and could usually be confirmed by observation with a microscope When it occured in flasks they were disposed of When it occured in 96 or 24 well plates the contaminating well was drained, and cleaned with alcohol This was usually sufficient to prevent spread to other wells Yeast contamination, which only occured on a few occasions, was treated similarly

Fungal contamination was very difficult to prevent due to the resilient nature of the spores Fungizone (amphotericin B) was used to control it, but this was not very effective, especially in the summer months When it occured in flasks they were disposed of When it occured in plates, uncontaminated wells could sometimes be transfered to an other plate, without spread of the contamination With all types of contamination, the incubator was scrubbed with alcohol before reuse

Mycoplasma contamination was a major problem They are very small (0 1-0 25 µm), and are very difficult to detect by direct light microscopy or to remove by filtration The parent myeloma often grew well in the presence of mycoplasma, but the hybridomas grew poorly, and could not be expanded The inability to expand

clones is often an indication of mycoplasma contamination. Cells were screened for mycoplasma prior to a fusion. When mycoplasma contamination occured all cells, media, and reagents were disposed of, and the laminar flows were decontaminated with formaldehyde. Cells could be salvaged by growing them as ascitic tumours, but only if there was sufficient cells (ie a confluent flask)(Carroll and O'Kennedy, 1988). Sp2's were cultured in antibiotic free media. Tylocine (Gibco), an anti-mycoplasma drug, proved ineffective in treatment, or prevention of mycoplasma infection.

II.7.8 GROWTH OF CLONES AS ASCITIC TUMOURS

Mice were injected with 0.5 ml of pristane (Aldrich). Ten days later confluent cells from a flask were injected ip. When the mice developed a swollen abdomen they were killed, 5 ml of saline was injected into the abdomen, and the ascitic fluid was removed by syringe. The ascitic fluid was centrifuged at 2,000 rpm for 10 min. The pellet, which contained the hybridoma cells, was resuspended in saline, and injected into other mice, or else it was frozen in liquid nitrogen. The supernatant was stored at -20 ^{o}C until it was processed further (see II.6.3). The time taken for ascitic fluid to develop depended on the number of cells injected, the batch of pristane, the individual mice used, and also, on the stability of the cell line used. On a second passage in mice, pristane was usually not necessary, as the cells were conditioned to growth in mice. However, the use of pristane on the second passage usually accelerated the growth of the ascites.

For growth of the ascitic tumour compatible mice had to be used, ie a mouse of the same strain as the parent myeloma, to prevent immunological rejection on the tumour. For most hybridomas this meant using Balb/c mice. The disadvantage of this is that they are very small, and thus, only a small amount of ascites is produced. This problem was overcome by using the F1 generation of a Balb/C-Schofield cross. These mice are much bigger, and produced much more ascitic fluid.

II.7.9 COUMARIN STUDIES

Cells were cultured in flasks, or grown as ascitic tumours in Schofield mice. The cells were then harvested, and diluted to the required concentration in media. These were then added to the wells of 24 well plates, 6 well plates, or 25 cm^2 flasks.

5mM stock solutions of coumarin, and 7-OHC were prepared in PBS (Dulbecco's A) The 7-OHC was autoclaved into solution, and the coumarin was dissolved in ethanol, and added to sterile PBS (final ethanol concentration was 1%(v/v)) Control solutions (diluents) were PBS, and PBS containing 1% ethanol The incubation volumes used were 1 5 ml for the 24 well plates, 5 ml for the 6 well plates, and 10 ml for the flasks

The 2 cell types used were SP2 myeloma and Landschutz tumour cells (LATC) The cells were usually grown in the presence of drug for 9 days , or until the control wells were confluent Each assay was performed in triplicate when 24 well plates were used, and in duplicate when 6 well plates or flasks were used Duplicate samples from each assay well were examined by fluoresence microscopy (II 7 5) and cell counts and viabilities determined

II 8 1 ELISA

Screening of hybridomas for antibody production was performed by Enzyme Linked Immunosorbent Assay (ELISA) 96 well microtitre plates were coated with 100 µl of either 50 mM Tris-HCl, pH 7 4, containing 150 mM NaCl, and 40 IU FVIII per litre, or 100 µl of coating buffer containing 1-10 mg of protein per litre, and incubated at 37°C for two hours The plates were then washed once with PBS-Tween, and then 150 µl of PBS-BSA, containing 50%(v/v) glycerol, was added The plates were sealed, and stored at 4°C until required If plates were required for immediate use, they were blocked with PBS-BSA for 30 min at 37°C

Antigen coated plates were washed 3 times in PBS-Tween before use and 50 µl of PBS-BSA was added to each well 50 µl of supernatant for analysis, and controls (media for the negative control and serum from the immunised mouse for the positive control) were added to the wells, and incubated overnight at room temperature The following day the plates were washed three times with PBS-Tween 100 µl of sheep anti-mouse Ig, β -galactosidase-linked F(ab')2 fragment (obtained from Amersham and diluted in PBS, containing 10mM $MgCl_2$, and 1mM EtSH) was added Incubation for 1-2 hrs at 37°C followed The plates were then washed 3 times with PBS-Tween, 100 µl of substrate (PBS, containing 3 mM ONPG, 10 mM $MgCl_2$, and 0 1 M EtSH) was added and the plates incubated at 37°C for 1-2 hrs The reaction was stopped by the addition of 35 µl of 1 M sodium carbonate The absorbances were measured on an ELISA plate reader (Bio-Tek, model EL 307) at 405 nm Each well from the fusion plate was assayed in duplicate

II 9 CHARACTERISATION OF ANTIBODIES

The characterisation of monoclonal antibodies consisted of three stages The first was to determine the isotype of the antibody, usually by ELISA The second stage was to determine the specific antigen, and if possible the epitope on the antigen to which the antibody binds This was determined by electrophoresis, electroblotting, and ELISA The third stage was to determine the properties of the antibodies These were studied by the effects of the antibody on the function of the antigen eg in a coagulation assay for FVIII

II 9 1 ANTIBODY ISOTYPING

Antibodies were typed using a commercial isotyping kit (Zymed Laboratories) This contained a series of rabbit anti-mouse immunoglobins, each specific for a different isotype The second antibody was peroxidase-conjugated goat anti-rabbit antiserum

Antigen-coated plates (see II 8 1) were washed 3 times with PBS-Tween, and 100 µl of purified ascitic fluid (see II 6 3), which had been suitably diluted in PBS-Tween, were added to each well The plates were incubated for 2 hrs at 37°C The plates were washed 3 times in PBS-Tween and 1 drop from a bottle of rabbit anti-isotype was added to the appropriate wells The plates were incubated at 37°C for 1 hr Sufficient wells were used to allow each isotype to be assayed in duplicate The plates were then washed 3 times in PBS-Tween, and 100 µl of enzyme-conjugated antibody (diluted in PBS-Tween) was added The plates were incubated at 37°C for 1 hr, washed 3 times in PBS-Tween, and 100 µl of substrate (perox buffer containing 40 mg OPD and 20 µl H_2O_2 per 100 ml) was added The plates were incubated at room temperature for 15 min, and the reaction was stopped with 35 µl of 20%(v/v) sulphuric acid The absorbances were read on an ELISA plate reader (Biotek model EL307) at 490nm

II 9 2 FUNCTIONAL TESTS

To test the effect of the antibody on the function of the antigen, change in coagulation time was measured

0 1 ml of purified ascitic fluid was incubated with 0 1 ml of a 100% normal plasma solution, and left for 3 hr at 37°C The APTT

assay was then carried out as described in II 2, and the effects of the antibody on coagulation time determined Alternatively, 0 1 ml of a 1/100 dilution of antibody was added to 0 4 ml normal plasma, and incubated for 4 hr at 37°C This was then diluted to give a reading in the APTT assay

II 9 3 COMPETITIVE ELISA

The polyclonal antibodies to the diazepam conjugate were characterised by a competitive ELISA This measured the ability of different benzodiazepines to inhibit the binding of the antibody to conjugate coated on a 96 well plate

The benzodiazepines were dissolved in DMSO (5 mg/ml) and then diluted in PBS-Tween 25 µl of benzodiazepine and 75 µl of antibody were added to each well of a 96 well plate coated with conjugate The final concentrations of the benzodiazepines were 300, 30, and 3 µg/ml and that of the antibody was 100 µg/ml The control well contained antibody and DMSO diluted to give the same concentrations as in the test wells Conjugate was also used as a control, and BSA was used as a test sample The conjugate and BSA were diluted in PBS-Tween Samples were incubated for 2 hr at 37 °C and washed The effects of the benzodiazepines on binding of the antibody were assessed using the β-galactosidase ELISA assay (see II 8 1)

II 10 1 AGAROSE ELECTROPHORESIS

A 1% (w/v) agarose gel was prepared in barbitone buffer (see II 2 4) and boiled to dissolve it The molten agarose was then poured onto a glass plate (84x94 mm) and allowed to set An LKB sample application foil was laid across the gel and 2-4 µl samples were applied to the gel Samples were diluted in barbitone buffer, containing 0 05% (w/v) bromophenol blue The gels were placed on the cooling plate of an LKB multiphor flatbed electrophoresis apparatus (2117) The gel was connected to the buffer troughs containing barbitone buffer, by paper wicks The gels were run at 20 V/cm until the bromophenol blue band had reached the end

After electrophoresis gels were fixed and stained in Coomassie blue The gels were rinsed in distilled water prior to destaining

II 10 2 CROSSED IMMUNOELECTROPHORESIS

This was similar to the Laurell electrophoresis, except that the sample was electrophoresed in a horizontal direction prior to electrophoresis into the antibody containing gel in a vertical direction

A 1% (w/v) agarose gel containing 50 µl of anti-human serum antibody (Miles) was poured (see II 2 4) After this set two-thirds of the gel was cut away, and an agarose gel containing anti-FVIIIR was poured in its place (see II 2 4) Half of this section of the gel was removed, after it had set

A sample of FVIII was run in an agarose gel (see II 10 1) This electrophoresis strip was then cut out and placed across the antibody containing gel, in the region cut out This gel was laid in a horizontal manner, and all the gaps were filled with molten agarose The sample was then electrophoresed into the gel, and the gel was fixed and then stained (see II 2 4)

II 10 3 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Stock solutions of 0 75 M Tris, pH 8 8, 0 25 M Tris, pH 6 8, 30% (w/v) acrylamide - 0 8% (w/v) bisacrylamide, 1% (w/v) ammonium persulphate, and 10% (w/v) sodium dodecyl sulphate (SDS) were

prepared

For the resolving gels the stock solutions were mixed with distilled water to give a gel concentration of 10%T (T= total acrylamide concentration in w/v), or 7 5%T, in 0 375 M Tris, pH 8 8, containing 0 1% SDS The stacking gels were 4%T, in 0 125 M Tris, pH 6 8, containing 0 1% (w/v) SDS

Samples were dissolved in 10 mM Tris, pH 6 8, containing 8 M urea, 2% (w/v) SDS, and 0 001% (w/v) bromophenol blue Samples were then incubated at 37°C for 30 min, or boiled for 2 min with 50 µl EtSH/ml buffer The gels were then run in 0 025 M Tris - 0 192 M glycine, pH 8 3, at 100 mA using the Bio Rad vertical electrophoresis system Gels were then stained or blotted

II 10 4 WESTERN BLOTTING

Gels were soaked in 25 mM Tris - 192 mM glycine, pH 8 3 (blotting buffer) for 1 hr Nitrocellulose paper (0 45 m, Schleicher and Schuell) was then placed on the gel, and clamped in the cassette This was then blotted in the Bio Rad Transblot apparatus overnight at 100 mA This was followed by 1 hr at 200 mA

The blots were then blocked in PBS-BSA, containing 0 1% (w/v) Tween, for 1 hr The monoclonal antibody was then diluted in blocking buffer, and incubated with the nitrocellulose for 1 hr at 37°C The blots were washed in PBS-Tween for 1 hr Alkaline phosphatase-conjugated sheep anti-mouse IgG, was then added at a 1/1000 dilution in blocking buffer, and left for 1 hr at 37°C The blots were washed for 1 hr in PBS-Tween and were incubated in 10 mM Tris, pH 8 9 After 10 min substrate was added (0 5 M Tris, pH 8 9, containing 4 mM $MgCl_2$, 0 12 mM nitro blue tetrazolium, 0 46 mM 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt, and 4%(v/v) N,N-Dimethylformahide) The blots were then washed in water and dried on tissue paper

II 10 5 DOT BLOTTING

This involved the direct application of protein to the nitrocellulose paper, rather than electroblotting it from a gel

Samples were diluted in PBS, and 2-5 µl were applied to nitrocellulose paper that had been soaked in PBS and dried The samples were allowed to dry and the nitrocellulose was blocked,

probed, and stained, as in Western blotting (see II 10 2)

II 10 6 GEL STAINING

Two different stains were used for polyacrylamide gels

(a) Coomassie blue Gels were soaked in 0 5% Coomassie brilliant blue R250 in destaining solution for 1 hr They were then washed in destaining solution (45% (v/v) ethanol and 10% (v/v) glacial acetic acid) until the background was clear

(b) Silver staining For gels with low protein concentration a silver stain, based on the Bio Rad method was used Ultrapure water was used for all stages of the silver stain Gels were soaked overnight in 40% (v/v) methanol and 10% (v/v) acetic acid Gels were then incubated in oxidising solution (0 1% (w/v) potassium dichromate, containing 28 6 µl concentrated nitric acid per 100 ml) for 10-15 min The gels were then washed for at least 90 min in water After washing the gels were incubated in 0 2% (w/v) silver nitrate for 1 hr followed by washing for 2-3 min with water The gels were then soaked in 3 changes of developer (0 3 M sodium carbonate, containing 50 µl formalin/100 ml) Development was stopped by pouring off the developer and adding stopping solution (5% (v/v) glacial acetic acid)

While silver staining is useful for detecting low levels of protein, certain proteins give a negative image, ie a clear band, with the silver stain (Merril, 1984) In particular fibrinogen and its fragments give a clear band with silver staining These can only be visualised if the gel is allowed to over stain, giving a dark background

II 11 AFFINITY CHROMATOGRAPHY

Monoclonal antibodies were coupled to CNBr-activated Sepharose, and were then used to isolate the antigen in a pure form The antigen could then be identified by electrophoresis, or relevant assay

II 11 1 COUPLING PROCEDURE

Purified antibody was dialysed against coupling buffer (0 1M $NaHCO_3$, pH 8 3, containing 0 5 M NaCl) and its volume was made up to 5 ml/g gel CNBr-activated Sepharose was suspended in 1 mM HCl for 15 min The gel was then washed with 200 ml of 1 mM HCl in a scintered glass funnel, and dried by suction The gel was then washed with with 100 ml of coupling buffer

The gel was suspended in the antibody solution and mixed at room temperature for 1-2 hr The gel was dried by suction and the absorbance (280 nm) of the solution was checked to ensure that all the protein had bound The gel was washed in 100 ml of coupling buffer, and dried by suction and incubated in 0 1 M Tris-HCl, pH 8 0, for 1 hr to block any remaining unreacted sites on the activated sepharose The gel was then washed in low pH buffer (0 1 M sodium acetate, pH 4 0, containing 0 5 M NaCl) followed by high pH buffer (0 1 M Tris-HCl, pH 8 0, containing 0 5 M NaCl) 3 times The repeated changes in pH are responsible for the elution of protein that is not strongly bound to the gel The coupled gel was stored at 4°C in 0 025 M Tris, pH 7 3, containing 0 15 M NaCl, and 0 02% (w/v) sodium azide

The concentration, in mg/ml, of the purified antibody solution was calculated by multiplying the measured absorbance of the solution at 280 nm by 0 74 (Hudson and Hay, 1980) The antibody was diluted to 5 mg/ml gel 1 g dry gel swells to 3 5 ml

II 11 2 ELUTION FROM THE GEL

The antibody-coupled gel was suspended in PBS, and poured into a 10 ml syringe The sample was applied to the column in the reverse direction and left for 15 min The column was then washed in the forward direction with PBS until the eluted buffer showed no traces of protein The sample was then eluted from the column with elution buffer (0 2 M glycine-HCl, pH 2 5) by gravity flow,

and 1 ml fractions were collected The pH of the fractions were raised to pH 7-8 with a Tris solution The remainder of the protein was eluted with elution buffer containing 10%(v/v) dioxane Again the pH of the fractions were raised The fractions containing protein were pooled, and dialysed against PBS

During the elution the presence of protein was detected using a micro Bradford assay The Bradford reagent (Bio Rad) was diluted 1 4 with distilled water, and 200 µl was added into the wells of a 96 well microtitre plate 10 µl of the eluted buffer was added to each well, and incubated for 5-60 min at room temperature A blue colour indicated the presence of protein

A second procedure was also used for FVIII, based on the method of Fass et al , (1982) Koate containing 0 25 M CaCl2 was applied to the antibody-linked column When all the unbound protein was eluted the column was washed with 1 column volume of 2 M NaCl This was followed by a wash with 1 column volume of histidine buffer (10 mM histidine, 5 mM $CaCl_2$, 1 mM PMSF, and 0 1 M NaCl, pH 6 0) The protein bound to the antibodies was eluted with a 1 1 mix of histidine buffer and ethylene glycol The eluted protein was pooled and dialysed against PBS overnight

II 12 1 PATIENT SELECTION

A sequential trial was established to test the clinical effectiveness of coumarin The patients were divided into 3 broad groups, based on diagnosis chronic brucellosis (50%), cancer, mainly breast carcinoma and melanoma (30%), and the remainder had either chronic infections (including toxoplasmosis, mycoplasmosis and mononucleosis), or chronic fatigue syndrome, also known as myalgic myeloencephalopathy (ME) When the results from the trial were assessed, the number of patients had reached 2,137 Patients treated in this study had given their informed consent and were treated at St Laurences Hospital, Dublin, now Beaumont Hospital, Dublin, The Missionary Hospital, Drogheda, and The James Connolly Memorial Hospital, Blanchardstown, Dublin

II 12 2 CLINICAL EVALUATION OF PATIENTS

All patients had regular medical examinations, with full blood counts (FBC) and liver function tests (LFTs) every 3 months This included determination of serum bilirubin (both total and direct), serum transaminases, both Alanine Aminotransferase (ALT/GPT) and Aspartate Aminotransferase (AST/GOT) and serum alkaline phosphatase (AP) These tests were performed in the hospital that the patient regularly attended

II 12 3 DOSE REGIMENS

The dose of coumarin administered to each patient was adjusted to give the maximum clinical response, measured in terms of an improvement in the patients condition Doses ranged from 25mg/day for chronic infections, to 2 g/day for advanced renal cell carcinoma and glioma The majority of patients received 100 mg/day for 1 month, followed by 50 mg/day for 2 years The coumarin used in the trial was donated by Schaper and Brummer (W Germany)

II 12 4 EVALUATION OF HEPATOTOXICITY

The role of coumarin in a case of hepatitis was assessed with a scheme used by Stricker and Spoelstra (1985) They used 3 criteria viz the specificity of the clinicopathological (CP) pattern, the presence of a temporal relationship, and the exclusion of other possible causes (see V 1 5)

(i) The CP pattern is defined as the clinical, histological and biochemical data, along with any other data which may help to define the toxicity A highly specific CP pattern is one that is sufficiently unique, to enable it to be differentiated from other causes of hepatitis Examples of this are alcoholic cirrhosis, deposits of certain agents at the site of injury, toxic levels of the agent in the blood, etc A specific CP is one that is frequently caused by drugs Its presence does not prove that it was caused by a drug, but it is a strong indicator of drug toxicity Examples of this are persistent rash, with hepatitis, or persistent fever, with hepatitis Both these could be caused by viruses, but if they persist after the appearance of the jaundice, they are more likely to be due to a drug reaction

(ii) A temporal relationship implies that the hepatitis appears within 6 weeks of starting treatment with the suspect agent Certain drugs need up to several months before hepatitis appears The LFTs should return to normal promptly, after stopping the drug There should be a prompt recurrence of hepatitis, on retreatment

(iii) Other possible causes, eg cancer and viral infections should be excluded

The model used by Stricker and Spoelstra (1985), uses different levels (A-G) to assess the role of a drug in hepatitis

LEVEL A The presence of a highly specific CP pattern This indicates a definite case of hepatotoxicity

LEVEL B The patient is rechallenged with the suspect drug A strong temporal relationship indicates a definite case of hepatotoxicity, and the absence of a temporal relationship indicates an unlikely/ possible relationship The absence of a clear temporal relationship suggests a possible/probable cause In level C-G there is no rechallenge of the patient

LEVEL C Mainly cholestatic, acute hepatic injury

LEVEL D Mainly hepatocellular, acute hepatic injury

LEVEL E Chronic intrahepatic cholestasis

LEVEL F Vascular disorders

LEVEL G Liver tumours

In level C and D the presence of any 2 of the 3 criteria indicates a probable cause (eg specific CP pattern and a clear temporal relationship) The presence of only 1 of the criteria indicates a possible cause

SECTION III

MONOCLONAL ANTIBODIES TO BLOOD COAGULATION FACTORS

III 1 1 BLOOD COAGULATION

Blood is the major transport system in the body It is responsible for the transportation of nutrients, and hormones to the cells, and the transport of waste away from the cells It also serves to transport cellular components, ie the erythrocytes which carry oxygen, leucocytes which fight infection, and platelets Any malfunction of this transport system can have very serious consequences A loss of blood, such as haemorrhage due to trauma, causes hypotension, which can eventually lead to shock This hypotension leads to reduced perfusion of many organs, which can result in damage to sensitive tissue, and ultimately to fatal brain damage A blockage in the blood supply to a specific area, such as that due to a thrombus (a clot), or an embolism (a fragment of a thrombus), leads to ischaemia In the extremities this leads to necrosis, and even gangrene In the brain it leads to a stroke, the severity of which depends on the location of the ischaemia In the heart the result is ischaemic heart disease, which can lead to myocardial infarction

Due to the importance of maintaining adequate perfusion of all tissue, there are 3 mechanisms for regulating blood flow The first is the heart/nervous system This pumps the blood and regulates the pressure The second is the coagulation/haemostatic system which prevents blood loss from the system The third is the fibrinolytic system which removes any unwanted clots from the system

The coagulation system is found in the plasma It consists of a number of proteins arranged in a cascade system which convert fibrinogen to fibrin, allowing it to polymerise, forming a clot These proteins are numbered with Roman numerals, which denote their order of discovery, and not their sequence in the cascade (Wright, 1962) There is no number VI Certain factors are usually refered to by their name eg fibrin(ogen) (FI), (pro)-Thrombin (FII), tissue thromboplastin (FIII), and calcium (FIV) The letter "a" after a number indicates an activated form of the protein

The coagulation system can be divided into 3 stages There are 2 initiation/amplification stages, followed by an activation/ coagulation stage The latter involves the conversion of prothrombin to thrombin by FXa, in the presence of FVa, calcium, and Platelet factor 3 (PF3) Thrombin then converts fibrinogen into fibrin which results in clot formation (see fig III 1 and below) These 3 stages form the cascade system (Macfarlane, 1964)

There are 2 initiation pathways The first is the extrinsic system (Zur and Nemerson, 1981) Damaged tissue releases tissue thromboplastin which in the presence of FVII, and calcium, activates FX The role of the extrinsic system in coagulation is uncertain, but it is thought that its activation produces small amounts of thrombin rapidly This thrombin then activates FV, and FVIII which are essential for the fuctioning of the intrinsic system Kallikrein, which is produced at the early stages of the intrinsic system, also activates FVII In this role the small amounts of thrombin produced act to accelerate the intrinsic system Unlike the intrinsic system PF3 is not required for activation of the extrinsic system This is because FIII has phospholipids associated with it (see Fig III 2)

The second initiation system is the intrinsic system (Griffin, 1981) This is activated by a 'foreign surface' eg collagen, sebum, glass, and low pH This activates a small amount of Hageman factor (FXIIa) This converts prekallikrein (Fletcher factor) to kallikrein, which in turn convers FXII to FXII fragments (FXIIf) FXIIf activates prekallikrein, and plasminogen proactivators Both FXIIa, and FXIIf activate FXI in the presence of Fitzgerald factor (high molecular weight kininogen, a precursor of bradykinin) FXIa converts Christmas factor to FIXa FIXa activates FX in the presence of FVIIIa, PF3, and calcium (see Fig III 3) While <u>in vitro</u> both the intrinsic, and extrinsic coagulation pathways are separate, <u>in vivo</u> there is probably no distinction between the two

The heart of the coagulation system is the activation of thrombin Not only does thrombin convert fibrinogen to fibrin, it also activates FV and FVIII which are essential for clot formation FXIII, which is responsible for cross linking fibrin clots, is also activated by thrombin The complicated cascade system serves

2 main functions Firstly, there are a number of different control points which allows fine control of the system Secondly, the large number of steps enables the signal to be amplified In the intrinsic system there are 6 steps to the activation of FX If there is a 10 fold increase in signal at each step, there will be a 1 million fold increase overall

The mechanism which controls blood loss (haemostasis) requires 3 components viz, the coagulation system, platelets, and vascular smooth muscle If there is a small hole in a vessel wall, platelets easily lodge in it Clot formation then occurs which plugs the hole With larger holes, platelets adhere to the collagen at the edge of the wound von Willebrands Factor is necessary for this These platelets secrete factors that initiate platelet aggregation (ADP), as well as factors which cause a localised vasoconstriction, thereby reducing blood flow Platelet aggregation (involving vWF) is then followed by coagulation, which plugs the hole Factors released from platelets are essential for coagulation, eg PF3, FV, and PF4 (heparin neutralising factor) Thrombosthenin, released by the platelets, causes retraction of the fibrin strands This draws the edges of the wound together, and also increases the mechanical strength of the clot Haemostasis is measured by the bleeding time In some disorders the coagulation time can be normal, and yet the bleeding time is prolonged (Williams et al , 1977)

Not only does FXIIa activate the intrinsic coagulation system, it also activates plasminogen activators in the plasma These then activate the fibrinolytic system by converting plasminogen to plasmin Plasmin breaks down both fibrinogen, and fibrin (see below), and thus removes unwanted clots(see Fig III 4) During clot formation, plasminogen becomes incorporated into the thrombus When plasminogen activators diffuse into the clot they convert the plasminogen to plasmin, which then proceeds to hydrolyse the thrombus Both coagulation, and fibrinolysis are subject to control by inhibitors of their respective systems (Williams et al , 1977)

III 1 2 FIBRIN(OGEN) STRUCTURE

Fibrinogen is a globular protein with a molecular weight of 340,000 It is converted to its active form (fibrin) by the action of thrombin The amino acid sequence for fibrinogen has

been determined, as has its structure The structure, and function of fibrin(ogen) is reviewed by Doolittle (1981a, 1981b)

Fibrinogen consists of 3 different polypeptide chains The alpha (Aα) chain has 610 amino acids and a Mr of 67,000 The beta (Bβ) chain has 461 amino acids and a Mr of 56,000 The gamma (γ) chain has 411 amino acids and a Mr of 47,000 (Doolittle, 1981b) Fibrinogen consists of 2 of each of these chains and thus , it is $A\alpha_2B\beta_2\gamma_2$ (see Fig III 5) Electron microscopy studies showed a trinodular structure for fibrinogen (Hall and Slayter, 1959) The dimensions of the nodules were calculated to be 500 nm for the central globule, 650 nm for the terminal globules, and 150 nm (thickness) for the connecting arms (see Fig III 6) The length of the complete molecule was calculated to be 4,750 nm There are 29 disulphide bonds in the molecule

One Aα, Bβ, and γ chain are held together by 2 rings of disulphide bonds which are in the N terminal region This is known as the N terminal DiSulphide Knot (NDSK) (Blomback et al , 1968) In the NDSK region each chain has the following sequence cys-X-Y-Z-cys The first cys from the A chain is bound to the last cys in the γ chain The second cys in the Aα chain is bound to the first cys in the Bβ chain, and the last cys in the Bβ chain is bound to the first cys in the γ chain - this is the disulphide ring (see Fig III 7) The first cys in the Aα chain is amino acid 45, while in the Bβ chain it is 76 and in the γ chain it is 19 The second disulphide ring occurs at cys 161 (Aα chain), 193 (Bβ chain), and 135 (γ chain) The region between the 2 disulphide rings is coiled in an alpha helical structure

The C terminal region of the Bβ chain and the γ chains are each folded into separate globules The Aα chain is hydrophobic and remains in an uncoiled form These form the terminal globules (Doolittle, 1981a) Fibrinogen is a dimer, with the N terminal regions of the 3 chain monomer joined together by 3 disulphide bonds One of disulphide bonds joins the Aα chains, and other two join the γ chains There are 2 different carbohydrate chains on each of the monomers (Gaffney, 1972) These 2 chains account for approximately 10,000 daltons (see Fig III 8)

III 1 3 FIBRIN(OGEN) FUNCTION-CLOT FORMATION

The function of fibrinogen is in the formation of clots The first

step is the conversion of fibrinigen to its active form - fibrin This is then followed by the polymerisation of the fibrin monomers into a clot, which is then stabilised

The enzyme responsible for activating the fibrinogen is thrombin This is a protease which cleaves off the terminal region of the $A\alpha$ and $B\beta$ chains These are called fibrinopeptides A and B Fibrinopeptide A is 16 amino acids long, while fibrinopeptide B is 14 amino acids long The resulting molecule is the fibrin monomer and is $\alpha_2\beta_2\gamma_2$ (Laurent and Blomback, 1958)

Fibrinogen has an overall charge of minus 26 The central globule has a charge of minus 8, while the terminal globules have a charge of minus 4 The connecting chains are neutral When the fibrinopeptides are removed the central globule loses its negative charges and becomes plus 5 (Doolittle, 1981a)

During activation by thrombin, the two fibrinopeptides are released at different rates, with fibrinopeptide A being released before B The regions exposed by the fibrinopeptides are responsible for the polymerisation of fibrinogen (Ferry, 1952, Laurent and Blomback, 1958) and The $A\alpha$ chain region exposed by removal of fibrinopeptide A is attracted to a portion of the γ chain carboxyl region by an electrostatic interaction This is then elongated to form a long polymer which is 2 molecules thick The region of the B chain exposed by removal of fibrinopeptides B is probably involved in lateral polymerisation of the fibrin chains At this stage the clot is very fragile, as it is only held together by electrostatic bonds The polymer is strengthened by the action of factor XIII (FXIII), which is activated by thrombin This forms covalent bonds between suitably sited lysines and glutamines (Pisano et al , 1971) The initial cross linking occurs at the carboxy terminal region of the γ chains The γ chains lie in an antiparallel manner and there are 2 cross links, 8 amino acids apart (Chen and Doolittle, 1970) Cross linking also occurs between the $A\alpha$ chains, but at a much slower rate (McKee et al , 1970) While the γ cross linking only links 2 fibrin monomers, each $A\alpha$ chain is linked to at least 2 different $A\alpha$ chains The glutamines involved in the $A\alpha$ chain cross linking are 38 amino acids apart in the middle of the $A\alpha$ chain, while the lysines are near the carboxy terminal The resulting cross-linked polymer is mechanically stronger and is resistant to enzymatic

digestion

III 1 4 FIBRIN(OGEN) FUNCTION - CLOT LYSIS

Unwanted clots are digested by an enzyme called plasmin, which is the active form of plasminogen This acts on fibrin and fibrinogen at the same rate Plasmin succeeds in breaking the stabilised fibrin clots by attacking their weak point This is the alpha helical connectors between the globules At this point the clot is only 3 chains thick (ie the Aα, Bβ, and γ chain) After breaking a few of these bonds the clot is split into a number of intermediate fragments, allowing the clot to be solubilised Plasmin continues to digest the clot until only small core fragments are left Stabilised clots are digested at a slower rate than unstabilised clots

Plasmin digestion of fibrin(ogen) has been the subject of many investigations, and was important in the determining of the structure of fibrin(ogen) (Marder, 1971) The initial action of plasmin is the formation of fragment X, which has a Mr of 250,000 (Nieuwenhuizen and Gravesen, 1981) This fragment is formed by the removal of two Mr 45,000 pieces from the carboxyl portion of the Aα chains The next fragment is the Y fragment, Mr 150,000 This is formed by the removal of one D fragment, Mr 90,000 (Haverkate et al , 1979), plus a small connector region, from the carboxy terminal of the X fragment The D fragment, consists of part of 1 A^{α} chain, 1 B^{β} chain (N terminal part), and part of 1 $^{\gamma}$ chain (N terminal part) The final fragment is fragment E, Mr 50,000 This consists of two N terminal fragments of the A^{α} chain, 2 fragments of Bβ, and 2 N terminal fragments of the $^{\gamma}$ chain The Y fragment is D_2E, and there are 2 D fragments and 1 E fragment in each fibrinogen molecule (see Fig III 6) The molecular weight of the D fragment is different if calcium is used in its preparation (Haverkate and Timan, 1977) Thus, D(cate) has a Mr of 93,000, while the Mr of D(EGTA) is 80,000 D fragments prepared in the presence of calcium have strong anticoagulant activity, while those prepared in the presence of EGTA have no anticoagulant properties (Haverkate et al , 1979) Plasmin digestion of fibrin yields a D_2 dimer It is probably due to protection of one of the plasmin sites by the calcium, since in the presence of EGTA, D monomers are formed Fibrinogen has 3 calcium binding sites, 2 of them are in the carboxyl terminal of the γ chain, which is located in the D(cate) fragment

(Nieuwenhuizen and Gravesen, 1981) Table III 1 summarises the structures of the fibrin(ogen) fragments (from Koppert et al , 1986)

III 1 5 FIBRIN(OGEN) DISORDERS

Disorders of fibrinogen can either be congenital, or acquired The congenital disorders are classed as quantitative, ie hypofibrinogenaemia, or afibrinogenaemia (depending on severity), and qualitative, ie dysfibrinogenaemia

With congenital afibrinogenaemia, there is no fibrinogen present, but in some cases levels of 5 mg/100 ml have been detected (normally 200-300 mg/100 ml) Patients suffer from internal bleeding bouts, and prolonged bleeding after minor traumas The disorder is transmitted as an autosomal trait Treatment involves replacement therapy In hypofibrinogenaemia there is usually no bleeding bouts, but there can be prolonged bleeding after surgery (Williams et al , 1977, Bloom, 1981)

A large number of dysfibrinogenaemias have been identified In each case there is usually a modification to the fibrin(ogen) molecule, but in some cases there is an associated hypofibrinogenaemia Dysfibrinogenaemia is usually due to a dominant autosomal trait, and they are usually named after the place in which they were first identified In a few cases the actual alteration has been identified Fibrinogen Detroit has an arginine, at position 19 of the $A\alpha$ chain, replaced by a serine Fibrinogen Zurich has abnormal fibrin monomer aggregation, fibrinogen Bethesda I has defective fibrinopeptide release, and fibrinogen Oklahoma has defective fibrinogen cross-linking Patients often have prolonged coagulation time Treatment is not usually necessary (Bloom, 1981)

The acquired disorders of fibrinogen are known as defibrination syndromes This defibrination is due to disseminated vascular coagulation (DIC) There are 3 problems associated with DIC The first is consumption of coagulation factors which, along with the increase in production of fibrin degradation products, leads to prolonged bleeding Secondly, fibrin can be deposited in capillaries which would block blood flow, leading to ischaemia Thirdly, loose strands of fibrin deposited in blood vessels may not cause a blockage, but they can damage the red blood cells as

they pass by The severity of the last two effects depends on the effectivness of the fibrinolytic system (Williams et al , 1977, Brozovic, 1981)

DIC only occurs in conjunction with other serious disorders In particular disorders of pregnancy such as toxaemia, or retained dead foetus Other disorders which cause DIC are gram-negative endotoxinaemia, gram-positive septicaemia, liver disease such as cirrhosis, heatstroke, extensive burns, and certain malignant diseases Therapy usually involves continuous infusion of heparin This prevents coagulation, thereby preventing further depletion of coagulation factors This can be accompanied by replacement therapy In particular, cryoprecipitate is effective in this as it provides both fibrinogen, and FVIII Platelets may also need to be administered If there is serious bleeding due to inhibition of coagulation by high levels of fibrin degradation products, a firinolysis inhibitor can be used Aminocaproic acid (EACA) can be used for this, but its use can be hazardous (Brozovic, 1981)

III 1 6 FIBRIN(OGEN) ASSAYS

There are 2 main fibrinogen assays The first involves the addition of thrombin to a plasma sample Thrombin acts directly on fibrinogen, and is not dependent on any other factors The thrombin time is the time taken for a clot to form, and this is a measure of the amount of fibrinogen present (Clauss, 1957) A variation on this is the gravimetric assay This involves removing the clot, drying it, and weighing it This gives a direct measure of the fibrinogen present The second method uses antibodies to fibrinogen in an ELISA, or RIA This is especially useful for studying fibrin(ogen) degradation products

III 1 7 FACTOR VIII COMPLEX

The study of factor VIII (FVIII) deficiency disorders indicated that FVIII had 2 distinct properties - coagulation (thrombosis) and the prevention of blood loss (haemostasis) Subsequent studies indicated that FVIII consisted of 2 proteins, one which controls coagulation (FVIII) and the other which controls haemostasis (von Willebrands factor - vWF) The nomenclature of these proteins has often been the source of confusion The system used here is the most widely used at present (Hoyer, 1981)

FVIII C - the portion of the complex with procoagulant

activity

FVIIIR/(vWF) - the portion of the molecule with haemostatic properties (von Willebrands factor)

FVIII CAg - the antigenic portion(s) of FVIII C

FVIIIR Ag - the antigenic portion(s) of vWF

FVIIIR RC - the portion of vWF that binds ristocetin resulting in platelet aggregation

For convenience, the term FVIII is used to refer to the complex of FVIII C and vWF when neither property is being being referred to specifically

FVIII C and vWF are both seperate and distinct proteins, but they do interact with each other The FVIII complex has a molecular weight of $1\text{-}20 \times 10^6$, with FVIII C comprising about 1% of the total The 2 proteins can be dissociated with high ionic strength buffers, with 0 25 M calcium being used generally While the 2 are seperate, immobilised polyclonal antisera to FVIII will remove both FVIII CAg and FVIIIR Ag from plasma This is not due to the antisera binding to FVIII CAg since the FVIII CAg can be eluted from the column using calcium On the other hand immobilised antibodies to FVIII CAg only remove this antigen, and do not deplete plasma of FVIIIR Ag This is thought to be due to destabilisation of the complex on binding the antibodies Under normal conditions the concentrations of both FVIII C and vWF fluctuate together, but in certain diseased states, eg haemophilia, their concentrations can vary independently of each other Thus, while the 2 proteins are seperate from each other, they also interact strongly together (Hoyer, 1981)

III 1 8 FVIII C - STRUCTURE

In 1984 Vehar et al , Toole et al , Gitschier et al , and Wood et al , reported the successful cloning of the FVIII C gene, and the subsequent determination of its amino acid sequence

The gene is located on the X chromosome, and it consists of 186 kbp (kilo base pairs), and is about 0 1% of the X chromosome There are 26 exons ranging in size from 69 bp to 3,106 bp, the latter is thought to be the largest known exon The introns range in size from 207 bp to 32 4 kbp The completed mRNA is 96 kbp Due to its large size it is estimated that transcription would take about 3 hr at a rate of 15 neucleotides per second

The mature protein is a single polypeptide chain containing 2,332 amino acids, and it has a calculated molecular weight of 264,763 The apparent molecular weight on SDS-PAGE is 330,000, which is in agreement with the calculated molecular weight allowing for glcosylation SDS-PAGE yields a number of bands for FVIII C Vehar et al , (1984) report 2 major protein pools The first has a Mr of 90,000-210,000, and the second has a Mr of 70,000-80,000 Fay et al , (1982) reported a Mr of 72,000 band under non-reducing conditions, and a Mr of 100,000 band under reducing conditions

Vehar et al , (1984) found that the tryptic sequences from the Mr 90,000-210,000 pool of peptides indicated that they were all derived from the N terminal of the parent molecule Similarly the Mr 70,000-80,000 pool were all found to be derived from the carboxy-terminal of the parent molecule The difference in apparent Mr between the reduced and non-reduced forms indicates intrachain disulphide bonds (see Fig III 9)

Molecular cloning data indicated that there were 2 repeating homologous regions in FVIII C The first is the A domain which is approximately 330 amino acids long The second is the C domain which is 150 amino acids long There is also a B domain of 980 amino acids There are 3 A domains, 2 C domains, and only one B domain (see Fig III 9) The C domains are at the carboxy- terminal end

Thrombin, which is responsible for activating FVIII C, cleaves the molecule into 2 fragments The Mr 90,000 fragment contains the A1 and A2 domains, and the Mr 80,000 consists of A3-C1-C2 The Mr 80,000 can be further cleaved to a Mr 73,000 fragment The Mr 90,000 fragment is cleaved to Mr 50,000 and Mr 43,000 fragments on prolonged exposure to thrombin The Mr 50,000 fragment can be further cleaved to an Mr 30,000 and an Mr 20,000 fragment (see Fig III 9)

The A domains were found to be approximately 30% homologous with the A domains of ceruloplasmin which consists of 3 A domains, but is lacking in B, and C domains Both ceruloplasmin and FVIII C bind metals Ceruloplasmin binds 6 copper ions, while calcium ions are bound by FVIII C (Mikaelsson et al , 1983) The amino acids responsible for some of the copper binding sites in

ceruloplasmin are conserved in the A1 and A3 domains of FVIII C, indicating that there is probably a similar binding mechanism involved There are also a number of similarities between FVIII C and FV (Kane and Davie, 1988) In each case they act in conjunction with another vitamin K-dependent protein (FIXa in the case of FVIII C, and FXa in the case of FV) in the presence of calcium and phospholipid The proteins they act on (prothrombin in the case of FV, and FX in the case of FVIII C) are also vitamin K-dependent proteins FVIII C and FV are single chain polypeptides with molecular weights greater than 300,000, and they are both activated by thrombin to give 2 polypeptides - Mr 90,000 and Mr 80,000 in the case of FVIII C, and fragments D and E in the case of FV)

III 1 9 FVIII C - FUNCTION

FVIII C acts as a cofactor in the activation of FX by FIXa Both calcium and phospholipid are necessary for the reaction FVIII C on its own has no effect, it only acts as a catalyst Activation of FVIII C to Mr 80,000 and Mr 90,000 by thrombin is necessary for activity This is thought to occur at Arg(372)-Ser(373) Ware et al , (1988) used a monoclonal antibody which inhibited coagulation, and identified its binding site as residues 338-362 on the Mr 90,000 fragment This would indicate that the Mr90,000 fragment is FVIIIa But, if the mechanism of activity of FVIII C is similar to that of FV, then both fragments would be necessary for activity, as is the case of FV Prolonged exposure of FVIII C to thrombin inactivates it Activated protein C, and FXa also inactivate FVIII This cleavage is thought to occur at Arg(336)-Met(337)

III 1 10 von WILLEBRANDS FACTOR - STRUCTURE

Like FVIII C, the structure of vWF was determined by molecular cloning (Sadler et al , 1985, Lynch et al , 1985, Verweij et al , 1985) and by protein sequencing (Titani et al , 1986) The cDNA was 8 9 kbp long, and it encodes for a single polypeptide chain of 2,813 amino acids, with a Mr of 309,000 The mature vWF has a Mr of 225,000, and is 2,050 amino acids long During maturation the 22 amino acid signal peptide, and a 741 amino acid peptide are cleaved off This peptide is called von Willebrands antigen II (vWAg II) (Fay et al , 1986)

The vWF precursor protein consists of 4 homologous domains There are 3 A domains, each of about 311 amino acids There are 2 B domains, and 3 C domains, which are quite short There are 4 D domains, which are 350-400 amino acids long vWAg II consists of the first 2 D domains from the N terminal The lead sequence of the N terminal of the mature protein is part of a D domain, called D' (Girma et al , 1987) (see Fig III 10)

The mature vWF is a single chain polypeptide chain with 2,050 amino acids, and 22 probable glycosylation sites with 18 7% carbohydrate resulting in a molecular weight of 270-280,000 (Chopek et al , 1986, Titani et al , 1986) On SDS-agarose gels under non-reducing conditions, vWF forms a series of multimers which have a range of molecular weights up to Mr $12x10^6$ (Hoyer, 1981) On SDS- PAGE gels, under reducing conditions there is a single band of Mr 240,000 (Shapiro et al , 1973, Fowler et al , 1985) The basic unit of the multimers is probably a dimer of the single polypeptide chain Two of the polypeptide chains are held together by strong disulphide bonds at the carboxy-terminals These dimers are held together in a linear polymer (Fowler et al , 1985, Chopek et al , 1986) Research indicates that the monomer is the active form of vWF The multimers probably enable localised high concentrations of the vWF to occur

III 1 11 vWF - FUNCTION

While the major function of vWF is in the aggregation of platelets, it has a number of functional sites, such as platelet binding, collagen binding, FVIII CAg binding, which enable it to carry out its function (reviewed by Girma et al , 1987)

The binding of vWF to platelets is mediated by 2 membrane bound glycoproteins In the presence of ristocetin, vWF binds to glycoprotein Ib (GpIb), which leads to aggregation This has been confirmed by the elimination of binding in the presence of a MoAb to GpIb MoAbs which prevent aggregation bind to a Mr 46,000 fragment on vWF between residues 449 and 728 (Fujimura et al , 1986) This has been shown to be the GpIb binding site GpIb is involved in platelet - vessel wall interactions

vWF also binds to platelets in the presence of thrombin or ADP This binding was found to involve glycoprotein IIb-IIIa

(GpIIb-IIIa) This was confirmed by MoAbs to GpIIb-IIIa The postulated binding site of GpIIb-IIIa is at residues 1,744 to 1,746 (Titani et al , 1986)

At high shear rates, such as those found in much of the circulatory system, vWF is necessary for platelet adhesion to vessel walls This is due to the binding of vWF to collagen in the vessel walls Using MoAbs the binding sites for type III collagen were localised to amino acids 542-622, and 948-998 The former is also part of the GpIb binding region These 2 peptides correspond to the A1 and A3 domains These 2 regions have a homologous sequence at 567-621, and 969-992, which is probably the collagen binding site (Roth et al , 1986)

Again, using MoAbs, it has been shown that vWF binds to cultured endothelial and smooth muscles cells, which does not involve collagen The binding site involved is unknown, except that it is on an region of molecular weight 80,000 on the carboxy terminal region (deGroot and Sixma, 1986) The portion of vWF that binds FVIII C was found to be located on an N terminal fragment with a molecular weight of 34,000, between residues 1 and 910 (Takahashi et al , 1987) A Mr 80,000 fragment from FVIII C is also involved vWF has been found to bind to heparin, the site for this is the same as that for GpIb These binding sites are shown in Fig III 11

III 1 12 FVIII COMPLEX - DISORDERS

Congenital disorders of FVIII C are called haemophilia This term is used to refer to two different disorders Haemophilia A (here referred to as haemophilia) is due to a deficiency of FVIII C, while haemophilia B (Christmas disease) is due to a deficiency of FIX

Haemophilia is due to a sex-linked trait, since the gene is found on the X chromosome The disease usually only affects male offspring of carrier females Females are only affected if they are homozygous offspring of a carrier, and a haemophiliac The incidence of haemophilia is 1 per 10,000 in males It appears that the gene also occurs by mutation at a rate of 1 per 300,000 This is thought to be the origin of haemophilia in the European royal families The mutation apparently occurred in Queen Victoria who gave birth to 2 carriers, and 1 haemophiliac There was no

previous incidence of haemophilia in her family

Haemophilia can be categorised into 3 types The first type, severe haemophilia, has no detectable FVIII CAg, nor FVIII C (less than 1% of normal) A few of these patients have low FVIII CAg levels, but no FVIII C This group of patients have no FVIII due to an absence of the gene Those with low levels of FVIII CAg could have a mutation that prevents activity of the molecule The other 2 groups, moderate, and mild haemophilia, have variable FVIII CAg levels, which usually reflect the FVIII C levels FVIIIR Ag levels are usually normal (Hoyer, 1981)

In the plasma of some haemophiliacs material that antigenically cross reacted with antihaemophilic factor, was identified with human antibodies These patients were referred to as CRM+ The problem with the use of heterologous serum for these studies is that the most antigenic portion of the FVIII complex is FVIIIR Ag Thus, in many cases these CRM+ patients have no FVIII CAg, but normal vWF (Hoyer, 1981) Moabs against FVIII CAg are needed for accurate studies Studies have shown that at least 85% of carriers have low FVIII C levels, and normal FVIIIR Ag levels This is due to the absence of one of the FVIII C genes

The clinical manifestations of haemophilia depend on its severity The most common means for identifying haemophiliacs, especially the mild, or moderate cases, is by prolonged bleeding after dental extractions Other problems are haematomata (bleeding into the tissue), peripheral neuropathies (due to pressure on a nerve from intramuscular bleeding), and haemarthroses (bleeding into soft tissue surrounding a joint) Patients with mild, or severe haemophilia rarely suffer from haemarthroses Patients with severe haemophilia often suffer from permanent disability, and disfigurement from haemarthroses The main treatment for haemophilia is care in the prevention of injury that could result in a bleeding bout In cases of severe bleeding, such as in haemarthroses, replacement therapy can be used FVIII concentrates are also given prior to surgery (Williams et al , 1977, Bloom, 1981)

Acquired haemophilia is due to the production of IgG to FVIII C This can occur in patients undergoing replacement therapy, or spontaneously in healthy patients The IgG produced can be weak

(type II), producing only mild inhibition, or it can be strong (type I), depending on the epitope the antibodies are directed to (Gawryl and Hoyer, 1982) The reason for the occurrence of these antibodies is unknown Treatment involves replacement therapy Patients with strong inhibitors can often require many plasma volumes to stop the bleeding Sometimes sufficient FVIII cannot be infused to overcome the inhibition, in which case exchange plasmapheresis may be helpful But, even this only removes the circulating IgG (60-75%), and FVIII concentrates may be needed to supplement plasmapheresis Since the anti-FVIII antibodies are often species specific, bovine, or porcine FVIII may be useful, but these can only be used once or twice as they are very antigenic Another possibility is infusion of vitamin K-dependent factor concentrates These appear to initiate coagulation without FVIII Immunosuppressive therapy has been used for long term maintenance of patients with FVIII inhibitors (Williams et al , 1977, Brozovic, 1981)

von Willebrand's disease (vWD) is due to a congenital deficiency of FVIIIR The disease is not sex-linked (Bloom, 1981) There are 3 types of vWD In type I vWD both FVIII CAg and FVIIIR Ag are reduced, but the levels are similar The bleeding time is prolonged, and there are often associated bleeding episodes In type II vWD there is only a slight reduction in FVIII CAg and vWF, but a larger decrease in FVIIIR RC In severe vWD (type III) there is a marked reduction of both FVIII CAg and FVIIIR Ag This is a homozygous form of the disease and is very rare (Takahashi et al , 1984) In type I vWD the multimer pattern of vWF in agarose electorphoresis is unchanged from the normal but it is reduced in quantity In type II vWD there is an absence of the large multimers of vWF usually present In vWD IIa only small multimers are present Type IIb is similar to type IIa except that there are intermediate forms of the multimers present also Type IIc is also similar to type IIa, but there are aberrant triplet structures present in the multimers (Ruggeri and Zimmerman, 1981, Ruggeri et al , 1982, Fulcher et al , 1983) In type II vWD the patients platelet-rich plasma (PRP) has reduced aggregation when ristocetin is added But in type IIB vWD, patient's PRP has increased reactivity to ristocetin The reduction in FVIII CAg levels is secondary to the vWD It is probable that vWF stabilises FVIII C, or protects it from proteases Thus, while the abnormal FVIIIR may not cause platelet aggregation, it still

appears to bind, and protect FVIII C Treatment involves replacement therapy, and prognosis is usually good (Bloom, 1981)

There is also an acquired form of vWD, known as von Willebrand's Syndrome (vWS) This is due to the production of inhibitor antibodies directed against vWF (Fricke et al , 1985) In some patients the antibodies also inhibit the FVIII C molecule as well

III 1 13 FVIII COMPLEX - ASSAYS

FVIII assays are divided into 2 types The first measure FVIII C, and the second measure FVIIIR The FVIII C activity of 1 ml of plasma from a large donor pool (normal plasma) is defined as 1 International Unit (IU) Similarly, the FVIIIR Ag, and the FVIIIR RC activity of 1 ml of normal plasma is 1 IU The levels of FVIII CAg in plasma have been estimated at 50 ng/ml, ie 50 ng/IU Similarly, the concentration of FVIIIR Ag in normal plasma has estimated to be between 5 and 10 µg/ml (Hoyer, 1981)

The assays used for FVIII C are functional assays since antisera to FVIII only recognise FVIIIR Ag Also, since the concentration of FVIII CAg is so low, it would be difficult to measure The activated partial thromboplastin time (APTT) is the main assay used (Williams et al , 1977) This uses FVIII-deficient plasma from haemophiliacs to provide all the essential coagulation factors The plasma is stored in a chelating agent to prevent coagulation The reagent that activates the plasma is kaolin (a "foreign surface"), and phospholipid (similar to PF3) Once the system has been activated, calcium is added to allow coagulation to occur The time taken for coagulation to occur is directly proportional to the amount of FVIII C present A new chromogenic assay for FVIII C has been developed This uses a synthetic peptide which is hydrolysed by FVIIIa This forms a chromogenic substrate The absorbance is proportional to the FVIII C concentration

vWF is measured by the addition of ristocetin to test plasma, and the rate of platelet aggregation is measured by a platelet aggregometer (Williams et al , 1977) This is proportional to the vWF concentration ELISA assays using sera against FVIII are also used to measure directly vWF concentration So too does rocket immunoelectrophoresis (Zimmerman et al , 1975) In "normal" patients the vWF level will be the same as the FVIII C level, ie 1

IU/ml Platelet aggregation, APTT, ELISA, and the bleeding time are used together in the diagnosis, and characterisation of bleeding disorders

III 1 14 SYNTHESIS OF BLOOD FACTORS

Fibrinogen has been shown to be synthesised in liver parenchymal cells (Hamashima et al , 1964) It is probably stored in reticuloendothelial cells (Williams et al , 1977) von Willebrand Factor is synthesised in endothelial cells (Jaffe et al , 1973, Levine et al , 1982) The site of synthesis of FVIII CAg has been difficult to detect, but using hybridisation probes it has been been possible to detect FVIII mRNA, indicating a site of production Wion et al , (1985) located the mRNA in hepatocytes, lymph nodes, and spleen, pancreas and kidney cells FVIII CAg was also detected in in hepatocytes using an immuno-gold stain (Zelechowska, 1985)

TABLES AND FIGURES

TABLE III 1

This table shows the amino acids present in fibrin(ogen) and their plasmin degradation fragments The figures represent the numbers of the start and end amino acids The N-terminal amino acid on each chain is no 1

FRAGMENT	Amino acid sequence		
	A^{α} chain	B^{β} chain	γ chain
Fibrinogen	(1-610) 2	(1-461) 2	(1-410) 2
Fibrin monomer	(17-610) 2	(15-461) 2	(1-410) 2
X	(1-208) 2	(1-461) 2	(1-410) 2
Y	1-208/1-78	1-461/54-122	1-410/1-58
D(cate)	111-197	134-461	86-410
D(EGTA)	111-197	134-461	86-303
E	(1-78) 2	(54-122) 2	(1-58) 2
NDSK(fbg)	(1-51) 2	(1-118) 2	(1-78) 2
NDSK(fb)	(17-51) 2	(15-118) 2	(1-78) 2
fibrinopeptide A	1-16	-----	-----
fibrinopeptide B	-----	1-14	-----

X, Y, and E plasmin degradation products of fibrinogen

D(cate) plasmin degradation product D of fibrinogen prepared in the presence of calcium

D(EGTA) Plasmin degradation product D of fibrinogen, prepared in the presence of EGTA

NDSK N-terminal disulphide Knot of fibrinogen (fbg) and fibrin (fb)

Fig III.1 CLOT FORMATION

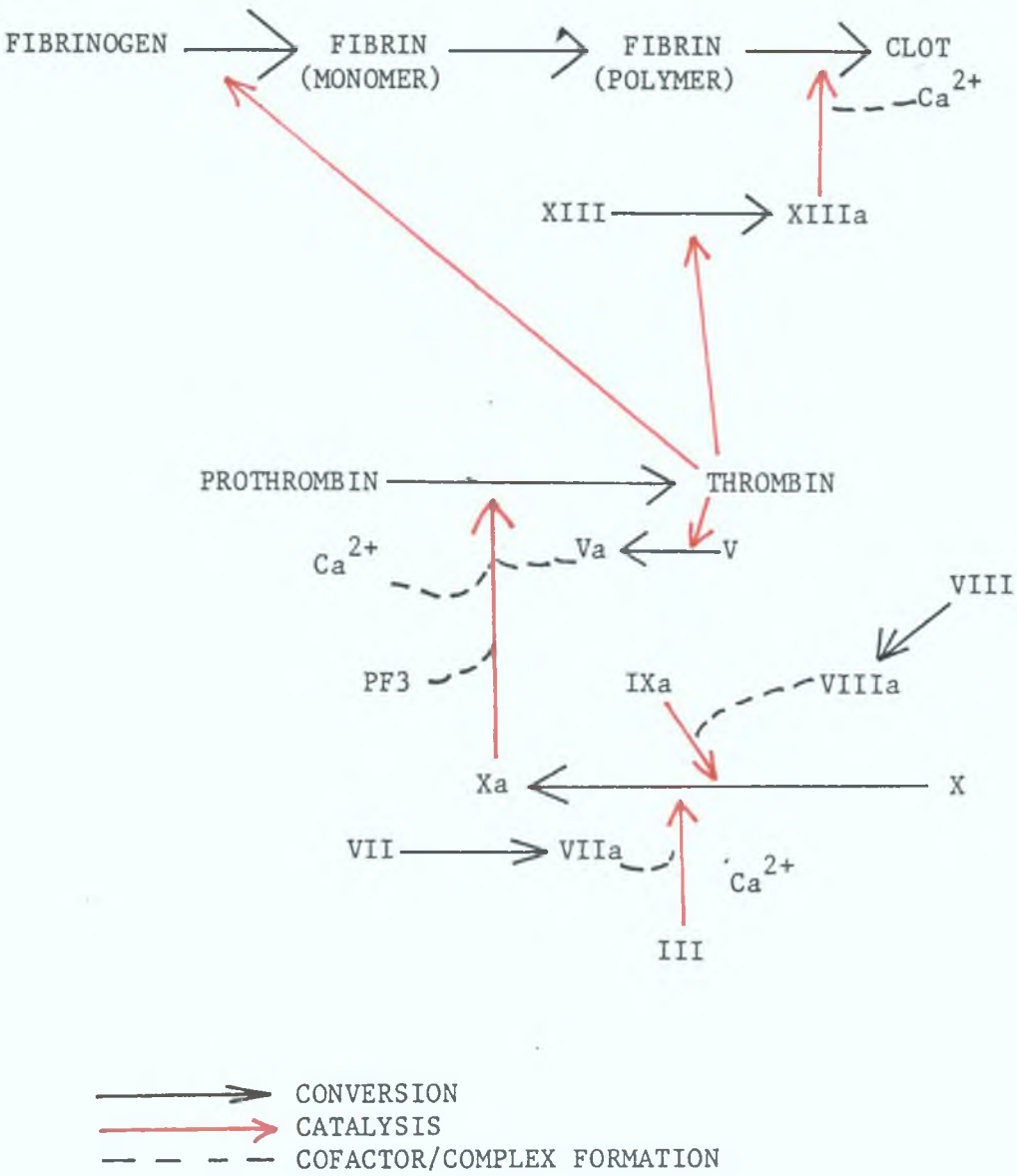


Fig III.2 THE EXTRINSIC PATHWAY

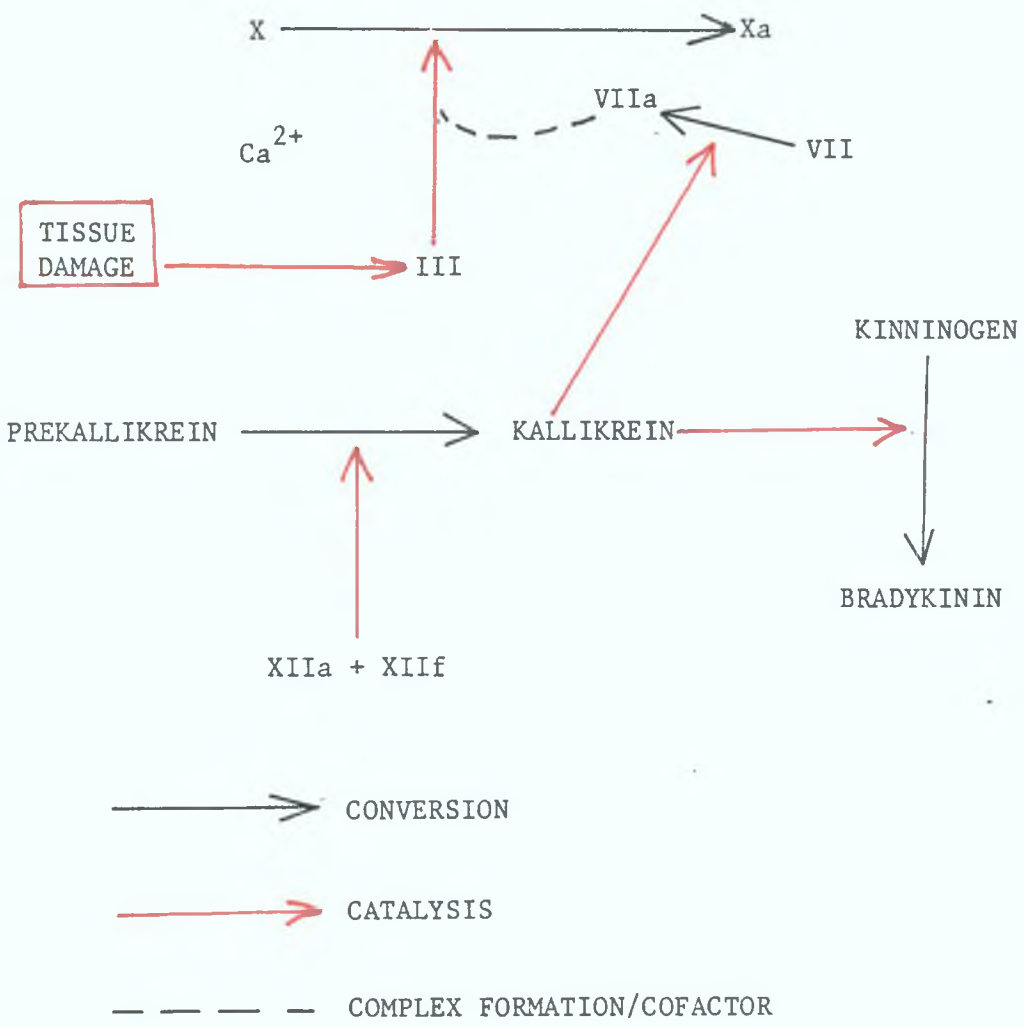


Fig III.3 THE INTRINSIC PATHWAY

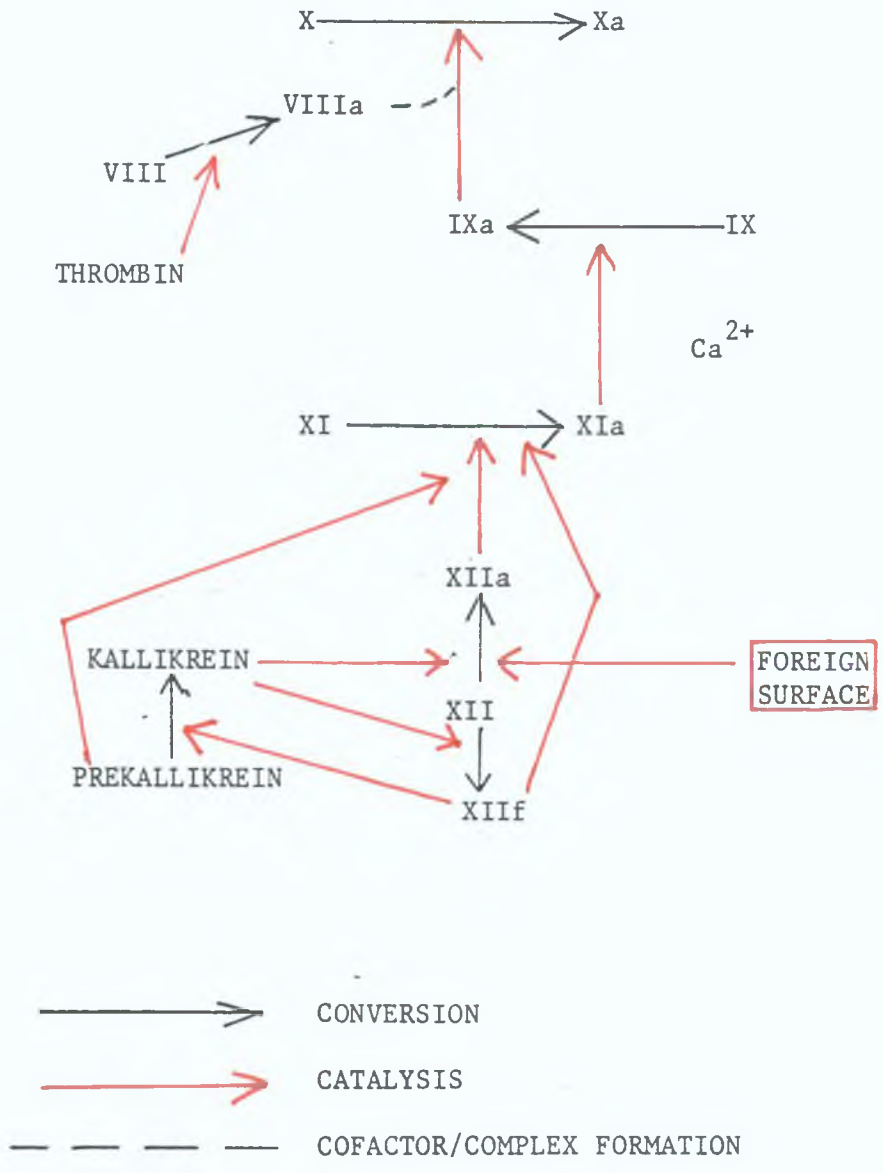


Fig III.4 FIBRINOLYSIS

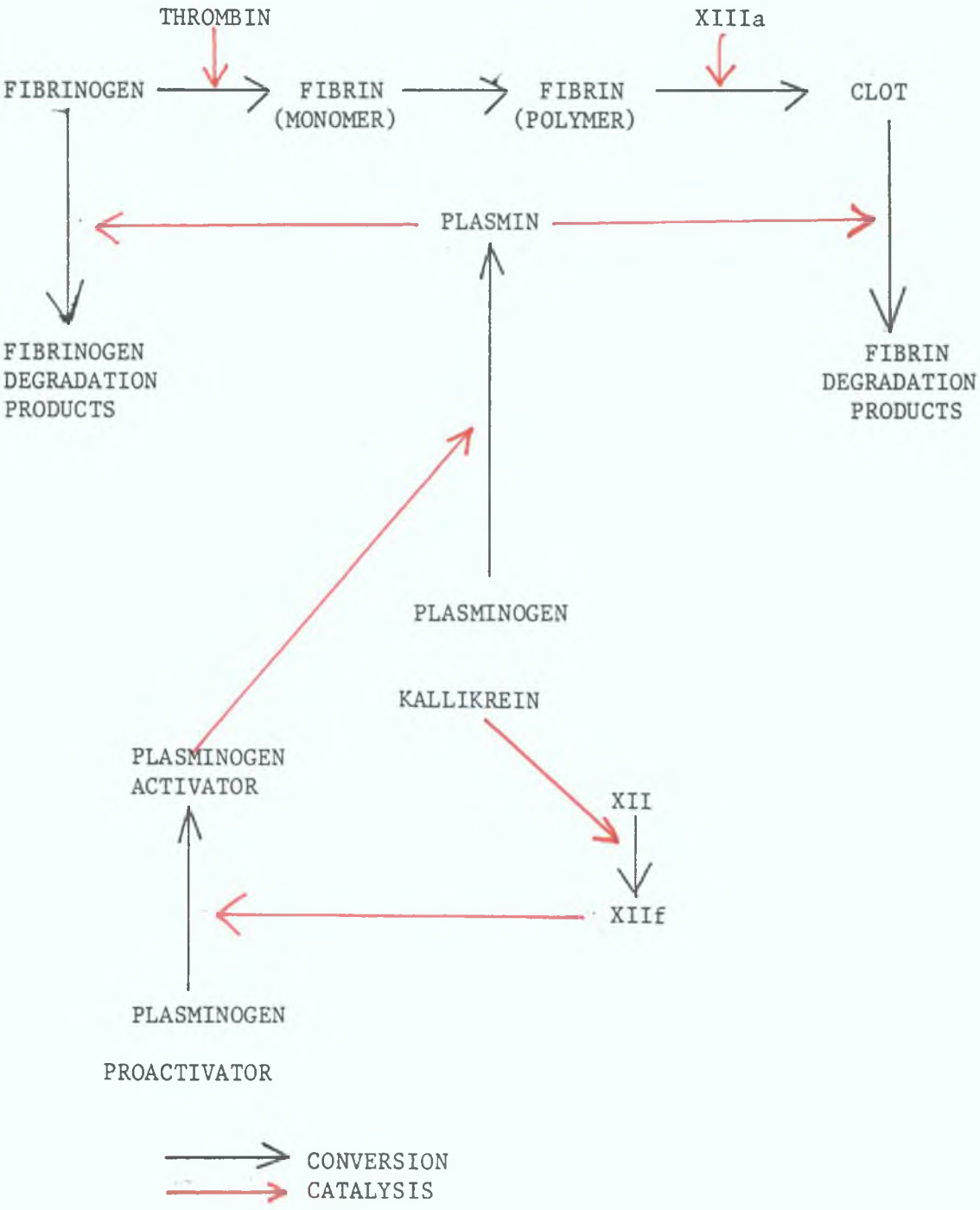


FIG III.5 Showing the structure of the fibrinogen molecule, and the molecular weights of its' chains (Doolittle, 1981a)

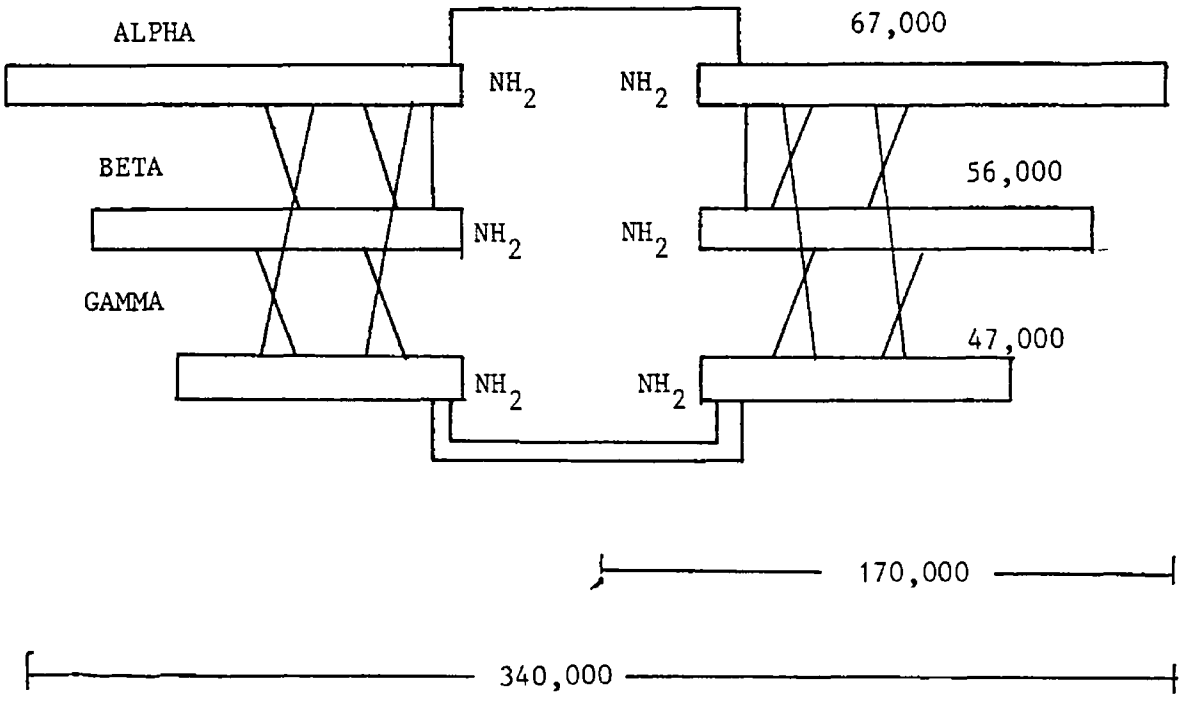


FIG III 6 Showing the structure of fibrinogen and it's plasmin degradation products The figures in parentheses are the molecular weights of the fragments (Doolittle, 1981a)

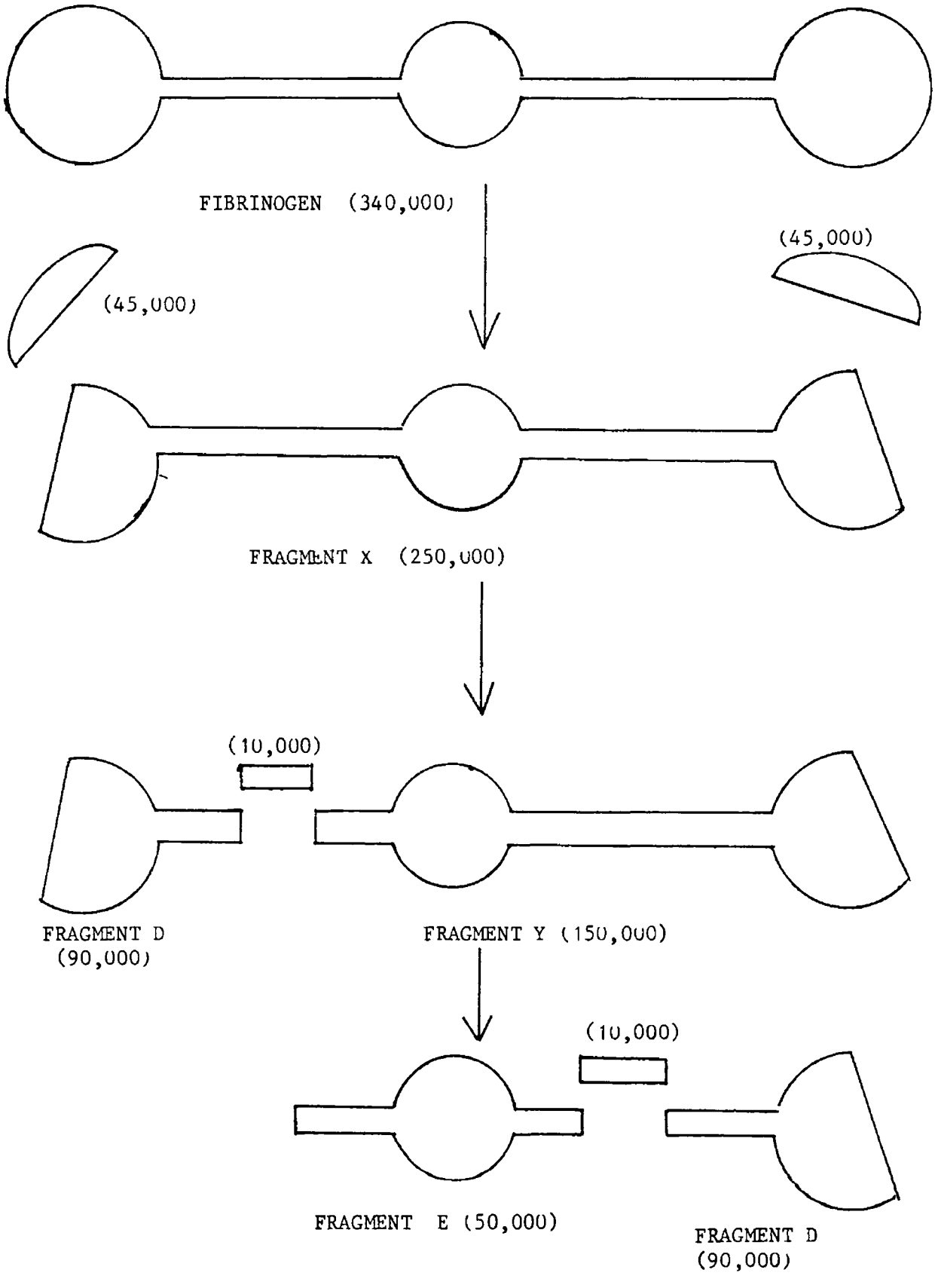
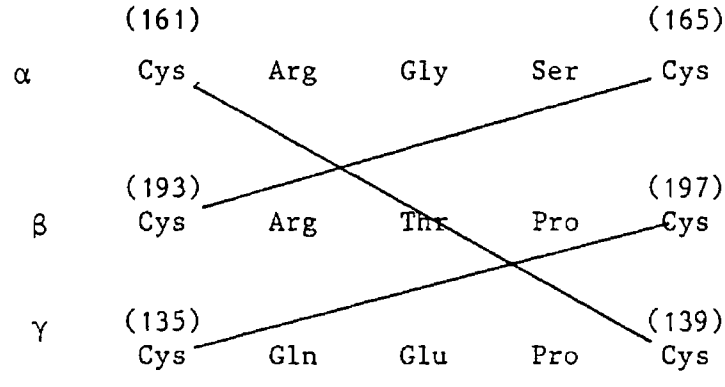


FIG III 7 Showing the structure of the disulphide bonds in the N-terminal disulphide knot (Doolittle, 1981a) The numbers in parentheses represent the position of the amino acids in the fibrinogen chains



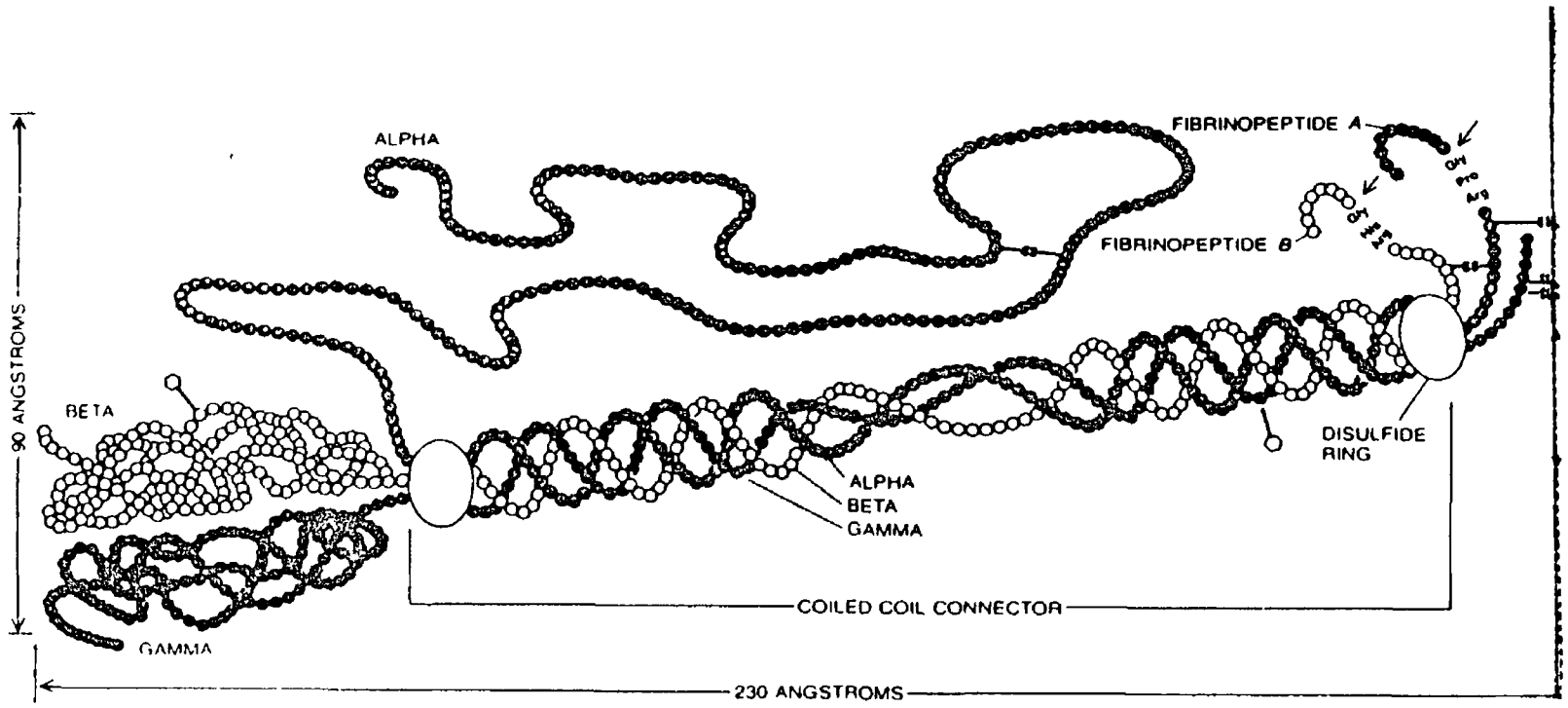
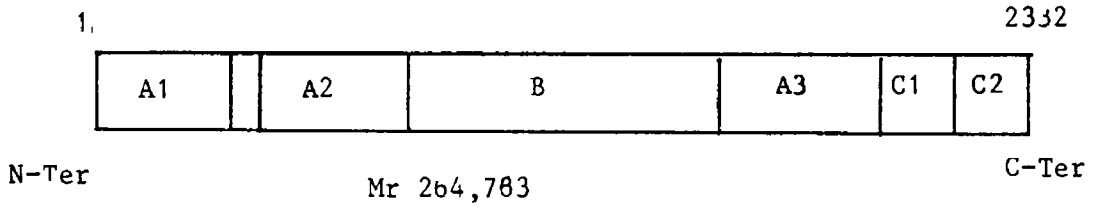
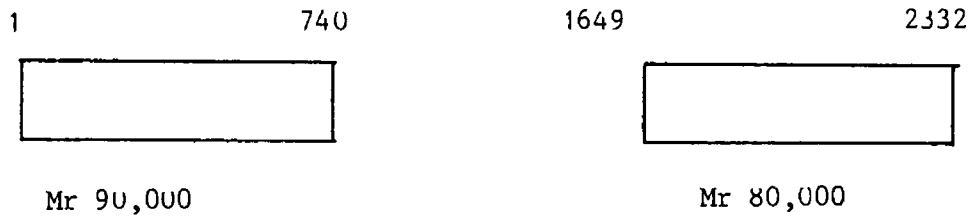


FIG III 8 This figure shows the structure of one half of the fibrinogen molecule The molecule is a dimer, with each half consisting of 1 Aα chain (grey), 1 Bβ chain (white), and 1 γ chain (black) The carbohydrate residue is indicated by a hexagon The thrombin cleavage sites are indicated by arrows The structure of the disulphide rings is given in Fig III 7 (Doolittle, 1981a)

FIG III 9 Showing the homologous regions in the FVIII.C molecule and its' thrombin degradation fragments The figures represent the positions of the amino acids on the molecule Also shown are the molecular weights of the fragments (Vehar et al, 1984)





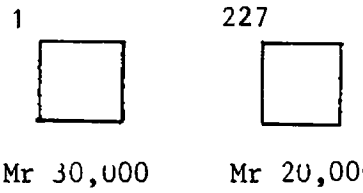


FIG III 10 Showing the homologous regions in the precursor vWF molecule (Girma et al , 1987)

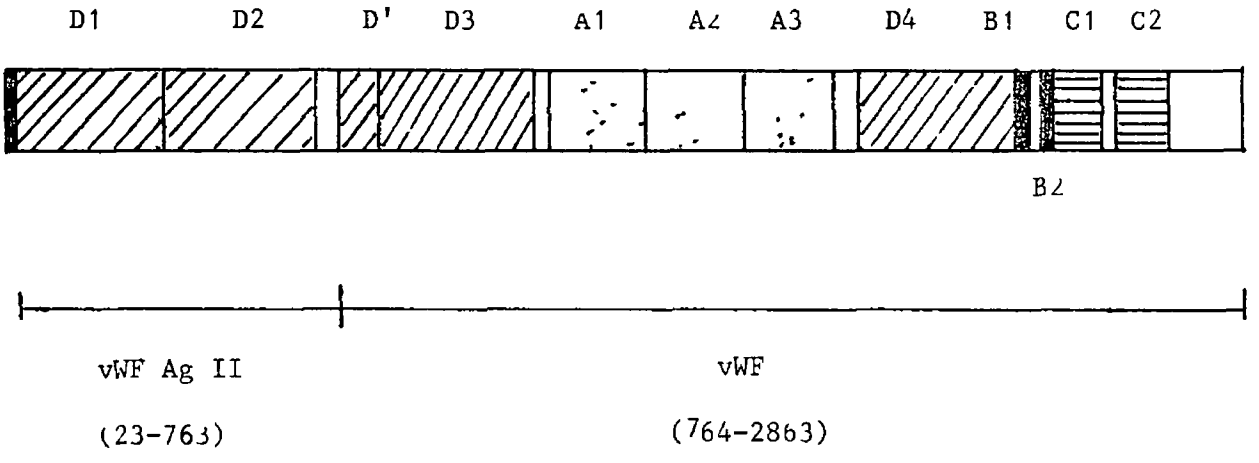
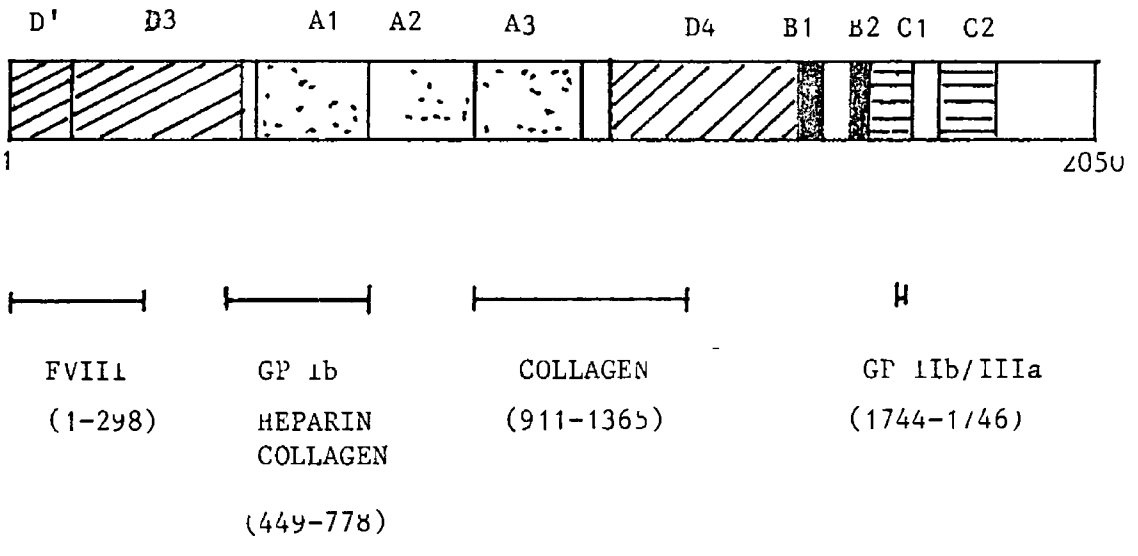


FIG III.11 Showing the homologous regions of the mature vWf molecule and its' functional binding sites (Girma et al, 1987)



III 2 1 PURIFICATION OF FVIII

Ethanol cryoprecipitation of 800 ml of plasma yielded a partially purified FVIII concentrate of 1 77 IU/ml PEG precipitation of this FVIII fraction gave 4 ml of a high purity preparation of 8 IU/ml These FVIII C levels were assayed by the APTT assay This represented only 6% of the FVIII C activity of the starting plasma 65% of the FVIII C activity remained in the plasma A second batch of 450 ml, produced 4 ml of a high purity FVIII preparation of 10 IU/ml This represented 8 8% of the total FVIII, and 21 5% remained in the plasma (see Table III 2)

Fig III 12 shows agarose electrophoresis of the different FVIII fractions As can be seen there is no noticeable difference between normal and FVIII-deficient plasma The cryoethanol precipitate (I) shows a decrease in the number of proteins present especially those of low molecular weight (at the top of the gel) There are two major contaminants present, one of high molecular weight, and the other of low molecular weight The high purity fractions (H1 and H2) have a further decrease in the low molecular weight fractions There is a total absence of the major low molecular weight band, but there is still some of the major high molecular weight band present The PEG 1 precipitate (F) shows only one band of high molecular weight (other than the protein remaining at the sample application point), while the remaining supernatant after PEG precipitation (R) shows only one small molecular weight band The increase in the staining in the high purity FVIII, as compared to the partially purified form, is probably due to the increased protein concentration The high purity fractions are 8 2 and 10 IU/ml while the partially purified form is only 1 77 IU/ml

The presence of FVIII in both the cryoethanol precipitate and the high purity FVIII was confirmed by Laurell rocket immunoassay (see Fig III 13 and III 14) No FVIII was detected in the first PEG precipitate The presence of FVIII in the FVIII deficient plasma is due to vWF The commercial polyclonal antibodies detect the vWF portion of the FVIII complex, which is usually present in haemophilia, instead of the FVIII CAg portion

Fig III 15 shows agarose electrophoresis of both the high purity FVIII and FVIII-deficient plasma One of the lanes containing the high purity FVIII was cut out and used in crossed immuno-electrophoresis This was then electrophoresed in a perpendicular direction into a gel containing anti-vWF and a second gel containing anti-human serum Only one peak is formed This is in the anti-vWF gel, and it corresponds to the main peak in the single dimension electrophresis This indicates that the major protein in the high purity sample is FVIII and that any other contaminating protein is only present in small quantities In the single dimension electrophoresis there is a second protein band which travels in the opposite direction to FVIII This is probably the immunoglobulin fraction, as this normally travels to the cathode This fraction will not form a peak in the anti-human serum gel as it will run towards the cathode, ie away from the gel, in the crossed immunoelectrophoresis

To further purify FVIII chromatography a Sepharose Cl 6B column was used Both PEG precipitated cryoprecipitate, and koate were used The profiles obtained from both of these were similar (see Fig III 16 and III 17) 4 protein peaks were obtained in each case The first, K1, was the void volume peak and corresponded to the major FVIIIR Ag peak The second, K2, probably contained fibrinogen The FVIIIR Ag peak trails off slowly This was probably due to fragments of vWF, which were not part of the FVIII complex, due to proteolysis

III 2 2 PURIFICATION OF FIBRINOGEN FRAGMENTS/CHAINS

After production of the fibrinogen fragments in the presence of EGTA the mixture was applied to a Sepharose column Instead of obtaining the 3 expected peaks, corresponding to the X, Y, and combined D and E fragments, only 2 peaks were obtained These corresponded to the combined X, and Y fractions, and the combined D, and E fractions The reason for the incomplete separation of the X and Y fragments was due to the size of the column Nieuwenhuizen and Gravesen (1981) use a 230cm column, this is compared to a 76 cm column used here The molecular weights of the X and Y fragments are too similar to be separated on a column of 76 cm

After fibrinogen was split into its chains the mixture was applied to a CM-cellulose column The chains were eluted with a linear gradient of sodium acetate Fig III 18 shows the profiles of the eluted protein and of the gradient There was only 1 peak obtained instead of the expected 3 The reason for this is unknown

III 2 3 MONOCLONAL ANTIBODY PRODUCTION

8 fusions were performed using Koate as the antigen There was no growth in 5 of the fusions One of the fusions produced clones, but these subsequently stopped producing antibody All the clones obtained were from 2 fusions There was a total of 1,344 clones from these fusions 306 of these were positive by ELISA using Koate as the antigen A total of 26 of these remained positive and were expanded into flasks 5 of these were cloned out, and further characterised (see Table III 3)

In fusion AHF2, 28% of the clones were positive, and 14% survived the first expansion 6% remained positive, and 2 6% survived and grew in flasks In fusion AHF3 only 19% of the clones were positive, 12% survived the first expansion, 3 25% were positive after the second screening, and 1 4% survived to grow in flasks The percentage of positives from AHF2 that survived was almost twice that of AHF3

III 2 4 ANTIBODY ISOTYPING

Antibodies were typed using a commercial kit Table III 4 shows the results of the isotyping of the antibodies AHF1 1 had very weak binding by both anti-IgA and IgM It was bound strongest by anti-IgG1, IgG2a and IgG2b It was bound very weakly by anti-IgG3 This would indicate that the antibody is an IgG, and is a mixture of G1, G2a and G2b subclasses Anti-IgG2a bound strongly to AHF2 1a, while the binding by anti- IgM, IgG1, IgG2b, and IgG2b were weaker It had virtually no activity with anti-IgG3 and IgGA Thus, it is probably an IgG 2a Meanwhile AHF2 1b, which was cloned from the same clone as AHF2 1b and was bound strongly by IgG1 AHF2 2 was bound strongest by IgG1, but it also showed binding by IgG2a This is probably a mixture of IgG1 (mainly) and IgG2a AHF2 3 was bound strongest by IgG2b and weaker by IgG1 This is also a mixture, mainly IgG2b but also some IgG1 AHF2 4 was bound strongest by IgG1, with some binding by IgG2a Again this is probably a mixture of IgG1 with some IgG2a

also present All antibodies were bound much stronger by the anti-k chain antibody rather than by the anti-λ chain antibodies Table III 5 summarises the isotypes of the antibodies The isotype which gave the highest absorbance was taken as the isotype

III 2 5 PURIFICATION OF ANTIBODY

Antibodies that were grown as ascitic tumours were purified by ammonium sulphate precipitation A solution of 10-15 mg of purified antibody/ml was usually obtained Titres were determined by ELISA against immobilised Koate, and the results are shown in Table III 6 A second batch of AHF1 1 was prepared and its activity is summarised in Table III 7

The titre was defined as the concentration of antibody that produced an absorbance equal to half it's maximum absorbance, in ELISA This would be on the linear portion of the concentration-response curve The maximum dilution was defined as the lowest concentration that gave the maximum absorbance This would be on the plateau of the concentration-response curve For the second batch of AHF1 1 the titre was 100 ng/ml, and the maximum dilution was 1 µg/ml The titre for the first batch of AHF1 1 was 2 µg/ml, for AHF2 1a, 2 µg/ml, and that for AHF2 1b was approximately 200 ng/ml The maximum dilutions were 20µg/ml for all 3 antibodies The activity profile of AHF1 1 was a bell shaped curve At concentrations of 1 mg/ml and above there was no activity of the antibody detectable (see Fig III 19)

III 2 6 STABILITY STUDIES ON ANTIBODIES

Samples of some antibodies were stored at a concentration of 200 µg/ml in 0 1 M Tris, pH 8, at 4°C These were tested for activity during storage These results are summarised in Table III 8

After 6 weeks the binding of all 3 antibodies had dropped, but it was higher in subsequent weeks This was probably due to the ELISA rather than to the antibodies It could have been caused by a decrease in the activity of the batch of enzyme-labelled second antibody used, or due to reduced binding of Koate to the 96 well plates for that assay By week 16 there was a drop in binding by all 3 antibodies This can be seen as a drop in the absorbance at an antibody concentration of 20 µg/ml Thus, after 16 weeks the absorbance for a 20 µg/ml antibody solution for AHF1 1 was reduced

form 0 3 to 0 18, while the value for AHF2 1a was reduced from 0 98 to 0 36, and the value for AHF2 1b was reduced from 1 27 to 0 69 After this initial drop the antibodies remained stable for at least 39 weeks

III 2 7 SCREENING OF ANTIBODIES BY ELISA

Antibodies were screened by a number of different ELISA assays Table III 9 compares the binding of different concentrations of AHF1 1 and AHF2 1b to different fractions of Koate by ELISA AHF1 1 preferentially binds to the void volume fraction obtained following chromatography of Koate on a Sepharose column (K1) It has much weaker binding to the first fraction after the void volume (K2) AHF2 1b bound strongly to both fractions but it showed stronger binding to the K2 fraction

Table III 10 compares the binding of 6 antibodies to different proteins, immobilised on a 96 well plate The binding was determined by ELISA AHF1 1 bound to Koate (K0), and it's K1 fraction It did not bind to BSA and only very weakly to the Koate K2 fraction It also bound to Fibrinogen obtained from Sigma All the other antibodies bound strongly to Koate (K0), fibrinogen and Koate fraction K2 They showed weaker binding to Koate fraction K1, and they showed no binding to BSA

To further characterize the antibodies their ability to bind to vWF was determined by ELISA Normal plasma or Koate were added to plates coated with commercial anti-FVIII and allowed to bind, similar to the ELISA for vWF After washing the plates the were then probed with either labelled anti-vWF antibody (see II 2 3), or else AHF1 1 or AHF2 1b were then added to the wells and then probed with labelled anti-mouse Ig (see section II 8 1) When normal plasma was added to the wells the immobilised anti-vWF antibodies only bound FVIII This coated the plate with pure FVIII only The binding of FVIII was confirmed after probing with the labelled anti-vWF, as the absorbances after probing were off scale Binding of AHF1 1 to the FVIII on the plate was not detected by probing with labelled anti-mouse Ig (see Table III 11) However, AHF2 1b bound to the FVIII if Koate was used to coat the plate, but not if normal plasma was used

III 2 8 SCREENING OF ANTIBODIES BY COAGULATION ASSAY

AHF1 1, AHF2 1a, and AHF2 1b were tested for their effect on the

activated partial thromboplastin time (APTT) Unpurified ascitic fluid containing these antibodies was used The results are summarised in Table III 12

AHF1 1 had only a slight effect on the APTT AHF2 1a, and AHF2 1b both slightly decreased the APTT This was probably due to contaminating calcium ions rather than to any effect of the antibody The commercial anti-FVIII antibody had only a slight effect This was because it is anti-FVIIIR Ag and not anti-FVIII CAg

III 2 9 AFFINITY CHROMATOGRAPHY

Purified AHF1 1 was coupled to a CNBr-activated Sepharose column and this was then used to isolate FVIII Initially 20 ml of normal plasma was applied to a column of about 2 ml, containing approximately 7 mg of antibody The protein eluted with glycine, pH 2 5 was assayed by APTT assay The protein eluted had no procoagulant activity at all However, electrophoresis under both reducing and non-reducing conditions showed one band, with Mr 79,500 and 61,900, respectively The plasma applied to the column could not be assayed for APTT because it contained heparin as an anticoagulant

To try and elute active FVIII from the column a different method was used Koate was applied to the column in 0 25 M $CaCl_2$ This dissociates the FVIII C from the vWF, which should improve binding of FVIII to the column The FVIII was eluted from the column using ethylene glycol-histidine buffer The applied sample, the protein washed off the column (ie did not bind to the immobilised antibody) and the eluted protein, which had bound to the immobilised antibody, were assayed for FVIII activity (see Table III 13) While no active FVIII could be detected in the protein that was eluted from the column with ethylene glycol-histidine, there was a loss of activity in the Koate after passage through the column, compared with the sample applied to the column Since the APTT assay requires calcium free plasma for the assay there is a problem comparing samples of different dilutions, since their calcium levels are different Thus, the only valid comparison is between the applied Koate and the column washings when both are diluted at 1/50 For the 1/20 and 1/10 dilution of the washing there is no FVIII concentration calculated This is because the high calcium concentration would

interfere with the results and any readings from a standard curve would be inacccurate

III 2 10 ELECTROPHORESIS

Koate run on a 7 5% SDS-PAGE system under non-reducing conditions produced 2 bands when stained with Coomassie blue (see Fig III 20) The first band was at the very top of the gel and consists of fibrinogen and vWF The second band had an apparent approximate molecular weight of 60-70,000 The precise molecular weight of this second band was difficult to determine for two reasons Firstly, it produced a "V" shaped band in the gel making it difficult to compare it with the markers Secondly, the markers stained very faintly with Coomassie blue which also made any comparisons difficult When Koate was run under reducing conditions three bands were detected when stained with Coomassie blue (see Fig III 20) One of these had a molecular weight of approximately 66,000 and the other two had molecular weights of around 50,000 These probably correspond to the fibrinogen chains rather than to the FVIII, since FVIII under reducing conditions produces a very faint, diffuse band which is difficult to detect even with silver staining (see Fig III 21)

A sample of protein, prepared by affinity chromatography of normal plasma on a Sepharose column with immobilised AHF1 1 antibody, was electrophoresed on 10% (w/v) polyacrylamide gel Under non-reducing conditions only one band with a molecular weight of 60,000 was detected when silver stained Under reducing conditions there was also only one band detected, but with a molecular weight of 75,800 (see Fig III 21) Under reducing conditions the band was much fainter than under non-reducing conditions, even though the same amount of protein was added to each lane

Fibrinogen run on a 7 5% (w/v) polyacrylamide gel under non-reducing conditions, and stained with Coomassie blue stained produced a single large molecular weight band at the top of the gel Under the same conditions the first plasmin digest pool produces two bands corresponding to fragment X and fragment D The latter appears as a dimer The second plasmin digest pool also produces two bands one corresponding to fragment D, the other, a very faint band corresponds to fragment E (see Fig III 22)

Fig III 23 shows the fibrinogen chains, and fragment E when stained with a silver stain They produce a negative image, which can only be seen if the rest of the gel is allowed to overstain

III 2 11 DOT BLOTTING

Dot blotting was used to screen the antibodies against different FVIII preparations (see Fig III 24) AHF1 1 showed only very weak binding to FVIII deficient plasma, normal plasma and serum It had slightly higher binding to Koate The reason for this lack of binding was probably due to the low level of FVIII CAg present in the dots There was about 100 pg of FVIII present in the normal plasma applied to the nitrocellulose, while the Koate would have about 500 pg This is at the limit of sensitivity for dot blotting

AHF2 1b, AHF2 2, AHF2 3 and AHF2 4 all bound to normal plasma, FVIII deficient plasma and to Koate None of them bound to normal serum This would indicate that none of them bound to FVIII CAg, and that they bound to a protein that was consumed during coagulation - probably fibrinogen These results are summarised in Table III 14

III 2 12 WESTERN BLOTTING

Fig III 25 compares the binding of AHF2 2, AHF2 3 and AHF2 4 to different fibrinogen preparations The lane with reduced fibrinogen (2) shows only 1 band with an apparent molecular weight of 55,000 when stained with AHF2 3 and AHF3 4 This band corresponds to the chain of fibrinogen AHF2 2 bound to none of the chains

All 3 antibodies bound to 3 bands in the lane containing the pooled D and E fragments (1) There was a major band at Mr 109,000 and 2 smaller bands at Mr 98,700 and Mr 94,600 These probably constitute the heterogenous population of D fragments AHF2 2 also bound to a band at Mr 72,000 AHF2 3 also bound to this band, but the binding was very weak AHF2 3 also bound to a band with Mr 48,000 This corresponded to the E fragment of fibrinogen Thus, AHF2 3 bound to both D and E fragments, AHF2 2 bound to the D fragment and a Mr 72,000 band, and AHF 2 4 only bound to the D fragment

Fig III 26 shows the binding of AHF2 1 to the fibrinogen preparations The lane containing the reduced fibrinogen molecule had 2 bands which corresponded to molecular weights 64,400 and 60,500 These bands correspond to the heterogenous population of Aα chains The lane containing the plasmin degradation fragments of fibrinogen show 2 bands with molecular weights 61,700 and and 48,100 This latter band corresponds to the E fragment

Fig III 27 shows a Western blot of fibrinogen, and its degradation products on 7%(w/v) SDS-PAGE AHF2 1b bound to fibrinogen, and the X, Y, D and E fragments AHF2 3 bound to fibrinogen, and the X, Y and D fragments

Fig III 28 shows the binding of AHF1 1 to different preparations of FVIII Lane 1 of the blot on the left shows binding of AHF1 1 to two different bands in Koate under non-reducing conditions These bands have apparent molecular weights of 61,900 and 103,300 Under reducing conditions there is only 1 band in the Koate preparation with Mr 79,500 (lane 2) Lane 3 and 4 contain the protein eluted with glycine, pH 2 5, from AHF1 1 immobilised on CNBr-activated Sepharose after the application of normal plasma to the column Lane 3, which was run under reducing conditions shows a very faint band at Mr 79,500 Lane 4, which was run under non-reducing conditions shows 1 band with Mr 61,900

Fig III 28 also shows the binding of AHF1 1 to different preparations of FVIII In lane 1 of the middle blot there are 2 bands of Mr 88,000 and Mr 79,900 when Koate, run on a reducing gel, was used In lane 2, containing Koate run under non-reducing conditions, there are 3 bands with Mr 75,000, Mr 112,000, and Mr 121,900 The blot on the right shows absence of binding of the second antibody to Koate run under either non-reducing or reducing conditions and is a negative control These results are summarised in Table III 15

TABLE III 2

This table shows the concentration of FVIII in the different fractions during purification of FVIII

FRACTION	% OF STARTING ACTIVITY		FVIII CONC (IU/ml)	
	RUN 1	RUN 2*	RUN 1	RUN 2 *
starting plasma	100	100	0 62	1 0
cryoethanol ppt	35 6	ND	1 77	ND
remaining plasma	64 5	21	0 4	0 21
PEG ppt 1	2	1 24	0 62	0 23
high purity	6	8 8	8 2	10
PEG supernatant	0 2	ND	0 16	ND

Run 1 800 ml plasma

Run 2 450 ml plasma

* During run 2 the starting plasma's FVIII level was not determined For calculation purposes it was assumed to be normal plasma ie 1 IU/ml

Cryoethanol ppt The fraction after cryoethanol precipitation

Remaining plasma plasma after cryoethanol precipitation

PEG ppt 1 The pellet from the first PEG precipitation

High purity The pellet from the second PEG precipitation

PEG supernatant the supernatant after the second PEG precipation

TABLE III 3

This table shows the number of clones produced in each fusion, the number of clones that were positive to Koate by ELISA, and their survival during expansion

FUSION NAME	NO OF CLONES	NO +ive CLONES	EXP 1	NO +ive CLONES	EXP 2	EXP 3	CONT
AHF2	576	163	80	33	17	15	24
AHF3	768	143	90	25	11	11	2

EXP 1 The number of positive clones that grew after expansion into a 24 well plate

EXP 2 The number of positive clones that grew after expansion into a 6 well plate (or into 3 wells of a 24 well plate)

EXP 3 The number of positive clones that grew after expansion into a flask

CONT The number of positive clones that were contaminated

TABLE III 4

Shows the binding of antiisotype antibodies to the monoclonal antibodies A negative control which contained PBS-Tween and no monoclonal antibody was used for each isotype These negative controls were used as blanks

ISOTYPE	AHF1 1	AHF2 1a	AHF2 1b	AHF2 2	AHF2 3	AHF2 4
IgA	0 07	0 09	0 10	0 07	0 06	0 06
IgM	0 08	0 15	0 18	0 05	0 03	0 07
IgG1	0 16	0 34	0 53	0 29	0 28	0 24
IgG2a	0 17	0 58	0 17	0 21	0 17	0 15
IgG2b	0 14	0 15	0 17	0 09	0 35	0 10
IgG3	0 01	0 01	0 04	0 01	0 02	0 02
k	0 19	0 52	0 49	0 29	0 33	0 27
λ	0 08	0 10	0 11	0 03	0 03	0 03

TABLE III 5

This Table shows the isotypes of cloned antibodies

ANTIBODY	ISOTYPE	LIGHT CHAIN
AHF1 1	IgG1/IgG2a	k
AHF2 1a	IgG2a	k
AHF2 1b	IgG1	k
AHF2 2	IgG1	k
AHF2 3	IgG2b	k
AHF2 4	IgG1	k

TABLE III 6

This table shows the absorbance for 100 µl of purified ascites at different concentrations in an ELISA assay using plates coated with Koate A negative control using no first antibody was used as the blank

	ANTIBODY		
CONCENTRATION	AHF1 1	AHF2 1a	AHF2 1b
20 µg/ml	0 30	0 98	1 27
2 µg/ml	0 10	0 37	1 09
200 ng/ml	0 04	0 09	0 44
20 ng/ml	0	0	0 09
2 ng/ml	0	0	0

TABLE III 7

Shows the concentration and binding (measured by absorbance in an ELISA on plates coated with koate) of AHF1 1 A negative control which had no first antibody was used as the blank

CONCENTRATION	ABSORBANCE
10 mg/ml	0 014
1 mg/ml	0 067
100 µg/ml	0 325
10 µg/ml	0 455
1 µg/ml	0 424
100 ng/ml	0 275

TABLE III 8

This table shows the binding (absorbance by ELISA on plates coated with Koate) of 3 antibody preparations at intervals during storage The antibodies were stored at a concentration of 200 μg/ml at 4°C in Tris, pH 8 0 Negative controls (wells containing second antibody only) were used as blanks in the assay

TIME (weeks)	CONC (μg/ml)	ANTIBODY		
		AHF1 1	AHF2 1a	AHF2 1b
0	200	ND	ND	ND
	20	0 30	0 98	1 27
	2	0 10	0 37	1 09
6	200	0 15	0 64	0 74
	20	0 09	0 31	0 76
	2	0 03	0 19	0 84
16	200	0 30	0 77	0 49
	20	0 18	0 36	0 69
	2	ND	0 11	0 55
26	200	0 34	0 91	0 51
	20	0 22	0 84	0 80
	2	ND	0 53	0 65
39	200	0 31	0 77	0 72
	20	0 18	0 55	0 90
	2	ND	0 35	0 75

ND= not determined

TABLE III 9

Shows the binding of 2 antibodies (unpurified ascites) in ELISA against the 2 Koate fractions The Koate fractions were prepared by chromatography on Sepharose The K1 fraction is the void volume fraction, and the K2 fraction is the first fraction after the void volume A negative control containing no first antibody was used as the blank

DILUTION	AHF1 1		AHF2 1b	
($X10^3$)	K1	K2	K1	K2
0 1	0 16	0 06	0 42	0 65
1	0 15	0 04	0 37	0 74
2	0 13	0 04	0 39	0 69
4	0 07	0 02	0 35	0 60
8	0 04	0 01	0 28	0 50
16	0 02	0 01	0 19	0 32
32	0 01	0 0	0 13	0 21
64	0 01	0 0	0 07	0 14
128	0 0	0 0	0 03	0 07
256	0 0	0 0	0 02	0 05

TABLE III 10

This table shows the binding (measured as absorbance) of 6 antibodies to different proteins by ELISA K0 is Koate, K1 is the void volume fraction eluted after Koate was applied to a Sepharose column, and K2 is the first fraction following K1 during chromatography Fib is commercially purified fibrinogen, and BSA is commercially purified Bovine serum albumin A negative control, containing no first antibody was used as the blank

	ANTIGEN				
ANTIBODY	K0	K1	K2	FIB	BSA
AHF1 1	0 50	0 20	0 09	0 14	0 01
AHF2 1a	0 55	0 24	0 24	0 29	0 01
AHF2 1b	0 90	0 37	0 70	1 03	0 01
AHF2 2a	0 95	0 39	0 71	1 04	0 01
AHF2 3a	0 87	0 22	0 61	0 87	0 00
AHF2 4a	0 70	0 17	0 47	0 74	0 01

TABLE III 11

Shows the binding of antibodies to FVIIIR Ag isolated from different preparations using plates coated with anti-FVIIIR Ag Binding was determined using ELISA Peroxidase-labelled anti-FVIII antisera in ELISA gave readings that were too high to read for both the normal plasma and Koate A negative control using no first antibody was used as the blank

SAMPLE	AHF1 1	AHF2 1b
NP	0 02	0 06
NP (1/2)	0 02	0 06
KO	0 05	0 27
KO (1/2)	0 02	0 09
DP	0 02	0 04
S	0 0	0 04

NP= Normal plasma DP= FVIII deficient plasma

S= Normal human Serum KO= Koate

Figures in parentheses are the dilution factors

TABLE III 12

This table shows the effect of antibodies on the activated partial thromboplastin time (APTT), expressed as the change in the coagulation time Neat ascitic fluid was mixed 1 1 with normal plasma diluted 1 in 10, and incubated for 3 hr at 37°C prior to the assay A control containing PBS only was used in each case

ANTIBODY	SIGMA	VERIFY
AHF1 1	+ 5 5	+ 4 5
AHF1 1 *	+ 3 0	ND
AHF2 1a	- 3 5	ND
AHF2 1b	- 5 5	ND
COMMERCIAL	+ 1 5	ND

* Purified antibody at a concentration of 0 1 mg/ml was mixed with normal plasma in a ratio of 1 4, and incubated at 37°C for 4 hr prior to the assay Samples were then diluted 1 in 20 for the assay

SIGMA Normal plasma obtained from Sigma
VERIFY Normal plasma obtained from Warner Lambert
COMMERCIAL Anti-FVIII antibody obtained from Serotec
ND Not determined

TABLE III 13

This table shows the coagulation time in an activatd partial thromboplastin time (APTT) assay for the applied sample, the protein washed off the column and the eluted protein The column was CNBr-activated Sepharose (2 ml volume) containing approximately 7 mg of coupled antibody (AHF1 1)

SAMPLE	DILUTION FACTOR	COAGULATION TIME (SECS)	FVIII CONC (IU/ml)
Koate	50	75	0 84
Washings	50	132 5	0 022
Washings	20	58	ND
Washings	10	10	ND
Eluate	1	150	< 0 01
Def plasma	1	150	< 0 01

Koate the applied Koate sample in 0 25 M $CaCl_2$

Washings the protein that did not bind to the column

Eluate the protein eluted from the column with ethylene glycol-histidine buffer This protein bound to the imobilised antibody

Def Plasma FVIII-deficient plasma

ND Not determined

TABLE III 14

This table shows the binding of 5 antibodies to Koate, plasma, FVIII deficient plasma and normal serum by dot blotting Binding is rated as strong (+++), medium (++), weak (+), very weak/uncertain (+/-), and no binding (0) as compared to a control which had no first antibody

ANTIBODY	KOATE	NORMAL PLASMA	DEF PLASMA	SERUM
AHF1 1	+	+	+/-	+/-
AHF2 1b	+++	+++	+++	0
AHF2 2	+++	+++	+++	0
AHF2 3	++	++	++	0
AHF2 4	++	++	++	0

TABLE III 15

This table shows the molecular weights of bands on western blots with 5 different antibodies 4 different preparations were used fibrinogen D+E fragments, fibrinogen chains, Koate reduced, and Koate unreduced All samples were run on SDS-PAGE (10%) and blotted onto nitrocellulose The blots were probed with alkaline phosphatase-labelled anti-mouse antibodies

	MOLECULAR WEIGHT OF BANDS			
	FIBRINOGEN		KOATE	
ANTIBODY	D+E	CHAINS	REDUCED	UNRED
AHF1 1	ND	ND	88,000 79,000	121,000 112,000 75,000
AHF2 1	61,700 48,100	64,400 60,500	ND	ND
AHF2 2	109,000 98,700 94,600 72,000	------	ND	ND
AHF2 3	109,000 98,700 94,600 72,000 48,000	55,000	ND	ND
AHF2 4	109,000 98,700 94,600	55,000	ND	ND

TABLE III 16

This table summarises the binding of 5 monoclonal antibodies The table shows the antigen as well as the chain, and the amino acid residues they may bind to

ANTIBODY	ANTIGEN	CHAIN	RESIDUE NUMBERS	SEQUENCE LENGTH
AHF1 1	FVIII C	light	1690-2332	642
AHF2 1	fibrinogen	Aα	1-78	78
AHF2 2	Fibrinogen	frag D	---	---
AHF2 3	Fibrinogen	Bβ	54-122	68
AHF2 4	Fibrinogen	Bβ	134-461	327

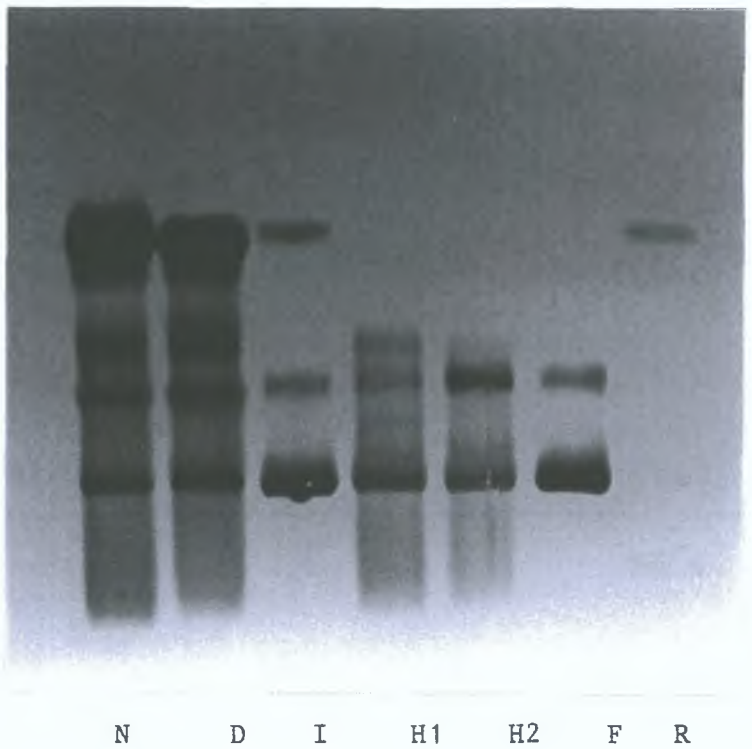


FIG III.12

Shows samples after electrophoresis in a non-denaturing 1% (w/v) agarose gel.

N: Normal plasma D: FVIII-deficient plasma

I: Partially purified FVIII (cryoethanol precipitate)

H1: Highly purified FVIII (8.2 IU/ml)

H2: Highly purified FVIII (10 IU/ml)

F: First PEG precipitate

R: remainder after PEG precipitation steps

FIG III.13

Shows sample analysed by Laurell rocket electrophoresis using anti-vWF antisera in the gel.

Lane 1+2: Cryoethanol precipitate dissolved in Tris (1/4 dilution)

Lane 3: High purity FVIII after PEG precipitation (1/20)

Lane 4: High purity FVIII after PEG precipitation (1/10)

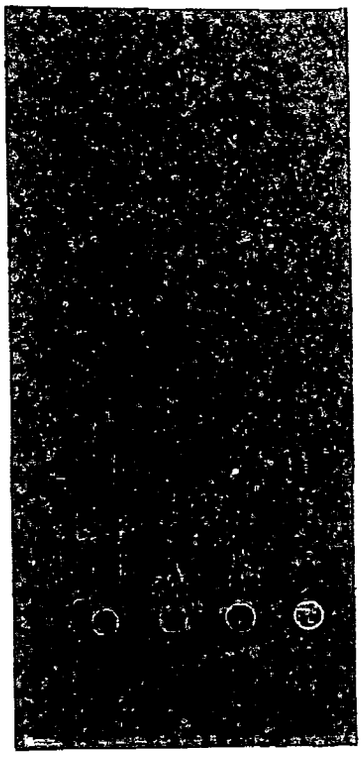


FIG 14

Shows samples analysed by Laurell rocket electrophoresis on a gel containing anti-vWF antisera

Lane 1 High purity FVIII, undluted
Lane 2 High purity FVIII, 1/10 dilution
Lane 3 High purity FVIII, 1/5
Lane 4 First PEG precipitate

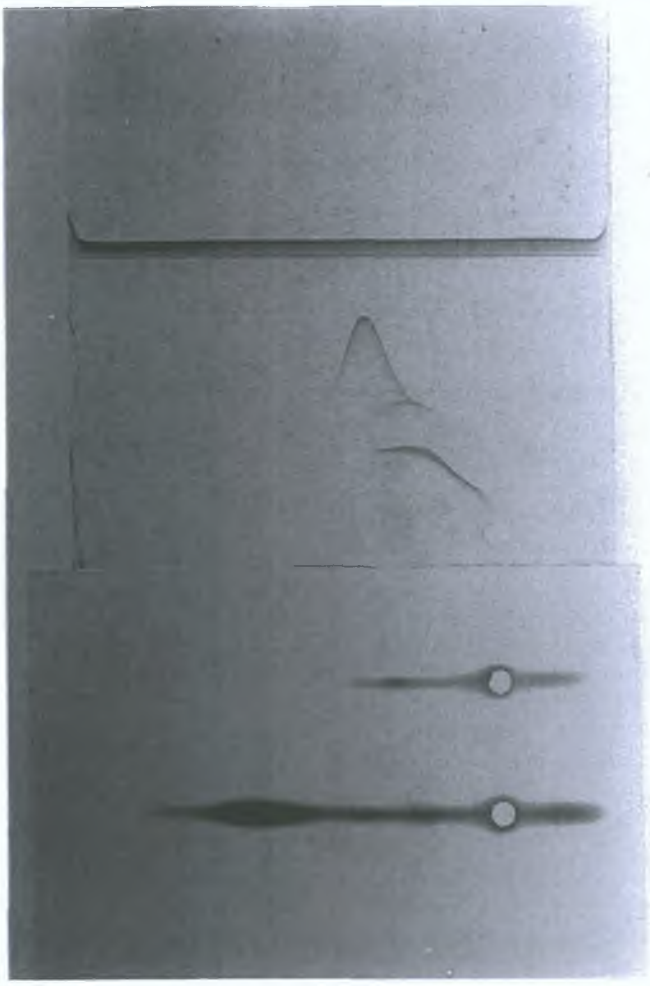


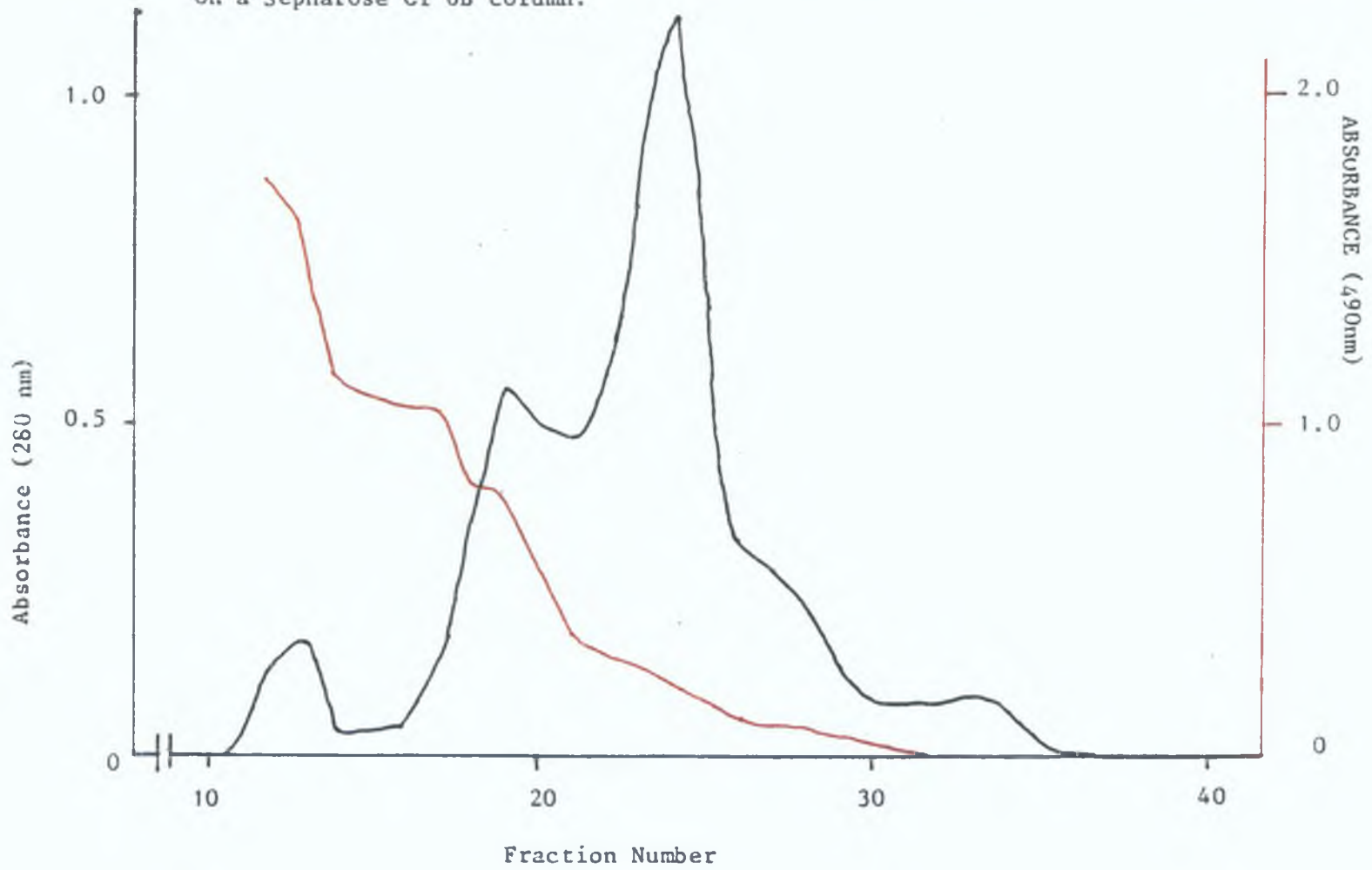
FIG III.15

Shows sample analysed by crossed immunoelectrophoresis. The first dimension was on a non-denaturing 1% (w/v) agarose gel. The second dimension was into a gel containing anti-vWF antiserum in the first layer, and anti-human-serum anti-sera in the second layer.

D: FVIII-deficient plasma

H: High purity FVIII

FIG III.16 Showing the absorbances at 280nm and at 490nm in a FVIIIR ELISA for the different fractions after the elution of 1 bottle of highly purified cryoprecipitate on a Sepharose Cl 6B column.



2.0
1.0
Absorbance (280nm and 490nm)
0
10
20
Fraction Number

FIG III.17 Showing the absorbance at 280nm and the absorbance at 490nm in a FVIIIR ELISA for the different fractions after elution of 1 bottle of Koate from a Sepharose Cl 6B column.

Absorbance at 280 nm

Absorbance at 490 nm

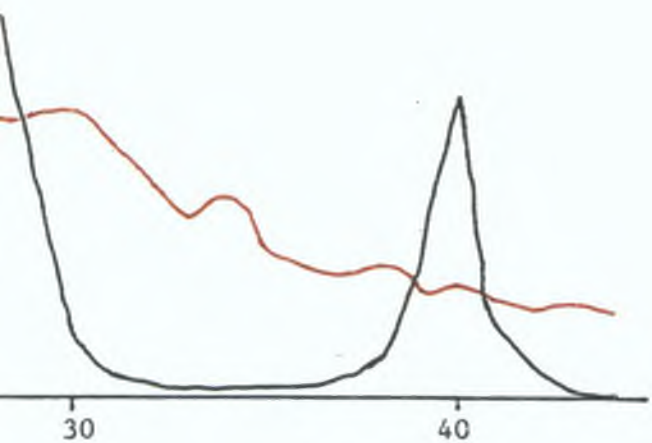
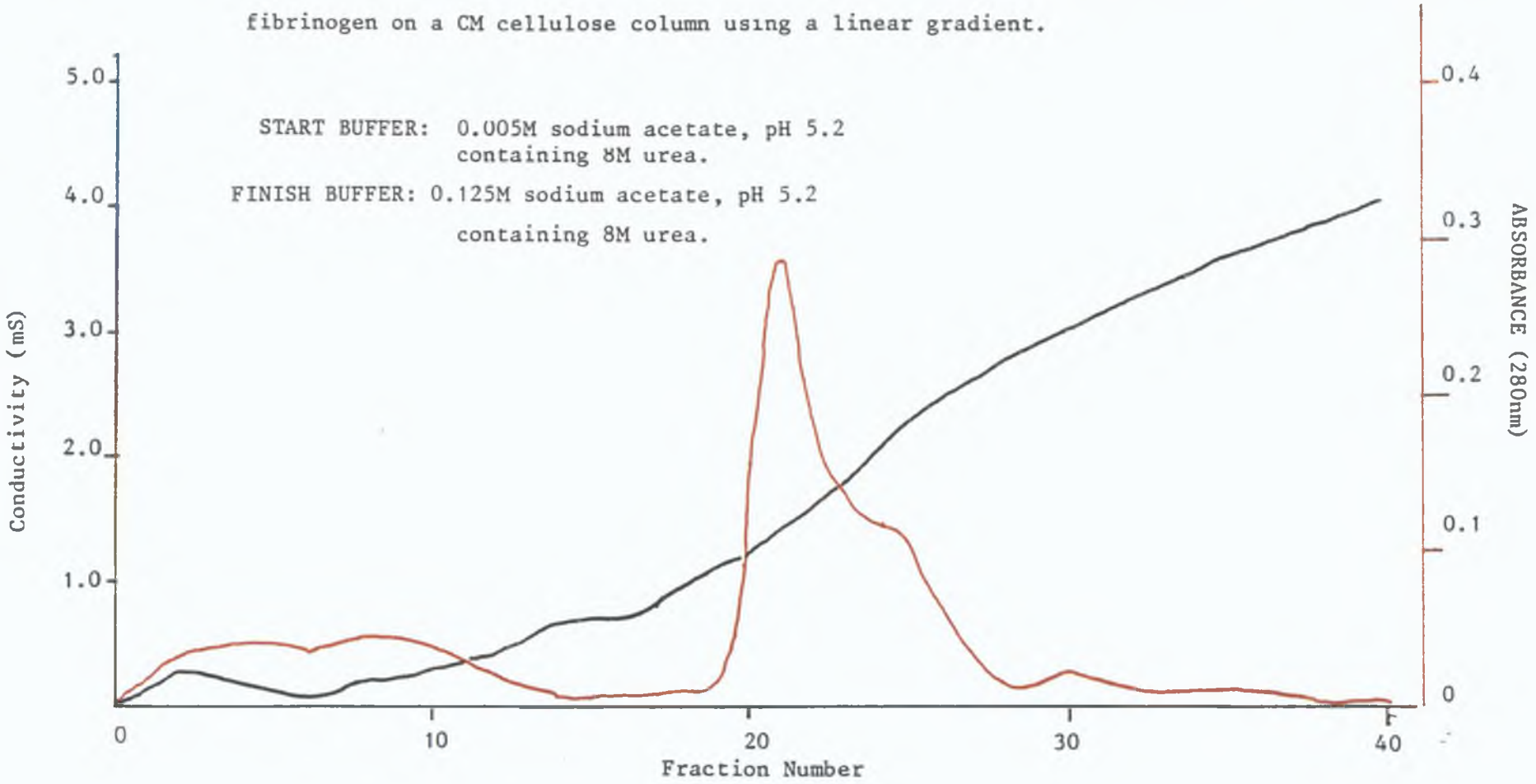


FIG III.18 Showing the absorbance at 280nm of the different fractions of carboxymethylated fibrinogen on a CM cellulose column using a linear gradient.



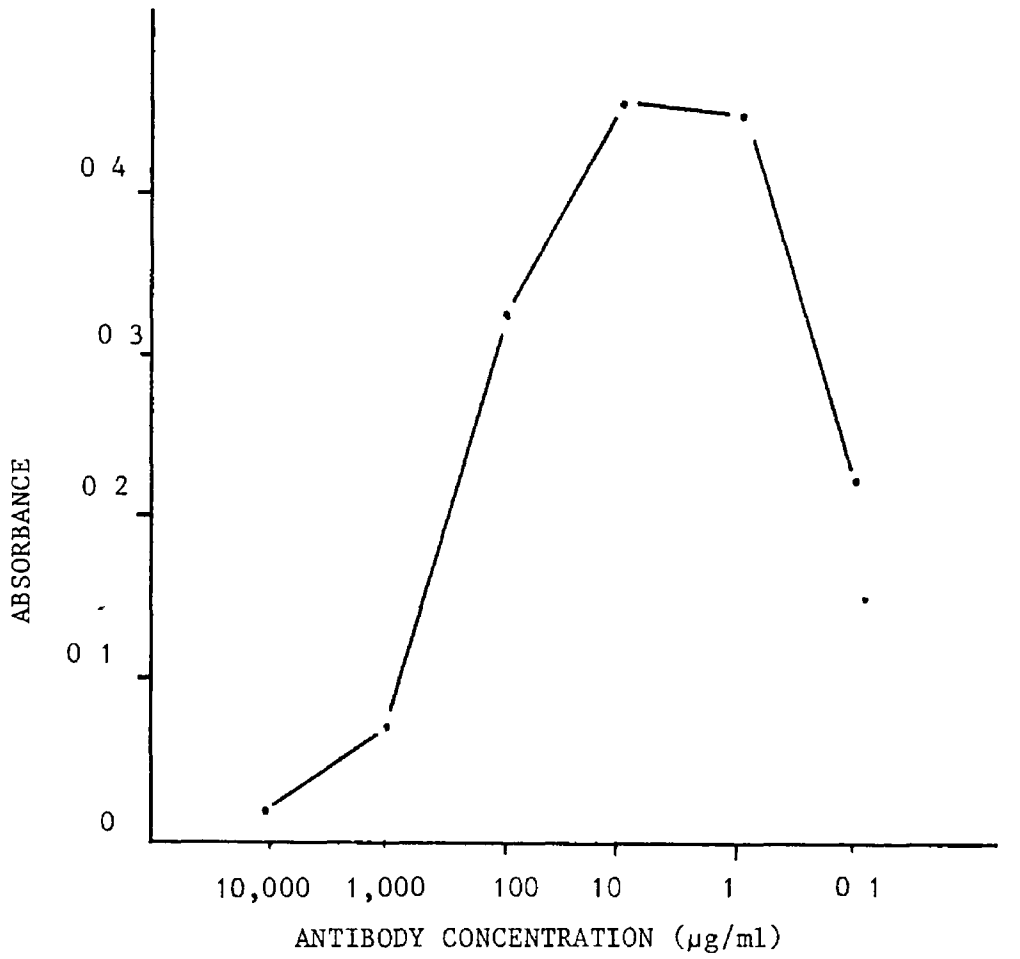


FIG III 19 This figure shows the absorbance values for different concentrations of AHF1 1 The values were obtained from an ELISA assay using plates coated with Koate at 405nm

FIG III.20

This figure shows the results of samples run on a 7.5% (w/v) SDS-polyacrylamide gel, and stained with Coomassie blue.

1: Koate under non-reducing conditions

2: Koate under reducing conditions

3: Molecular weight markers (too faint to see)

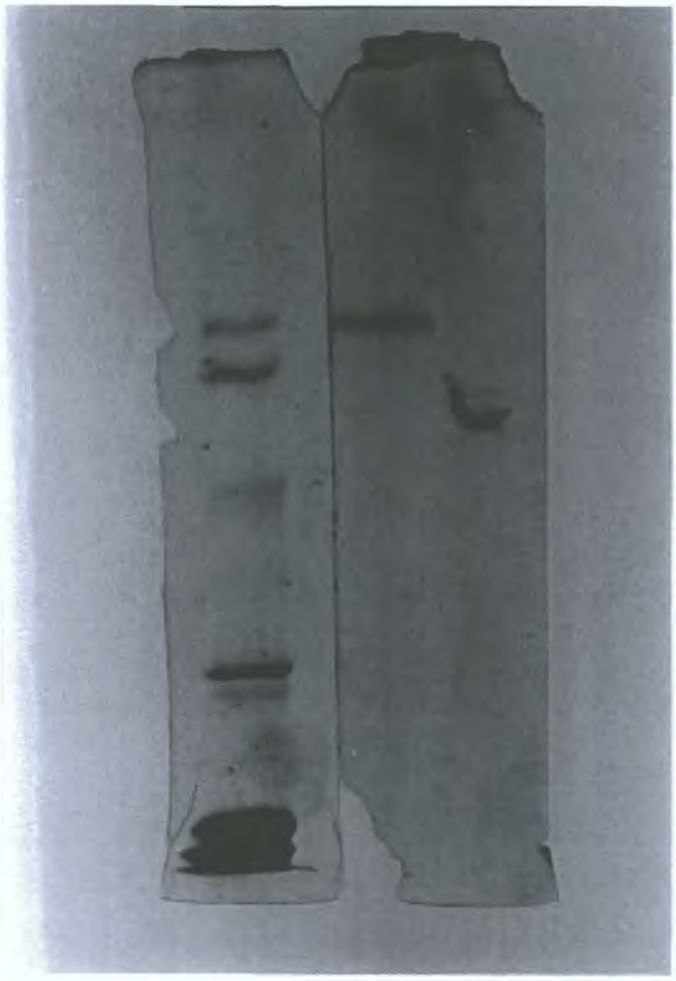


FIG III.21

Thios figure shows the results of electrophoresis on a 10% (w/v) SDS-Polyacrylamide gel, stained with a silver stain.

1: Molecular weight markers (the figures are the molecular weights in thousands)

2: Koate under reducing conditions

3: Koate under non-reducing conditions

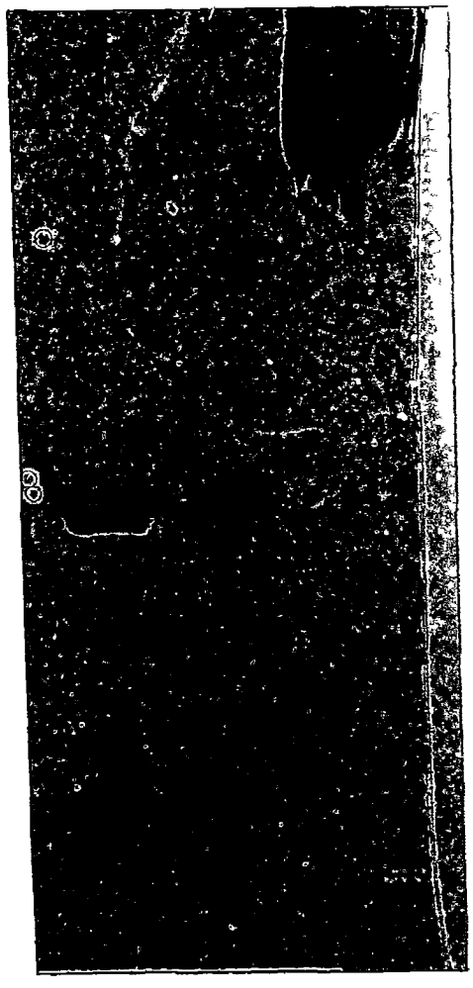


FIG III 22

This figure shows the results of samples electrophresed on a 7 5% (w/v) SDS-Polyacrylamide gel under non-reducing conditions, stained with Coomassie blue

1 D+E pool from plasmin digest of fibrinogen
2 X+Y pool from plasmin digest of fibrinogen
3 Fibrinogen

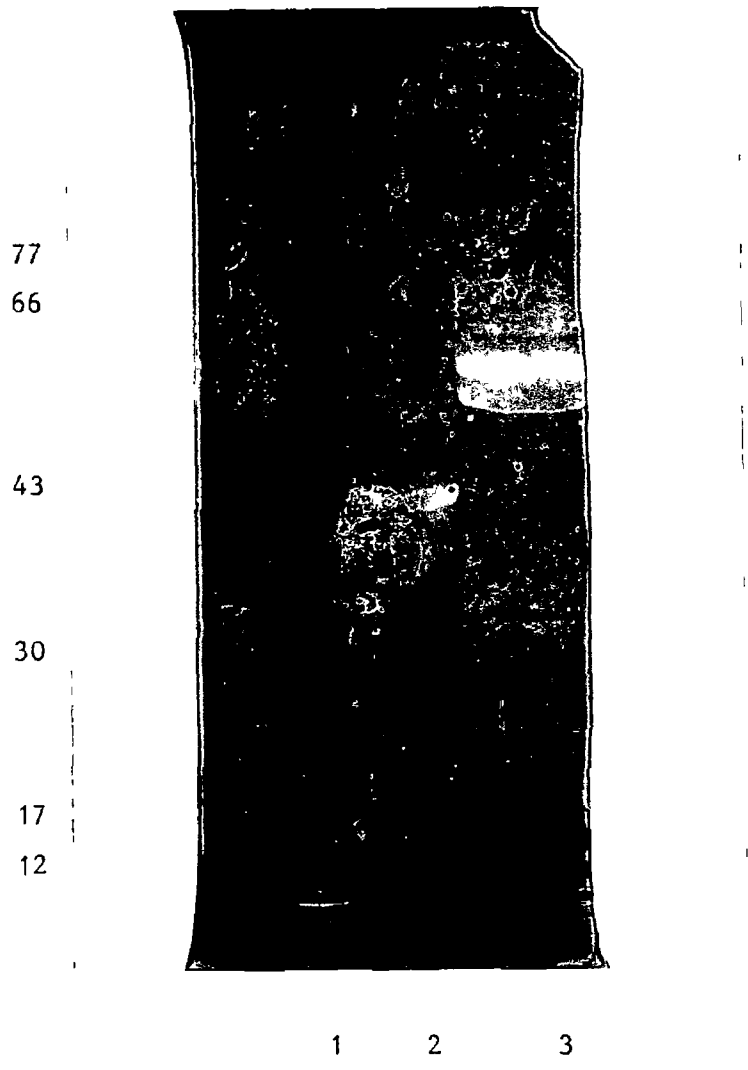


FIG III 23

This figure shows the results of electrophoresis on a 10% (w/v) SDS-Polyacrylamide gel, stained with a silver stain

1 Molecular weight markers (figures are the molecular weights in '000 's)
2 D+E pool of plasmin digest of fibrinogen
3 Fibrinogen under reducing conditions

All of the fibrinogen fragments, and chains produce a negative image with silver staining, ie they appear as clear bands, and can only be seen if the background is dark

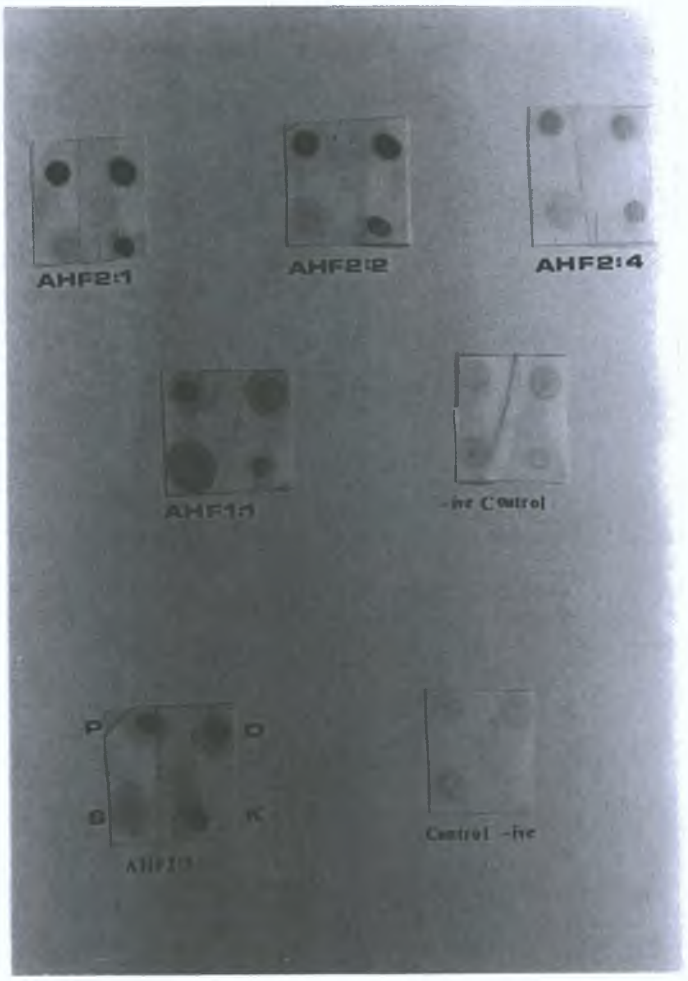


FIG III.24

This figure shows dot blots probed with different antibodies. The were then probed with an anti-mouse IgG labelled with alkaline phosphatase. The arrangement of the dots is the same on all the blots. The arrangement is shown for AHF2:3.

P: normal plasma
D: FVIII-deficient plasma
S: Normal serum
K: Koate
-ive: Control with no first antibody.

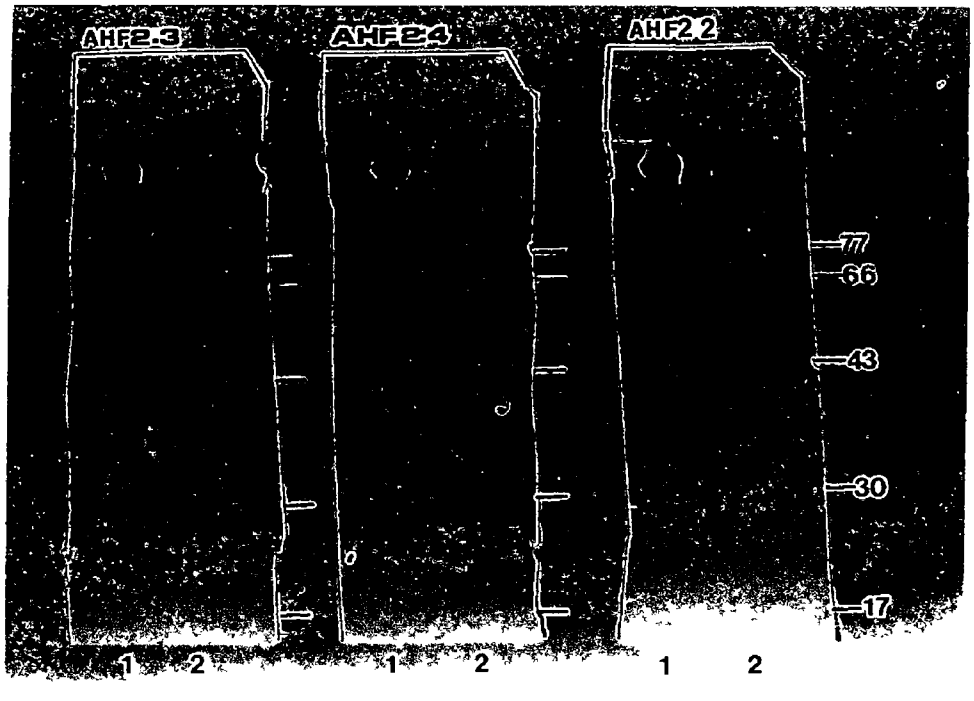


FIG III 25

This figure compares the binding of 3 different antibodies to different preparations of fibrinogen in a Western blot The original SDS-PAGE was on a 10% (w/v) gel The figures are the molecular weights of the markers in '000's and they indicate their position on the gel

1 D+E pool of the plasmin digest of fibrinogen

2 Fibrinogen under reducing conditions

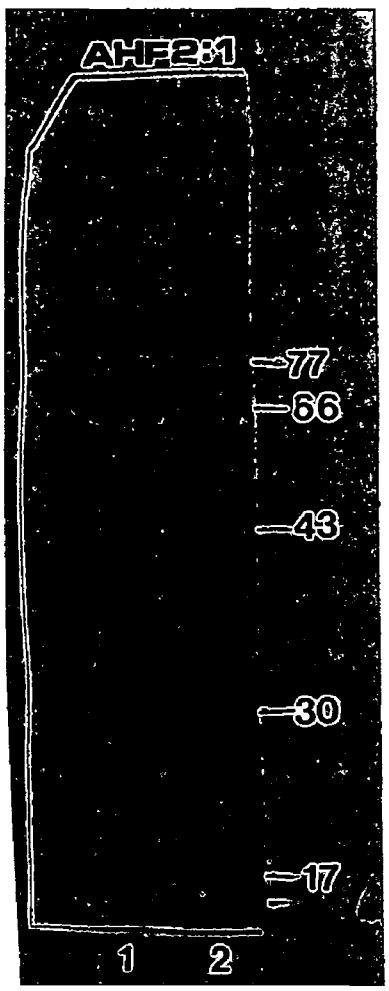


FIG III 26

This figure shows binding of AHF2 1 to two different preparations of fibrinogen in a Western blot The sample were electrophoresed on a 10% (w/v) SDS-PAGE system first The figures on the right are the positions and molecular weights (in '000's) of the markers

1 D+E pools of the plasmin digest of fibrinogen
2 Fibrinogen under reducing conditions

AHF2 1 AHF2 3

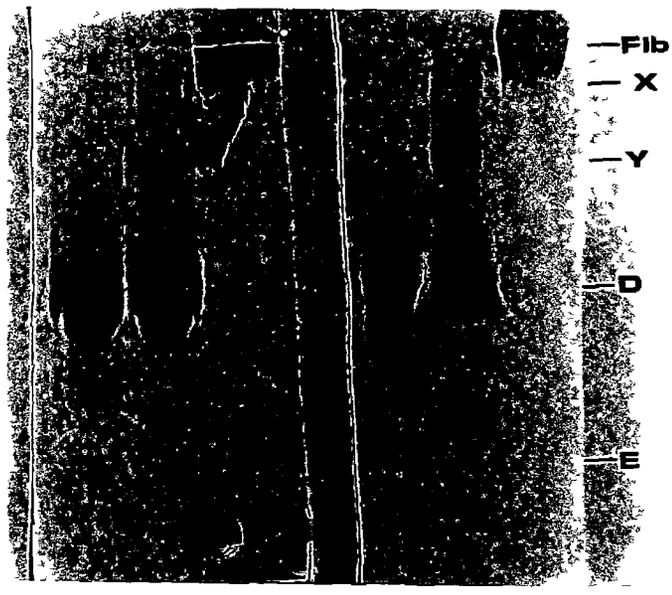


FIG III 27

This figure shows the binding of 2 antibodies to different fibrinogen preparations on a Western blot after electrophoresis on a 7 5% (w/v) SDS-PAGE system Samples were probed with alkaline phosphatase-labelled anti-mouse Ig The blot containing no first antibody, i e negative control, (not shown) was clear

1 D+E plasmin digest pool of fibrinogen
2 X+Y plasmin digest pool of fibrinogen
3 Fibrinogen under non-reducing conditions

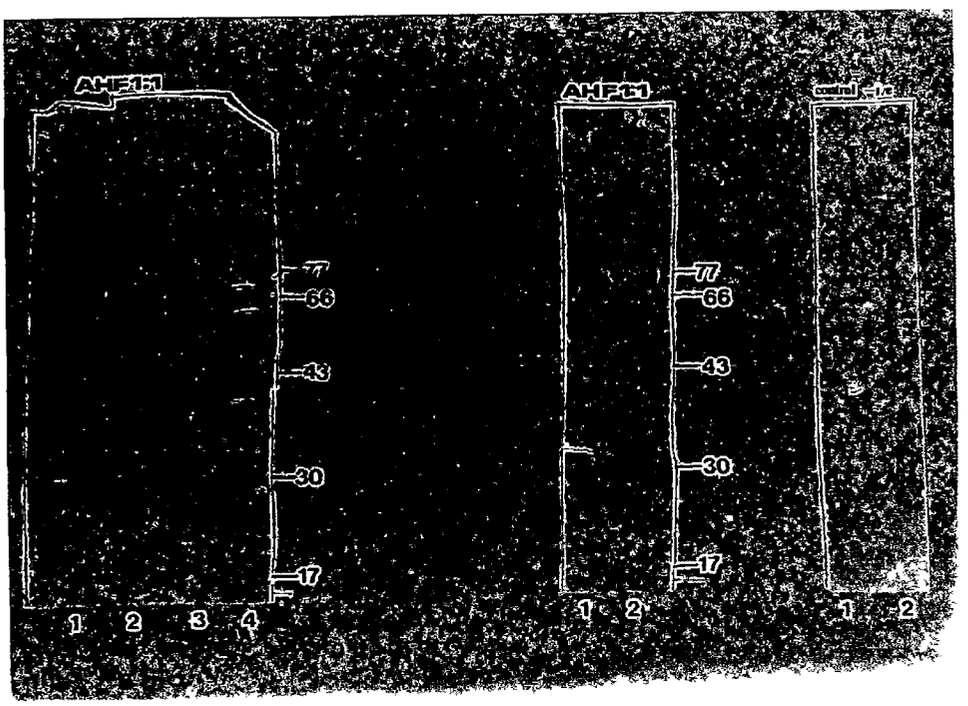


FIG III 28

This figure shows the binding of AHF1 1 to different Koate preparations in a Western blot Samples were electrophoresed on a 10% (w/v) SDS-PAGE system The blots were probed with alkaline phosphatase-labelled anti-mouse IgG The figures on the right indicate the positions and molecular weights (in '000's) of the markers

1 Koate under non-reducing conditions
2 Koate under reducing conditions
3 Protein isolated from plasma after elution from a column with immobilised AHF1 1- reducing conditions
4 Protein isolated from plasma after elution from a column with immobilised AHF1 1- non-reducing conditions

FVIII was purified from plasma and different FVIII concentrates This purified FVIII was used to immunise mice for the production of monoclonal antibodies From these fusions 5 antibodies were cloned and characterised

III 3 1 PURIFICATION OF FVIII

Newman et al , (1971) reported a yield of 62% on their purification of FVIII In the system used here the final yield was between 6 and 9% In run 1 the major problem was a low yield during the cryoethanol precipitation Only 35% of the FVIII was precipitated out Newman reported that the major factor in obtaining a high yield from plasma was the ethanol concentration In run 2 there was a 79% yield of FVIII based on a theoretical FVIII concentration of 1 IU/ml in the starting plasma During run 2, 5 8%(v/v) ethanol was used while in run 1 2 8%(v/v) ethanol was used Thus, increasing the ethanol concentration improved the yield of FVIII

The second major loss of FVIII occured during PEG precipitation The supernatant remaining after both PEG precipitations had very little FVIII activity (0 2% in run 1) The first PEG precipitate showed between 1 and 2% of the starting activity Fig III 13 and III 14 confirm this by the absence of FVIIIR Ag in a Laurell rocket assay Thus, since the FVIII activity was not in the first PEG precipitate nor in the supernatant, it must have been in the second PEG precipitate Since only 6-9 % of the FVIII procoagulant activity was present in this second precipitate the remainder must have been FVIII that had lost its coagulation activity Agarose electrophresis (Fig III 12) showed a strongly staining band in the high purity FVIII preparation This was identified as FVIIIR Ag by crossed immunoelectrophoresis (Fig III 15), indicating that there was a high level of FVIII present, a large percentage of which was no longer active

There are at least two reasons for the loss of activity of FVIII Firstly, FVIII is very susceptible to proteolysis which inactivates it This may be prevented to some extent by using a protease inhibitor in the separation procedure The second possibility is the formation of aggregates on storage as reported by Newman et

al , (1971) and Fulcher et al , 1983) Agarose electrophoresis of the FVIII preparations (Fig III 12) shows a band at the sample application well that stains heavily in Coomassie blue This indicates that there are some proteins with molecular weights so large that they could not run into an agarose gel FVIII will electrophorese on an agarose gel The results of an ELISA assay (see Table III 11) also suggest that aggregate formation occurs It was found that AHF2 1 bound to FVIIIR Ag bound to immobilised anti-FVIII antibody However, this only occured if Koate was used as the source of FVIII Since AHF2 1 was found to be an anti-fibrinogen antibody, this would indicate that on storage, FVIIIR and fibrinogen formed a complex Newman et al , (1971) recommend altering many of the conditions of the purification eg ethanol concentration, pH, and PEG concentration if the plasma has been stored for more than 3 months These modifications cater for the alterations of the physiochemical properties of FVIII after it aggregates The main problem with purifying FVIII was obtaining suitable sources of FVIII It proved to be very difficult to obtain fresh plasma for the purification procedure, due to the high demand for plasma Thus, "old" plasma had to be used despite the difficulties associated with this

As can be seen from Fig III 12, cryoethanol precipitation removed many of the low molecular weight proteins from plasma The two major contaminating proteins appear to be fibrinogen and albumin The first PEG precipitation appears to remove the fibrinogen, while the second PEG precipitation appears to precipitate the FVIII leaving the albumin in the supernatant The major contaminants in the FVIII fraction appear to be immunoglobulins (these run in the opposite direction to the other serum proteins) and fibrinogen The higher levels of contaminants in the higher purity fraction was due to the concentration difference between it and the low purity FVIII The intermediate purity FVIII for run 1 had a volume of about 100 ml, while the high purity fraction had a volume of about 4 ml, ie it was 25 times more concentrated

Crossed immunoelectrophoresis (Fig III 15) showed only 1 peak, representing FVIIIR There was no evidence of any other contaminants other than immunoglobulins Both PEG precipitated cryoprecipitate, and Koate, produced similar profiles when their absorbances were analysed at 280nm, following chromatography on a Sepharose column (see Fig III 16 and III 17) Both profiles show

4 major peaks FVIII, which was found in the first peak, was the smallest of these peaks The largest peak was probably fibrinogen This would indicate that cryoethanol precipitation of frozen plasma gave a higher purity than commercially available FVIII cryoprecipitate, since it only had only one band in electrophoresis

III 3 2 MONOCLONAL ANTIBODY PRODUCTION

Successs in the production of monoclonal antibodies can be difficult to achieve There are many factors involved, and usually it is very difficult to isolate a particular reason for an unsuccessful fusion There is evidence to indicate that one of the most important variables is the operator An other major problem is mycoplasma contamination This can cause a loss of antibody producing abilty in clones, inhibit growth of clones, and also prevent successful fusions (Carroll and O'Kennedy, 1988)

8 fusions were performed, but 5 of these produced no viable clones The specific cause(s) for this are not known One fusion produced 5 positive clones which subsequently ceased to produce antibody There are 2 possible reasons for this The first is overgrowth by non-producing clones This was unlikely as all the clones were cloned twice Westerwoudt (1986) suggests that cessation of antibody production is seldom due to overgrowth by non-secreting cells, but is probably due to loss of the ability to produce antibody Since the newly formed hybridomas have double the normal amount of chromosomes, they shed half of their chromosomes before they stabilise It is probably a 50 50 chance whether they loose the chromosome responsible for producing the antibody or the chromosome responsible for growth in culture Fusion AHF2 had a higher percentage of positive clones compared with AHF3 This difference was probably due to differences in the immune response of the mice used for the 2 fusions

III 3 3 ANTIBODY ISOTYPE ANALYSIS

Isotype analysis indicated that none of the clones produced had only 1 particular isotype In particular AHF2 1a and AHF2 1b were both from the second cloning of the same clone which was monoclonal, and yet AHF2 1a is predominantly an IgG2a, while AHF2 1b is predominatly an IgG1

The reason for these differences is due to the ability of a clone to change the isotype of its antibodies The constant region loci are arranged in the following order

$$(5') \quad \mu - \delta - \gamma_3 - \gamma_1 - \gamma_{2b} - \gamma_{2a} - \varepsilon - \alpha \quad (3')$$

This switching of isotypes can occur spontaneously in culture The highest frequency of changes is to to their nearest neighbour on the 3' end, but even larger shifts are possible (Kipps, 1985) Thus, shortly after cloning some of the cells in AHF2 1a probably changed the isotype of their secreted antibody from IgG1 to IgG2a The frequency of this shift was estimated at 10^{-6} by Kipps (1985) The reason that there is a mixture of isotypes in most of the clones is that they were not screened for isotype during cloning It is possible to have clones that only produce one particular isotype if they are repeatedly cloned and screened for a particular isotype Only those with a single isotype would be expanded, and further cloned

III 3 4 ANTIBODY STABILITY

Table III 6 shows the binding of 3 antibodies to Koate at differnt concentrations Both AHF1 1 and AHF2 1a have titres of 2 µg/ml, even though they are against different antigens AHF2 1b has a titre closer to 200 ng/ml This would suggest that the change in isotype from IgG1 to IgG2a also produced a lower titre Also the maximum absorbance that was obtained in ELISA is reduced for AHF2 1a Thus, the ability of the IgG2a to bind to the antigen is less than that of the IgG1

The binding of AHF1 1 to Koate in ELISA was found to be repesented by a bell shaped curve (Table III 7) Thus, at concentrations that are either above or below the optimum (1-10 µg/ml) there is a reduction in binding At low concentrations this occurs due to a lack of antibody At higher concentrations this is probably due to steric hindrance by other antibodies Thus, if the ratio of antibody to antigen is too high the antibody cannot bind properly This could be due to many antibody molecules partially binding, ie only one "arm" of the antibody can bind The second "arm" cannot bind because all the antigen sites are occupied Since these antibodies are not properly bound they are probably washed off during the ELISA

AHF1 1 and both AHF2 1 antibodies were stable on storage at 4°C After 9 months storage at a concentration of 200 μg/ml there was only a slight reduction in binding which occured during the first 3-4 months of storage

III 3 5 ANTIBODY CHARACTERIZATION AHF1 1

AHF1 1 was found to be predominantly an IgG2a with a k light chain ELISA indicated that it preferentially bound to the void volume fraction (K1) of Koate following Sepharose chromatography (see Table III 9) It did not bind to BSA and bound only slightly to fibrinogen and to the second Sepharose fraction (K2) of Koate (see Table III 10) This data would indicate that it bound to a large molecular weight component of Koate with a molecular weight of at least $4x10^6$ (this is the exclusion limit of Sepharose 6B) The obvious candidate for this was FVIII, as its molecular weight is several millions (Hoyer, 1981)

Western blotting using Koate as the antigen and AHF1 1 as the probe, showed that under non-reducing conditions AHF1 1 binds to 3 bands of molecular weights 121,000, 112,000 and 75,000 Some Koate preparations show bands at 103,000 and 61,900 Fay et al , (1982) report a molecular weight of 72,000 for FVIII Vehar et al , (1984) report two pools of protein when FVIII is run under non-reducing conditions One has a molecular weight of 80,000 while the other has a range of proteins from 90,000 to 210,000 This latter pool contains a number of bands, including single bands at 120,000 and 100,000 While the intact molecule has a molecular weight of 330,000, if thrombin is present in the preparation the Mr 90,000, and Mr 80,000 bands are formed due to proteolysis During prolonged exposure to thrombin the Mr 90,000 fragment is converted to Mr 50,000 and Mr 43,000 fragments The Mr 80,000 is similarly converted to a Mr 73,000 fragment (see Fig III 9) Thus, AHF1 1 probably binds to the thrombin degradation product of the Mr 80,000 fragment of FVIII Lollar et al , (1988) report a conversion of a Mr 76,000 band to a Mr 69,000 band after exposure of porcine FVIII to thrombin The Mr 61,900 fragment seen here is probably due to proteolysis of the FVIII, either by thrombin or some other protease

Under reducing conditions AHF1 1 was found to bind to two bands with molecular weights of 79,900 and 88,000 The larger apparent

molecular weight during electrophoresis under reducing conditions, indicates the presence of internal disulphide bonds in the protein Fay et al , (1982) found that FVIII forms a single band with an apparent molecular weight of 100,000 under reducing conditions

The Mr 90,000 fragment (heavy chain) is the N-terminal fragment of FVIII and the Mr 80,000 fragment (light chain) is the C-terminal fragment of FVIII Thus, it appears that AHF1 1 binds to the light chain only Since it also binds to the degraded form of the light chain its epitope must be on a region of 640 amino acids This region is between amino acids numbers 1,690 and 2,332

To confirm that the antibody AHF1 1 bound to FVIII CAg it was coupled to CNBr-activated Sepharose, and used to isolate its specific antigen The protein that was isolated had the same characteristics on SDS-PAGE, under both reducing and non-reducing conditions, as the FVIII in Koate, and as the void volume fraction from Koate (K1) However, this protein had no procoagulant activity This is not surprising when glycine, pH 2 5 was used to elute it, as FVIII is very unstable below pH 6 (Hoyer, 1981) When ethylene glycol-histidine, pH 6, was used to elute the FVIII there was still no activity This could be due to inactivation of the FVIII by thrombin or other proteases Certainly, western blotting of Koate showed a high proportion of low molecular weight ie Mr 60,000-70,000 in some preparations This is usually associated with inactivation of the protein The loss of procoagulant activity after passing Koate through the column (from 0 84 IU/ml to 0 02 IU/ml) is too large to be due to dilution, and thus, probably represents removal of FVIII C from the Koate due to binding to the immobilised antibody (see Table III 13)

ELISA was used to examine the possibility that AHF1 1 was binding to vWF rather than to FVIII CAg This would account for the absence of activity in the protein fraction binding to immobilised AHF1 1 and eluted from the affinity column Plates coated with anti-vWF antibody showed no binding of AHF1 1 Similarly, plates coated with AHF1 1, showed no binding of vWF Also, the molecular weight of vWF, under non-reducing conditions, is so large (usually greater than 10^6) that it has to be run on agarose Under reducing conditions it has an apparent molecular weight of

195, 000-240, 000 (Hoyer, 1981) The Western blots show that AHF1 1 does not bind to a protein of this molecular weight

ELISA indicated that AHF1 1 had weak binding to both the K2 fraction from Sepharose column chromatography, and to fibrinogen This could be due to a similar epitope on both proteins It is more likely that FVIII was a contaminant in both fibrinogen preparations Fig III 16 and III 17 show that the majority of vWF is in the K1 peak, but there is also some present in the K2 peak The molecular weights of monomeric FVIII C and vWF are 330,000 and 240,000 respectively These would coelute with fibrinogen which has a molecular weight of 340,000 There is also evidence that fibrinogen and FVIII form a complex (Fulcher et al , 1983) This was also found with AHF2 1 which binds to fibrinogen, and also binds strongly to K1 SDS-PAGE of the protein fraction isolated by affinity chromatography of normal plasma produced only 1 single band This band corresponded to FVIII There was no evidence of fibrinogen in the eluted protein This would indicate that AHF1 1 did not bind to any other proteins in plasma

Inhibition studies on plasma coagulation show that AHF1 1 had very little effect on the coagulation time (3-5 sec increase in coagulation time) This is to be expected as the FVIII C site was localised to residues 338-362 on the heavy chain (Ware et al , 1988) Binding of AHF1 1 to FVIII C may be hindered if the intact FVIII complex is used This is because the binding site for vWF to FVIII C is at residues 1,670 and 1,684 on FVIII C, which is adjacent to the binding site for AHF1 1 at residues 1,690 to 2,332 If AHF1 1 binds at the N-terminal of this fragment, its binding to the complex would be inhibited due to masking of the epitope If it binds to the C-terminal of light chain it would be less likely that the epitope would be masked

III 3 6 ANTIBODY CHARACTERIZATION AHF2 1

Two different versions of AHF2 1 were cloned out One of these (AHF2 1a) was an IgG2a with a k light chain, the other (AHF2 1b) was an IgG1 with a k light chain Both these antibodies were stable on storage at a concentration of 200 µg/ml, at 4°C, for at least 9 months

Both antibodies bound to the K1 fraction, and the K2 fraction of Koate, and to fibrinogen Neither of them bound to BSA This

would indicate that they bound to fibrinogen, especially since they always gave a higher maximum absorbance than AHF1 1 (1 2 v's 0 3) Dot blotting showed that AHF2 1 did not bind to serum indicating that the only plasma proteins that it bound to were those that were consumed during coagulation

Western blot analysis using reduced fibrinogen on SDS-PAGE shows two bands of molecular weights 64,400 and 60,500 These correspond to the fibrinogen Aα chain which has a molecular weight of 67,000 (Doolittle, 1981) However, this consists of a population of mixed molecular weights due to proteolysis of the chain

Western blotting from SDS-PAGE, using the pooled D and E fragments, showed that AHF2 1 bound to 2 bands of molecular weights 61,700 and 48,000 The reported molecular weight of the D fragment is between 80,000 and 90,000, while that of the E fragment is 50,000 Thus, it would appear that AHF2 1 binds to the E fragment, as well as a Mr 61,700 band the identity of which is unknown Western blotting of fibrinogen and its early degradation products on 7 5% (w/v) SDS-PAGE show binding to fibrinogen, and the X, Y, D and E fragments

Since AHF2 1 binds to both the E fragment and the Aα chain its epitope can be narrowed to a region of 78 amino acids This is the portion of the Aα chain that is present in fragment E (see Table III 1) These amino acids are Aα (1-78) Binding to X and Y would be expected since they contain both the D and E fragments Binding to the D fragment could be due to a similar epitope on both fragments, or due to pieces of the E fragment remaining on the D fragment during digestion

III 3 7 ANTIBODY CHARACTERIZATION AHF2 2

AHF2 2 was found to be an IgG1 with a k light chain It was found to bind strongly to Koate fraction K2 and fibrinogen It demonstrated weaker binding to K1, and it did not bind to BSA Dot blotting showed that AHF2 2 did not bind to serum, indicating that it must bind to a protein that is consumed during coagulation Since it also binds to FVIII deficient plasma, it cannot bind to FVIII Thus, it was probably an anti-fibrinogen antibody

Western blotting of reduced fibrinogen on SDS-PAGE showed no binding of AHF2 2 to any of the chains This would indicate that AHF2 2 only recognises the native protein Thus, its binding site probably incorporates more than 1 of the chains

Western blotting of the plasmin degradation products D and E of fibrinogen after SDS-PAGE show 4 bands when probed with AHF2 2 These bands have molecular weights of 109,000, 98,700, 94,600, and 72,000 The 3 larger molecular weight bands are probably D fragments, which are a heterogenous group of polypeptides (Haverkate et al , 1979) Western blotting of fibrinogen and its early degradation producs on 7 5% (w/v) SDS-PAGE shows binding by AHF2 2 to bands that correspond to fibrinogen, and fibrinogen fragments X, Y and D

Thus, AHF2 2 binds to the D fragment of fibrinogen Binding to X and Y fragments would be expected since they both contain the D fragment It only recognises the native protein

III 3 8 ANTIBODY CHARACTERIZATION AHF2 3

AHF2 3 was found to be an IgG2b with a k light chain It was found to bind strongly to fibrinogen and the K2 fraction from Koate It had weaker binding to K1, and did not bind to BSA Dot blotting showed that it bound to normal plasma and FVIII deficient plasma Thus, it appeared to bind to fibrinogen

Western blotting of reduced fibrinogen on SDS-PAGE showed that AHF2 3 bound to 1 fragment of molecular weight 55,000 This is obviously the Bβ chain which has a reported molecular weight of 56,000 (Doolittle, 1981a)

Western blotting with the plasmin degradation fragments of fibrinogen showed that it bound to 5 fragments These had molecular weights of 109,000, 98,700, 94,600, 72,000, and 48,000 The 3 high molecular weight fragments are probably the D fragments, while the low molecular weight fragment is probably the E fragment

Since AHF2 3 binds to the E fragment and to the Bβ chain its epitope must be in a region of 68 amino acids (see Table III 1) Thus, its epitope is located at Bβ (54-122)

Some of the antibodies bind to both the D and E fragments This could be due to a similar epitope on both fragments It could also be due to pieces of the E fragment remaining attatched to the D fragment during digestion

III 3 9 ANTIBODY CHARACTERIZATION AHF2 4

AHF2 4 was found to be an IgG1 with a k light chain It bound strongly to fibrinogen and Koate fraction K2 in ELISA It bound weakly to fraction K1 and did not bind to BSA Dot blotting showed that it bound to normal plasma and FVIII deficient plasma It did not bind to serum Thus, it was probably directed against fibrinogen

Western blotting of reduced fibrinogen on SDS-PAGE with AHF2 4 showed binding to one band with a molecular weight of 55,000 This band corresponds to the Bβ chain

Western blot analysis of the plasmin degradation products of fibrinogen on SDS-PAGE showed binding to 3 bands with molecular weights of 109,000, 98,700 and 94,600 These probably make up the D fragments of fibrinogen

Since AHF2 4 binds to the Bβ chain of the D fragment the epitope must be a 327 amino acid fragment located at B β(134-461)

III 3 10 CONCLUSION

Table III 16 summarises the details of the 5 antibodies that were characterised There are many uses for these antibodies The two main applications for monoclonal antibodies to blood factors are in analysis and purification

In recent years there has been a growing awareness of the hazards of using replacement therapy for factor deficient disorders, especially haemophilia The most recently identified risk is from HIV infected plasma This problem has reached crisis point as many patients are at risk from infected plasma (Friedland and Klein, 1987) Haemophiliacs are a very high risk group due to the necessity of receiving multiple blood transfusions A recent survey showed that all commercially available FVIII deficient plasma (usually obtained from haemophiliacs) was HIV positive (Jones et al , 1985) One solution to this problem is to use

genetically engineered FVIII While this has been produced and is due for routine use in the next few years, indications are that it will be much more expensive than the FVIII concentrates currently in use In addition, patients with FVIII inhibitors have to be treated with animal FVIII preparations

These problems can be overcome by using either human or animal FVIII concentrates The risk of infection with these preparations can be eliminated by using heat treated concentrates which are then affinity purified This results in a very high purity FVIII preparation with minimum risk of infection (Ronneberger, 1986) It also eliminates many of the other unwanted proteins found in FVIII concentrates These contaminating proteins can cause risks as the recipient may produce antibodies against them This can then result in the patient developing a second, acquired factor deficiency A commercial concentrate such as Koate is almost 90% fibrinogen Suitable monoclonal antibodies could remove inactive FVIII as well as other contaminants, which would greatly reduce the amount of protein that has to be administered to achieve the same results

The problem with using AHF1 1 for affinity purifying FVIII is that it binds to the light chain rather than to the heavy chain which contains the active site But, AHF1 1 will still remove the complete FVIII C molecule, which is probably the best way to administer it A more frequently used method for the affinity purification of FVIII is to use an anti-vWF antibody Since it removes vWF it will also remove FVIII C This has the added advantage that vWF stabilises FVIII C, and protects it from proteolysis However, when a pure source of FVIII is necessary the use of an anti-FVIII CAg antibody is faster and less expensive for isolation

A second use for AHF1 1 is in the assay of FVIII C levels The problem that must be overcome with any assay for FVIII C is that it's levels in normal plasma are estimated to be between 50 and 200 ng/ml (Hoyer, 1981, Bloom, 1983) If a 100 µl sample is used for the assay, a very sensitive assay would be necessary This would be at the limit of sensitivity for conventional ELISA This can be overcome by using RadioImmunoAssay (RIA) or some other amplification immunoassay system These assays would not replace the coagulation assay, since this measures the amount of active

FVIII present, which is the important value However, they can be used to determine if a FVIII deficiency is quantitative or qualitative It may also have a function in carrier detection, since carriers of haemophilia appear to have a lower level of FVIII C than the normal population (Hoyer, 1981)

AHF1 1 would also be very useful in studying the fuctions of FVIII Many of the active sites on FVIII and vWF were located by using monoclonal antibodies eg the localisation of the coagulation site (Ware et al , 1988) and the localisation of the vWF binding site on FVIII C (Foster et al , 1988)

There is no major role for anti-fibrinogen antibodies in the purification of fibrinogen This is because fibrinogen is present in high concentrations in plasma and it is relatively easy to purify The main use for these antibodies is in assaying plasma levels of fibrinogen and fibrin degradation products Kruskal et al , (1987) showed that there was a difference between the levels of fibrin degradation products in patients with stable angina and those with unstable angina and acute myocardial infarction There results show that in the latter there is an increase in the fibrin degradation products indicating a fibrinolytic process They suggest that this fibrinolysis may be involved in the conversion of unstable angina to acute myocardial infarction, and that anti-fibrinolytic agents may prevent this conversion Thus, routine screening of patients with angina for level of fibrinogen and fibrin degradation products would enable a differential diagnosis of stable and unstable angina, and might possibly prevent the development of acute myocardial infarction by early treatment with anti-fibrinolytic agents To be effective for this the antibodies would have to be specific for either the D monomer (derived from fibrinogen) or the D dimer (derived from cross-linked fibrin)

Fibrin specific antibodies also have a potential use in locating fibrin deposits in the vascular network Once detected, fibrinolytic therapy can be commenced, thereby reducing the risk of a stroke Brown et al , (1988) showed that solid tumours convert fibrinogen to fibrin which is necessary for their growth and proliferation Labelled monoclonal antibodies to fibrin could also be used to detect these tumours in vivo There is also a possible role for anti-FVIII CAg antibodies in tumour detection

Cancer of FVIII CAg producing cells such as hepatocytes and spleen cells (Wion et al , 1985), could also be detected with labelled anti-FVIII CAg antibodies

Further work necessary for the total characterization of these antibodies would involve the use of enzyme digests of FVIII C to determine specifically the binding site of AHF1 1 It would also be necessary to develop a suitable elution system to affinity purify active FVIII These require a large amount of relatively pure, and fresh FVIII, or large amounts of fresh plasma Both of these are expensive, and difficult to obtain Further work on the anti-fibrinogen antibodies is necessary to determine whether or not they are specific for fibrin or fibrinogen degradation products

The antibodies will also be used in the development of an adsorptive stripping voltametric system for the study and analysis of FVIII and fibrinogen (Smyth et al , 1988, Rodriguez-Flores et al , 1988a, 1988b) One of the benefits of this system is that it is thought that an immunoassay using an adsorptive stripping voltametric technique as the amplification step would be more sensitve than ELISA and RIA

SECTION IV

PRODUCTION OF ANTIBODIES

TO DIAZEPAM

IV 1 1 HAPTENS

The ability of the immune system to respond to antigens depends on their 'antigenicity' The antigenicity of a substance depends on a number of different properties, one of the most important being its size Molecules with a molecular weight of 1,000, or less, are poor antigens (Erlanger, 1980), and they are called haptens Haptens are usually conjugated to carrier proteins before immunisation If the carrier protein is sufficiently antigenic some of the antibodies will be directed against the hapten portion of the conjugate since it should form the major epitope Examples of commonly used haptens are peptide hormones, neurotransmitters, and drugs Often when antibodies to specific portions of a molecule are required it is easier to isolate the fragment, conjugate it to a carrier, and use this as the antigen

IV 1 2 CONJUGATION REACTIONS

The nature of the coupling reaction depends on available reactive groups on the protein and on the hapten There are 5 major reactive groups on proteins ε-amino groups (lysine residues), α-amino groups, phenolic hydroxyl groups (tyrosine residues), sulfhydryl groups (cysteine residues), and imidazole groups (histidine residues) It is important to choose a reactive group on the protein that is present in sufficient quantity In BSA there are 59 ε -amino groups, 7 sulfhydryl groups and only 1 α-amino group Thus, the ε-amino groups would be better suited to conjugate formation than the sulfhydryl groups (Erlanger, 1973)

The main criterion in choosing a conjugation reaction will be the available reactive groups on the hapten The 3 main reactive groups on haptens that are used for conjugation are carboxyl groups, amino groups, and hydroxyl groups (Erlanger, 1973) When there are no suitable reactive groups on a hapten they have to be inserted chemically

Landsteiner (1945) considered that 10 haptenic groups per molecule of BSA was optimal for antibody formation while Dixon (1982) considered an 18 to 1 molar ratio of hapten to protein to be optimum Another consideration is the orientation and

conformation of the hapten Since antibodies are very specific for the antigen, eg antidigoxin antibodies can distinguish between digoxin (OH group on C12) and digitoxin (H on C12) (Butler, 1973), the part of the molecule that the antibody has access to must be carefully selected Sometimes a spacer arm (a chain of carbon atoms) between the hapten and the carrier can improve the antigenicity of the hapten, and the subsequent specificity of the antibodies, by improving access to the molecule

IV 1 3 DIAZEPAM

Pharmacologically diazepam (Valium) is a tranquillizer (also termed antineurotic or anxiolytic drugs) Chemically, it is a benzodiazepine Benzodiazepines are the "classic" tranquillizers, thus, many other compounds with similar effects to diazepam are refered to as benzodiazepines even though their chemical structure would not justify this Hence the term "benzodiazepine" is used to refer to a class of compounds rather than to a chemical structure

The true benzodiazepine consists of a fused bicyclic nucleus (see fig IV 1) This nucleus consists of a benzene ring and a 7 membered, partially unsaturated heterocyclic ring, with 2 nitrogen atoms in the 1,4 positions (1,4-benzodiazepines) There is also an aromatic ring (usually phenyl) substituted in the 5 position Compounds with only one nitrogen, in either the 1 or 4 position, are technically benzazepines but have similar properties to the benzodiazepines Fig IV 2 shows the structures of the benzodiazepines used for immunisation, and characterisation studies

Hypnotics, sedatives and tranquillizers are very closely related groups of drugs Hypnotics act by producing sleep directly, while sedatives relax the patient which will enable them to fall asleep Tranqillisers are used to produce a calming effect in patients that are anxious or tense Benzodiazepines' anticonvulsant activity is used in the prophylactic treatment of epilepsy They also have central muscle relaxant effects which are used to treat status epilepticus and tetanus (Pharmaceutical Codex) Benzodiazepines also have strong behavioral effects, they have a calming effect in anxious states, and they have sedative effects As tranquillizers, the benzodiazepines have no anti-psychotic activity

The toxicity of benzodiazepines is very low The major problem associated with the chronic use of diazepam is the strong psychological dependence that can develop, and the withdrawal symptoms that can follow the cessation of long term therapy (Nutt, 1986) There are also a few very rare cases of hepatotoxicity to diazepam Certain severely disturbed patients become aggressive when administered diazepam (Pharmaceutical Codex) In acute exposure to diazepam, as in an overdose, there is little risk to the patient as a lot of diazepam has to be consumed before any serious problems arise

IV 1 4 GABAergic NERVES

Within the nervous system nerves do not make electrical contact with each other, instead there is a small gap, the synaptic junction, between each nerve fibre Chemicals called neurotransmitters are used to transmit signals across the synaptic junction Neurotransmitters bind to specific receptors on both the pre- and postsynaptic membranes If the binding of the neurotransmitter to its receptor causes a depolarization, resulting in an action potential, the presynaptic nerve is known as as excitatory nerve Conversely, if a hyperpolarization is caused, stabilising the membrane, then the presynaptic nerve is termed an inhibitory nerve A postsynaptic nerve would usually have a number of different afferent (incoming) nerves Some of these may be inhibitory and others may be excitatory The transmission of an action potential in the postsynaptic nerve depends on the sum of the depolarizing and hyperpolarizing signals Adrenalin and acetylcholine are both excitatory neurotransmitters The amino acid, γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter

Chemicals that bind to a receptor and mimic the response due to binding of the neurotransmitter are termed agonists Binding of agonist produces a response in the neuron Antagonists bind to the receptor but they have no effect other than preventing an agonist from binding Partial agonists bind to a receptor and initially cause a response which is short lived Subsequently they remain bound to the receptor and act as antagonists

Localisation studies indicated that GABAergic nerves are found in Purkinje fibres of the cerebellum, pyramidal cells of the cerebral

cortex and hippocampus, and the olfactory bulb GABA is the major inhibitory neurotransmitter in the brain with about 30% of the synapses being GABAergic (Haefely, 1983) GABA has two different receptors The main one is coupled to a chloride channel in the membrane (Bowery, 1982) Activation of these receptors increases the influx of chloride ions through the membrane of the cell In most neurons this flow of chloride ions is inwards, causing a hyperpolarisation In certain neurons there is an efflux of chloride ions causing a depolarisation This depolarisation is inhibitory because it reduces the amplitude of the action potential since it starts from a smaller potential (Haefely, 1983) The second type of receptor is linked to a calcium channel where it decreases the permeability to calcium thus causing an inhibition (Bowery, 1982)

Receptor studies have confirmed the differences in the 2 GABA receptors The chloride channel-linked receptors are inhibited by bicuculline, while the calcium channel-linked receptors are bicuculline-insensitive The bicuculline-insensitive receptors bind only a few of the GABA analogues In particular they bind baclofen, while the bicuculline-sensitive receptors do not The bicuculline-sensitive, chloride channel-linked receptors were termed GABAa while the bicuculline-insensitive, calcium channel-linked receptors were called GABAb (Bowery, 1982)

IV 1 5 GABA AND BENZODIAZEPINES

In 1967 Schmidt et al , showed that diazepam enhanced presynaptic inhibition Haefely et al , (1975) and Costa et al , (1975) showed that this was probably due to an enhancement of GABAergic inhibition Subsequently it was found that GABAergic neurons throughout the brain responded to benzodiazepines GABAergic neurons in the spinal cord, the dorsal column nuclei; the cerebellar cortex and the hippocampal pyramidal cells all showed enhanced activity in the presence of benzodiazepines This response was also found to the application of exogenous GABA (Haefely, 1983)

Adenosine, which is a putative inhibitory neurotransmitter found in purinergic nerves was also found to be effected by benzodiazepines The uptake of adenosine into cells appears to be inhibited This effectively increases the concentration of adenosine at purinergic synapses and thereby increasing the

inhibition due to the adenosine Certain benzodiazepine antagonists also act as weak adenosine antagonists (Phillis and O'Regan, 1988)

GABAergic nerves provide a control of the activity of neurons They prevent neurons from becoming overstimulated Thus, in certain pathological states such as epilepsy or anxiety, part of the GABAergic system does not function properly Benzodiazepines act by increasing the activity of the GABAergic nerves thereby overcoming the defect The role of adenosine is probably secondary to that of GABA

IV 1 6 THE BENZODIAZEPINE RECEPTORS

Braestrup and Squires (1977) and Mohler and Okada (1977) identified high affinity binding sites in the brain for ^{3}H-diazepam These binding sites were considered to be specific receptors Subsequently, there was a search for an endogenous ligand for the benzodiazepine receptor based on the idea that the existence of a receptor also indicates the existence of an endogenous ligand

Using ^{3}H-flunitrazepam, which covalently binds to the benzodiazepine receptor in the presence of uv light, it has been possible to localise and purify the benzodiazepine receptor (Mohler et al , 1980, Sigel et al , 1983) This established a direct relationship between the GABA and benzodiazepine receptors

Various ligands with affinity for the benzodiazepine receptor were synthesised CL 218872, a triazolopyridazine, and ethyl-β-carboline-3-carboxylate (β-CCE) both displaced ^{3}H-flunitrazepam from the receptor with a high potency These compounds differed from the benzodiazepines in many respects β-CCE has effects directly opposite to those of the benzodiazepines β-CCE not only antagonises the anticonvulsant effects of diazepam, it also has proconvulsant effects when bicuculline is used to induce seizures β-CCE antagonises the anxiolytic actions of diazepam and also has anxiogenic activity In cultured mouse spinal cord cells diazepam produces an increase in the opening of chloride channels by GABA, while β-CCE produces a decrease

Initially it was thought that since β-CCE opposes the actions of diazepam it must be an "antagonist" and diazepam an "agonist" for

the benzodiazepine receptor However, the problem with this is that both produce a response on binding to the receptor So the question arose as to which was the agonist and which was the antagonist The difficulty was overcome by considering diazepam to be an agonist and β-CCE an "inverse agonist" Other β-carboline derivatives had similar effects on the benzodiazepine receptor In particular methyl-β- carboline-3-carboxylate (MCC) and methyl 6,7-dimethoxy-4- ethyl-β-carboline-3-carboxylate (DMCM) both induced seizures when injected into mice Ro 15-1788 (an imidazodiazepine) has no intrinsic activity at the benzodiazepine receptor, but antagonises the anticonvulsant activity of the benzodiazepines and the convulsant activity of both MCC and DMCM Thus, it is a true antagonist

The concept of inverse agonists has caused problems in the world of pharmacology One way of conceptualising is to use the model proposed by Ehlert (1986) He uses a two state model for the benzodiazepine-GABA receptor complex similar to that proposed by Chin and Rosenberg, (1983) The open state is associated with an open chloride channel, and the closed state is associated with a closed chloride channel Both GABA and diazepam have a higher affinity for the open state GABA enhances the binding of diazepam, which in turn enhances the binding of GABA This is positive cooperativity The binding of GABA to the open state causes the chloride channel to remain open Benzodiazepines have no effect on their own, as their affinity for the open channel is low in the absence of GABA The ligands with a higher affinity for the closed state allosterically inhibit the binding of GABA This is termed negative cooperativity An antagonist, such as Ro 15-1788 (Fluazepil) would have equal affinty for both the open and closed states and act by blocking both the positive and negative cooperativity

Recent work has indicated that there may be 2 distinct receptor types for benzodiazepines, with different biological distributions The second class of receptors is mainly distributed outside the CNS, although a few localised areas in the CNS also have these binding sites Ro 5-4864 does not bind to the CNS benzodiazepine receptor, but it has a high affinity for the peripheral receptor Ro 15-1788 has a high affinity for the CNS receptors, but does not bind to the peripheral receptors At present there is no known function attributed to these peripheral

receptors, and thus, they may be acceptors rather than receptors (Haefely, 1983)

After the success in locating endogenous ligands for the opiate receptor, many researchers tried to locate an endogenous ligand for the benzodiazepine receptor Many compounds were isolated and a lot of interest was focused on β-carbolines Those that had the highest affinity for the benzodiazepine receptors were unlikely to occur in the CNS, and those that were likely to occur in the CNS had only weak affinity for the receptor (Haefely, 1983) The most likely candidate for endogenous receptor is a polypeptide called Diazepam Binding Inhibitor (DBI), described by Guidotti et al , (1983) The binding site was localised to an 18 amino acid fragment called ODN (Ferrero et al , 1984) This peptide has an anxiogenic effect Endogenous ligands may play an important role in modulating GABA neurons and thereby enhancing anxiety Benzodiazepines may act by both antagonising the actions of ODN and enhancing the actions of GABA (Cooper, 1985)

IV 1 7 ANTIBODIES TO DIAZEPAM

The work presented here involved synthesising diazepam conjugates to be used in the production of antibodies to diazepam Antibodies to diazepam have a number of useful functions The most important is for use in assaying diazepam levels in biological fluids Diazepam is reputed to be one of the most prescribed drugs in the world (Nutt, 1986) In one survey it was the fifth most common drug found in the urine of Los Angeles County probationers (Budd and Walkin, 1980) HPLC and GLC techniques, while very sensitive, are costly and time consuming Both these problems can be overcome by using immunoassays These can be in the form of Enzyme Multiplied Immunoassay (EMIT) (Budd and Walkin, 1980) or ELISA These techniques require little specialised equipment, are very sensitive, can easily assay a large number of samples, and lend themselves to automation Radio ImmunoAssay (RIA) (Dixon, 1982) and voltametric analysis of diazepam are other useful roles for antisera to diazepam Another use for antibodies to diazepam is in the production of antiidiotypic antibodies (Briles and Kearney, 1985) These antibodies bind to the recognition site of the anti-diazepam antibodies Since there is a limit to the number of possible conformations of proteins that will bind the same compound, the binding site on an anti-diazepam antibody must be similar to the

binding site on the diazepam receptor Thus, an antiidiotypic antibody would probably recognise the benzodiazepine receptor and could then be used for studying the receptor

Diazepam is a much abused drug and consequently there are many cases of overdose Antibodies have been shown to be very effective in reducing the plasma levels of many drugs in overdose situations eg with digoxin overdose (Butler, et al , 1973) They can often be better than using a receptor antagonist which may precipitate a life threatening withdrawl

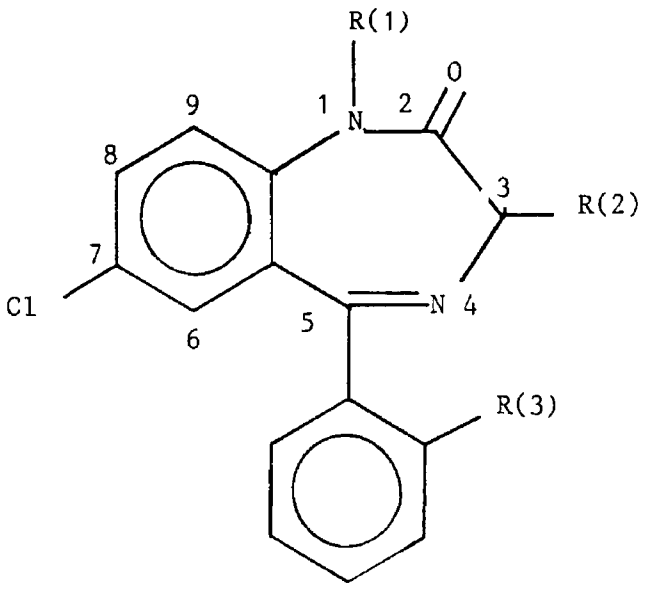
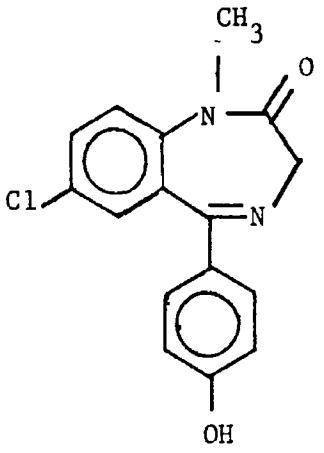
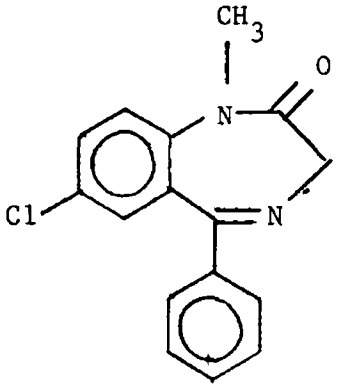


FIG IV 1 Showing the general structure of benzodiazepines, and the numbering system of the ring structure The R groups indicate the usual site for functional groups

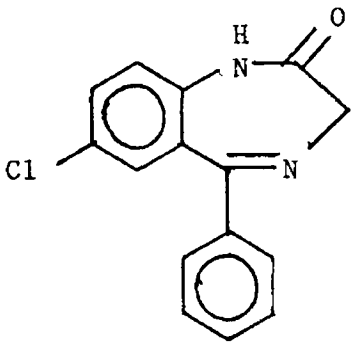
FIG IV.2 Showing the structures of some benzodiazepines



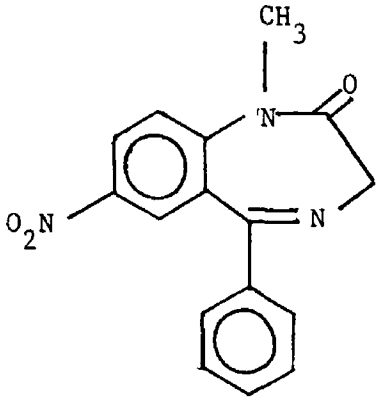
Ro 7-3551



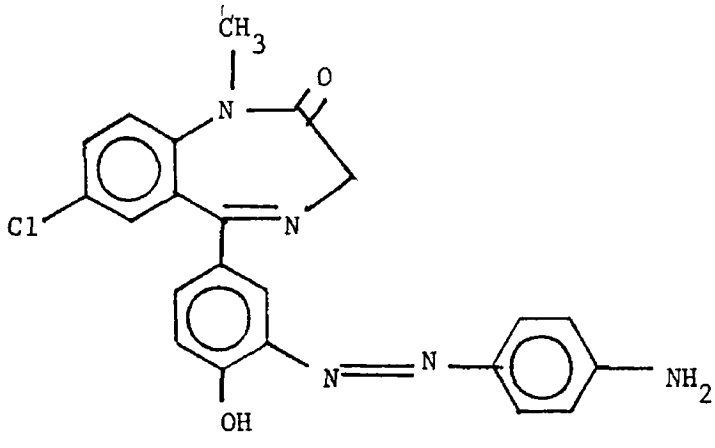
Diazepam (Ro 5-2807)



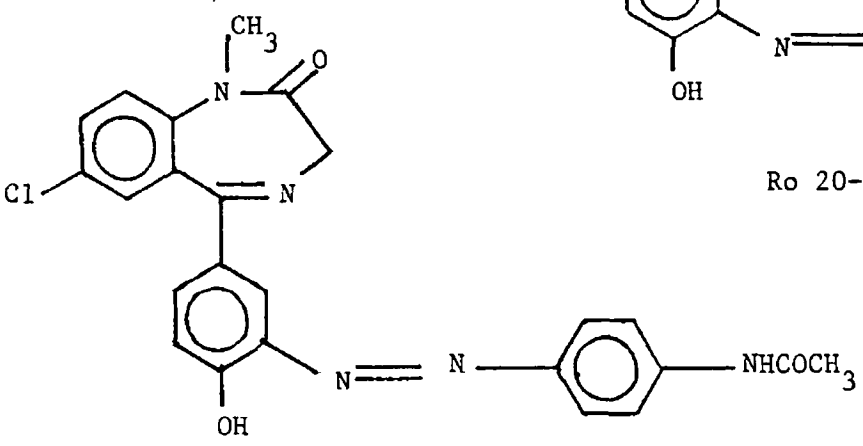
Ro 5-2180



Ro 5-3453

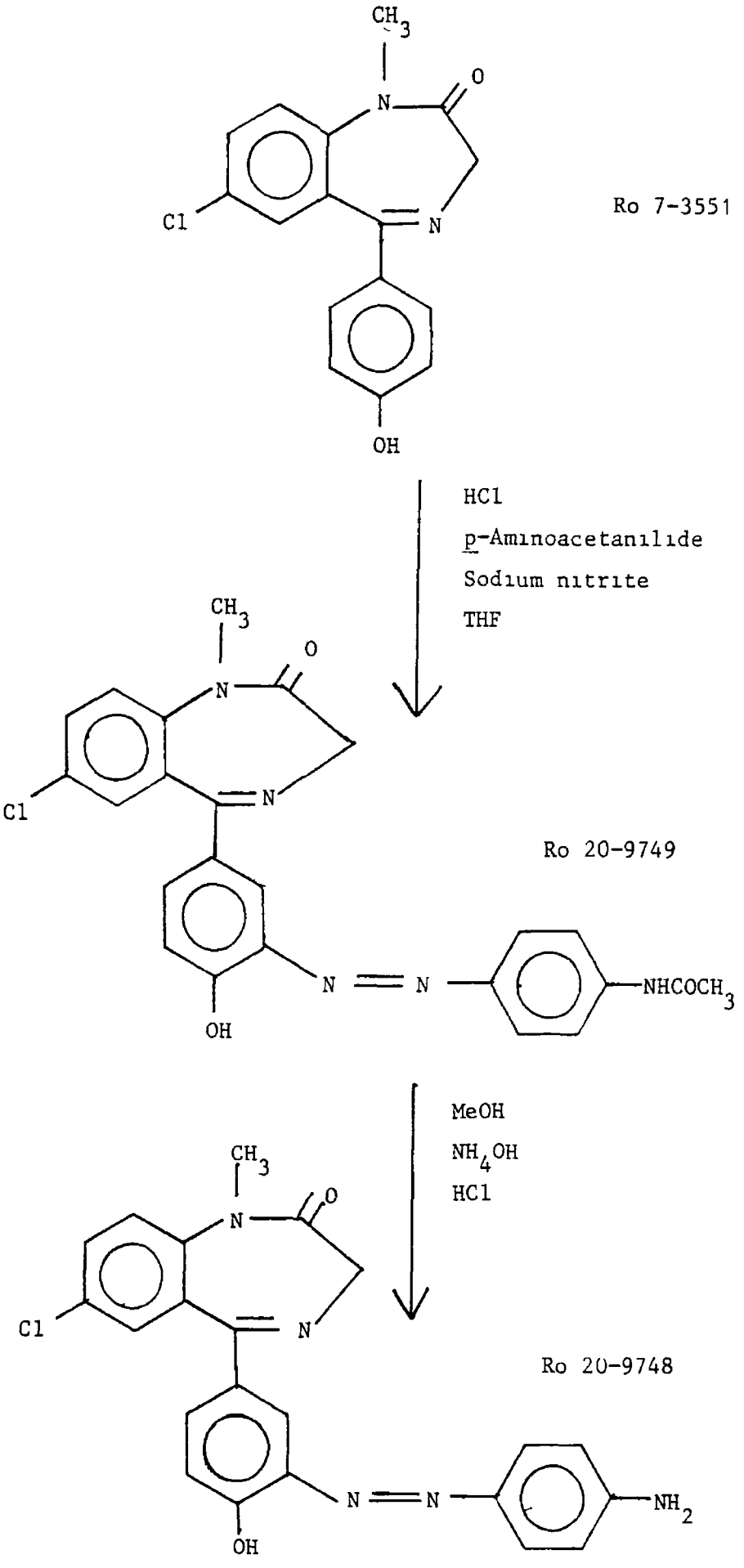


Ro 20-9749



Ro 20-9748

FIG IV 3 Showing the synthesis of the diazepam derivative used for conjugation to BSA



IV 2 RESULTS

A diazepam-BSA conjugate was synthesised and used for producing antibody-rich ascites fluid in mice This ascites fluid was purified and the binding of the antibodies to benzodiazepines studied

IV 2 1 CONJUGATE SYNTHESIS

The proposed synthetic pathway for the diazepam hapten is shown in Fig IV 3 This inserts a -NH_2 group onto the 5-phenyl group of the basic benzodiazepine molecule A 4-hydroxyphenyl derivative (Ro 7-3351) was used, as this directs the p-aminoacetanilide group to the 3-phenyl position This amino group was then used for a diazo coupling to BSA

When the method of Earley et al , (1979) was used only 31 mg of crystals were obtained when 400 mg of Ro 7-3551 were used This was a very low yield, representing between 5% and 10% yield Its IR spectrum (Fig IV 4) contains a broad band from 1660 to 1690 /cm This is the expected band for 2 C=O bonds There was insufficient product to proceed with the next stage of the reaction

Fryers revised method was then used This combined the synthesis of Ro 20-9749 and Ro 20-9748 500 mg of Ro 7-3351 was used and 1 25 g of crystals were obtained, which corresponded to impure Ro 20-9749 This was then converted to Ro 20-9748 by removal of one of the C=O groups and was purified using Florisil chromatography Crystallization from THF/ethanol gave 40 mg, and recrystallization gave 24 mg This was a very low yield (about 5%) The melting point was 254-257^{o}C, which is lower than the expected 265-268^{o}C The IR spectrum (Fig IV 5) shows a broad band at 3350-3450/cm which would be expected for the 2 -NH_2 groups, and a narrow band at 1670/cm which would be expected for a single C=O group The yield from this reaction was too low for synthesis of the conjugate

At this stage a sample of pure Ro 20-9748 was made available by Hoffman La Roche and was used for the conjugate synthesis 20 mg of this was coupled to 200 mg of BSA Attempts were made to couple Ro 20-9748 to fibrinogen by the same method, but in each case an

insolube complex was formed Similarly, an attempt to couple Ro 20-9748 to Keyhole Limpet hemocyanin (KLH) failed due to the inability to dissolve the KLH in the coupling buffer (pH 9) Consequently the diazepam-BSA conjugate was used for both immunisation and screening

IV 2 2 ASCITES PRODUCTION

A number of mice were immunised and were tapped to remove their ascitic fluid Eventually the surviving mice were killed and their ascitic fluid was collected The ascitic fluid was pooled and purified by ammonium sulphate precipitation

A total of 15 ml of ascitic fluid was obtained, with a total protein content of 387 mg (micro Bradford assay) The purified ascitic fluid had a total protein content of 69 mg and a concentration of 15 mg/ml The binding of the antisera to the diazepam conjugate was studied by ELISA (see Table IV 1)

The maximum binding for pure IgG was at 15 µg/ml and the titre was between 1 5 µg/ml and 150 ng/ml The maximum binding for the crude ascites was at a 1/100 dilution, and the titre was at 1/10,000

IV 2 3 CHARACTERISATION OF POLYCLONAL ANTIBODIES

To look at the role of binding of the antibody to BSA in the ELISA, ascitic fluid diluted in PBS-Tween was compared to that diluted in PBS-BSA (1% w/v)-Tween (see Table IV 2)

As can be seen from Table IV 2 the titre of the ascitic fluid diluted in PBS-Tween was between 1/50,000 and 1/100,000 However, the titre of the ascites when BSA was used in the diluent was between 1/5,000 and 1/10,000 This 10 fold drop in titre when BSA was used in the diluent was due to the removal of anti-BSA antibodies

For further studies it was necessary to purify the antibodies further This was achieved by using 2 CNBr-activated columns The first had BSA coupled to it and the second had the BSA- conjugate coupled to it The purified antibody was passed through the first column which removed anti-BSA antibodies Unbound antibody from this was then passed through the second column which bound the anti-diazepam antibodies These were eluted from the column with

glycine buffer pH 2 9 (see section II 11)

A total of 4 mg of purified antibody was applied to the column and 0 97 mg was eluted This represented a 25% yield The eluted antibody had a low titre (absorbance of 0 37 at 130 μg/ml) This purified antibody was used to examine binding to different diazepam derivatives

Derivatives of diazepam were dissolved in DMSO and used in a competitive ELISA to determine their ability to compete with the diazepam-BSA conjugate for binding to the antibodies The amount of antibody that bound was determined by ELISA (see Table IV 3)

Table IV 3 summarises the results of the competitive ELISA Conjugate, BSA, and different benzodiazepines were added to a microtitre plated coated with conjugate These competed with the immobilised conjugate for binding of the antibody It was found that the conjugate completly eliminated binding of the antibody to the immobilised conjugate, while BSA eliminated most of the binding Binding to Ro 20-9748 could not be determined as it came out of solution when the DMSO solution was diluted in PBS-Tween Ro 5-2180 and Ro 5-3453 had no effect on the binding of the antibodies to the immobilised conjugate Ro 5-2807 reduced the binding of the antibody to the immobilised conjugate by a small amount at 300 μg/ml, but not at the lower concentrations Ro 7-3351 reduced binding to the immobilised antibody at all concentrations

IV 2 4 MONOCLONAL ANTIBODY PRODUCTION

The diazepam conjugate was used to immunise mice for use in the production of monoclonal antibodies 14 fusions were performed with spleens from mice immunised with the diazepam conjugate In 8 of these fusions there was no growth of clones In one of these the lack of growth was probably due to a poor batch of serum that was found to have poor growth stimulating effects 1 fusion produced approximately 500 clones all of which were negative, and remained negative on repeated screening while the positive control gave a strong positive result 1 fusion produced clones but these died soon after

4 fusions produced positive clones 2 of these together produced 6 positive clones which on repeated screening were reduced to only

2 1 of these ceased to produce antibody and the other one was frozen down On thawing, this clone failed to produce antibody 1 fusion produced 12 positive clones on the first screening On the second screening there were only 2 positive clones which became contaminated 1 fusion produced 172 positive clones on the first screening On a second and third screening there were only 14 positive clones All 14 clones failed to expand into larger wells

TABLES AND FIGURES

TABLE IV 1

This table compares the absorbance values (405 nm) for different concentrations of crude ascitic fluid and the purified antibody, using diazepam-BSA conjugate as the antigen in ELISA The second antibody was a β-galactosidase-linked anti-mouse antibody

PURE IgG		ASCITES FLUID	
CONC	ABS	DILUTION	ABS
1 5 mg/ml	ND	10	1 9
150 µg/ml	ND	100	1 9
15 µg/ml	1 93	1000	1 77
1 5 µg/ml	1 24	10000	0 86
150 ng/ml	0 27	100000	0 14

TABLE IV 2

This table compares the absorbance readings from ELISA (405 nm) for ascitic fluid diluted in PBS-Tween with that diluted in PBS-Tween containing 1%(w/v) BSA

DILUTION	ABSORBANCE VALUES	
FACTOR	PBS-TWEEN	PBS-BSA-TWEEN
100	1 70	1 30
500	1 68	1 13
1 000	1 58	0 91
5 000	1 37	0 46
10 000	1 19	0 29
50 000	0 57	0 10
100 000	0 39	0 07

TABLE IV 3

This table shows the absorbance values (405 nm) for the binding of antibody to diazepam-BSA conjugate in the presence of benzodiazepine derivatives, BSA, or diazepam-BSA conjugate The values were determined by ELISA Drugs were dissolved in DMSO and made up in PBS-Tween and used at 3 different concentrations The control well contained DMSO and PBS-Tween without any drug BSA and the conjugate were made up in PBS-Tween only

DRUG/ ANTIGEN	ABSORBANCE VALUES		
	300 µg/ml	30 µg/ml	3 µg/ml
Ro 20-9748	ND	ND	ND
Ro 5-2180	0 17	0 16	0 11
Ro 5-3453	0 15	0 13	0 12
Ro 5-2807	0 12	0 10	0 10
Ro 7-3351	0 15	0 09	0 08
Conjugate	0 00	0 00	0 00
BSA	0 08	0 05	0 03
Control	0 19	0 11	0 10

ND= not determined

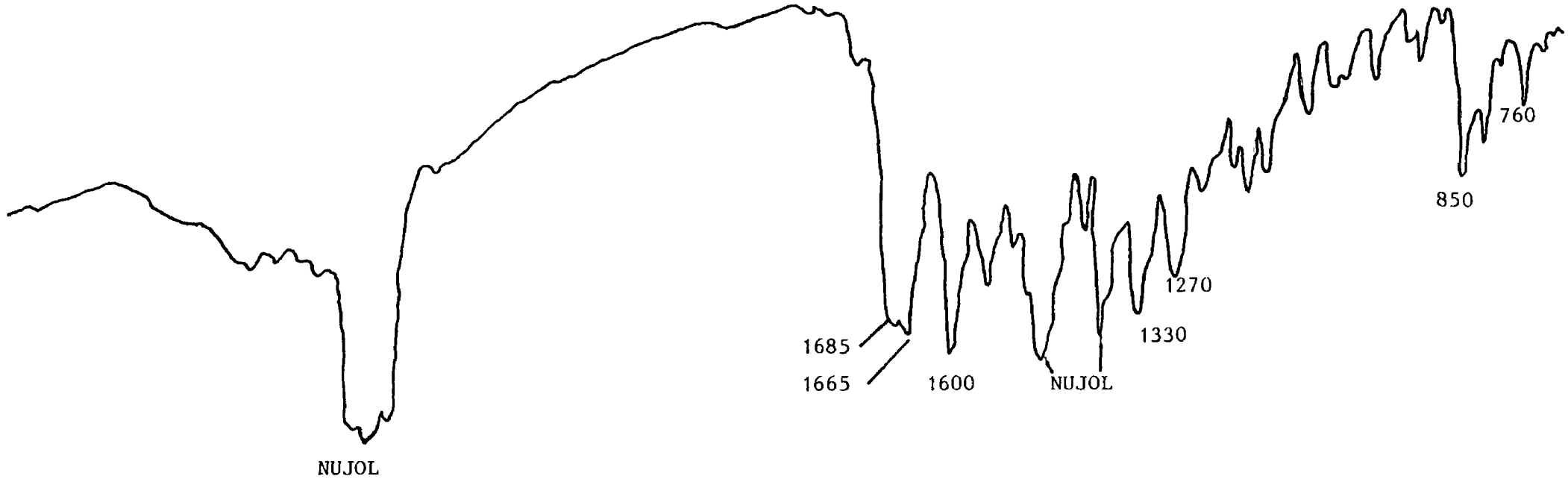


FIG IV.4 Showing the IR spectrum (prepared in Nujol) of Ro 20-9748, prepared as in text. The figures represent the frequency (cm^{-1})

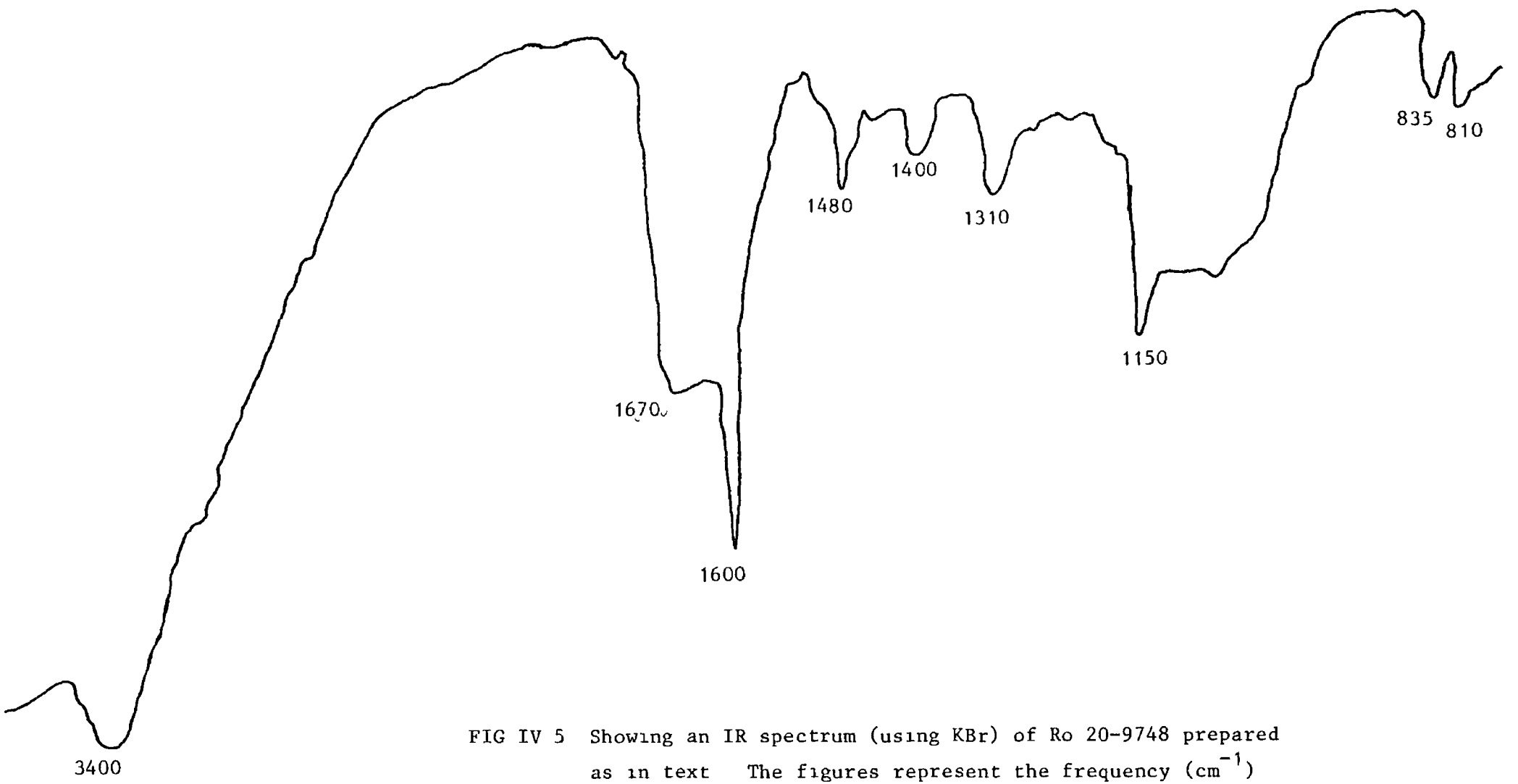


FIG IV 5 Showing an IR spectrum (using KBr) of Ro 20-9748 prepared as in text The figures represent the frequency (cm^{-1})

The production of diazepam derivatives for conjugation to BSA was quite successful, especially when the modified procedure was used, except for the low yield The final product had an IR indicating that it was probably Ro 20-9648 This IR agreed with that of Ro 20-9748 that was provided by Dr Fryer from Hoffman La Roche The mp was lower than that of Ro 23-9748 but this was probably a reflection on the purity of the compound The good agreement of the IR spectra is a stronger indication of identity than agreement of melting points

The production of polyclonal antibodies in rabbits, sheep, goats etc is a commonly used technique The major drawback is the expense of keeping these animals, especially since a large number have to be used, as not all animals will produce high titre antiserum The use of mice for the production of antiserum reduces this expense Since mice produce very little antiserum a modification is necessary to obtain sufficient antibody This involves producing the antibodies in ascitic fluid This method produced 69 mg of IgG with a working dilution of between 1 5 µg/ml to 150 ng/ml Thus, this technique can produce a reasonable yield of polyclonal antibody The major disadvantage is that not all mice respond Also, some of the mice die during the tapping procedure It is therefore essential that a sufficient number of mice be used to allow for this

Another solution to the production of antibodies is to produce monoclonal antibodies This method can give excellent results, but it is not always successful In this case 14 fusions yielded no clones In section III 3 the problems associated with monoclonal antibody production are dicussed

Another problem in the production of antibodies to conjugates is that a high percentage of antibodies will be directed against the carrier protein The ascitic fluid had a 10-fold decrease in titre when BSA was used in the diluent In fact, even in the affinity purified antibody most of the binding was eliminated by competition with BSA This indicates a high level anti-BSA antibodies Monoclonal antibodies can overcome this problem If clones are screened against a conjugate with a different carrier

protein, or are diluted in a solution of the carrier protein, anti-carrier clones can be eliminated The final number of stable clones directed against the hapten will be small, but if sufficient cells are used in the fusion this would not be a problem In previous fusions (see III 2 2) only about 8% of positive clones are stable With this success rate 12 positive clones to the conjugate would be necessary to obtain 1 stable positive clone If the number of antibodies against the carrier is 10-times greater than those against the hapten, then 120 positive clones against the conjugate would be needed to produce 1 clone that was positive against the drug portion

De Blas et al , (1985) produced monoclonal antibodies to benzodiazepines They used 3-Hemisuccinyloxy-clonazepam-BSA as the antigen The 4 monoclonal antibodies produced had high affinities for Ro 5-4864, which is a peripheral receptor ligand They also had higher affinities for derivatives with a methyl group in the 1 position, and a NO_2 or Cl in the 7 position The phenyl ring at position 5 was essential for binding, but addition of a Cl group to its 4 position did not effect binding

The characterisation studies on the polyclonal antibodies indicated that even after affinity chromatography to remove anti-BSA antibodies they are still responsible for the major part of the binding of the antiserum to the conjugate Ro 5-2180, Ro 5-3453 and Ro 5-2807 (diazepam) are all very similar in structure Ro 5-2180 and diazepam only differ in the substituent on the N(1) position of the heterocyclic ring In diazepam it is a methyl group, and in Ro 5-2180 it is hydrogen Neither of these had any effect on the binding of the antibodies indicating that the methyl group on the N(1) is not essential for the binding to the drug Ro 5-3453 is similar to diazepam except it has a $-NO_2$ group on the 7 position of the heterocyclic ring instead of the Cl This indicates that the Cl at position 7 is not important for binding Ro 7-3551 is also similar to diazepam, but it has a hydroxyl group at the 4 position of the 5 phenyl ring This does reduce binding of the antibody, indicating that this hydroxyl group is an important factor in the binding of the antibodies

Thus, the major epitope on the hapten is at the 5- phenyl position The hapten has a hydroxyl group on this phenyl group This hydroxyl group is necessary to direct the incoming

conjugating group (p-aminoacetanilide) to the 3-position To reduce the antibodies produced to this site it would be necessary to try and remove the hydroxyl group In fact the aminophenylazo-linker arm is probably also involved in the binding This illustrates one of the problems of the use of conjugates, namely the production of antibodies to the conjugate rather than to the hapten Thus, the polyclonal antibodies produced are more likely to bind to BSA or to Ro 7-3351, than to Ro 5-2807 (diazepam)

While the production of specific antibodies to diazepam was not achieved, the methodology for the production of polyclonal antibodies in ascitic fluid was successfully developed To ensure the production of antibodies directed against diazepam, and not the conjugate, the monoclonal antibody technique is necessary This technique enables a clone of cells to be selected, based on the ability of the secreted antibody to bind to a specific region of the antigen As well as their uses in immunoassay systems, antibodies to benzodiazepines have an important role to play in the production of anti-idiotypic antibodies These anti-idiotypic antibodies can be used to mimic the actions of the original drug as either an agonist or antagonist They can also be used to isolate the benzodiazepine receptor, and to locate and identify specific cell types in the CNS

SECTION V

COUMARIN AN *IN VITRO* STUDY OF ITS EFFECTS ON CELL GROWTH

V 1 1 HISTORICAL

Coumarin is a member of a class of compounds called benzopyrones These consist of fused benzene and alpha-pyrone rings (see fig V 1) Coumarin occurs naturally in plants and derives its name from the plant Coumarouna odorata Coumarin often exists as an odourless complex with sugars and acids, and is released by the action of acids, enzymes or uv radiation Thus, the smell of coumarin in hay only develops after drying the grass In 1820 Vogel purified coumarin from the tonka bean (Dipteryx odorata), and Perkin synthesised it in 1868 Plants containing coumarin have been used in folk medicine for such ailments as burns, leprosy, rheumatic disease and as antispasmodics

V 1 2 CHEMICAL PROPERTIES

Coumarin has a melting point of 70 6°C and a boiling point of 303 °C It has a molecular weight of 146 1 and it has a fragrant odour It is freely soluble in ethanol, chloroform and ether, and it is soluble in water at 0 25 g/ 100ml at 25°C

Coumarin is widely used in the perfume industry, due to its strong fragrant odour (Meuly, 1978) and it is also used as a sweetener and fixative in many perfumes It also used to enhance the fragrance of natural oils such as lavender, citrus and rosemary Coumarin is often used in combination with vanillin in soaps and detergents In some countries where there are less strict controls on food additives, coumarin is found with vanillin in confectionary Many tobaccos use coumarin as a flavour/odour enhancer Coumarin is also very effective in masking unpleasant odours, and is used in many industrial products, eg paints, plastics, rubbers etc It is especially effective against iodo-form, phenolic and quinoline odours Coumarin is also used in electroplating to reduce the porosity and increase the brightness of nickel, zinc and cadmium deposits

While coumarin has many synthetic and natural derivatives only a few have any economic importance 3,4-Dihydrocoumarin has a similar odour to coumarin and it is used in perfumes Unlike coumarin, it is permitted in foodstuffs and is used with vanilla,

butter, rum and caramel It is synthetised by hydrogenation of coumarin and it is also a metabolite 6-Methylcoumarin is also used as a flavour enhancer 7-Hydroxycoumarin (umbelliferone) has a blue fluorescence in solution and is used in sun screens and as a fluorescent brightener The most important of the derivatives is 4-hydroxycoumarin This is a metabolite of coumarin found in spoiled hay, and is also the precursor of dicoumarol and warfarin which, are vitamin K antagonists Warfarin is also used as a rodenticide (Meuly, 1978)

V 1 3 METABOLISM

The major phase I metabolic route of coumarin in animals is hydroxylation (Kaighen and Williams, 1961, Schilling et al, 1969) While hydroxylation can occur at all the possible ring positions, there are two major hydroxylation pathways that yield either 7-Hydroxycoumarin (7-OHC), or 3-Hydroxycoumarin (3-OHC) The 3-OHC is further metabolised by non-enzymatic ring splitting to o-Hydroxyphenyllactic acid (OHPLA) and o-Hydroxyphenylacetic acid (OHPAA) (see fig V 2) The major phase II metabolite is a glucuronide conjugate (Ritschel et al , 1977), which forms about 60% of the metabolites of coumarin in man (Moran et al , 1987)

There are species differences in the metabolites that are formed, but these are of a quantitative nature, rather than of a qualitative nature In primates (man and baboons) the major metabolite is 7-OHC (68-92%) (Schilling et al, 1969), with a small amount of OHPAA also formed In rats the major metabolite is OHPAA (12-27%), with small amounts of 3-OHC (1-2%) and very little 7-OHC (Kaighen and Williams, 1961) In rabbits the major metabolites are OHPAA (18-22%), 3-OHC (18-23%) and 7-OHC (10-16%), there are also trace amounts of 4-, 5-, 6-, and 8- Hydroxy-coumarin (Kaighen and Williams, 1961) (see Fig V 3) In mice there is a difference in metabolism in different strains The activity of coumarin- 7-hydroxylase in DBA/2J mice is much higher than that in C3H/HeJ mice (Wood and Conney, 1974) The coumarin hydroxylases are part of the microsomal enzyme system and are inducible enzymes The activity of coumarin-7-hydroxylase is much higher in human microsomes, than in rodent microsomes The coumarin-3-hydroxylase activity is very high in rodent micro-somes, and is absent in human microsomes (Cohen, 1979)

V 1 4 PHARMACOKINETICS

Ritschel et al , (1977) found that coumarin and 7-hydroxycoumarin-glucuronide (7-OHCG) had distributions that were best fitted by open, two compartment models The biological half-life for coumarin was 1 02 hr for the intravenous (iv) route, and 0 8 hr for the oral (po) route The biological half-life for 7-OHCG was 1 47 hr (iv), and 1 15 hr (po) 7-OHC was found to be best fitted by an open one compartment model Only 3 4% of the coumarin was present in the plasma as coumarin, the rest was converted to 7-OHC The 7-OHC was rapidly converted to its glucuronide conjugate (7-OHCG)- peak concentrations occured in less than 0 5 hr after iv administration of coumarin The levels of 7-OHC were never more than 2 2% of the 7-OHCG levels after iv administration There was no significant difference in the biological half life between oral, and intravenous administration of coumarin

This data indicated that coumarin was rapidly absorbed from the gut and and was metabolised by a first-pass effect (to the extent of 97%) to 7-OHC This in turn was rapidly converted to its glucuronide form There was evidence that the glucuronidation also occured in the gut and other tissues Further work by Ritschel and Hoffmann (1981) and by Ritschel (1984), compared the pharmacokinetics of oral coumarin and an oral, slow release preparation They found that on repeated dosage with the slow release preparation, higher plasma levels could be obtained Waller and Chasseaud (1981) found that the pharmacokinetics of coumarin in baboons was similar to that in humans

The levels of 7-OHCG in the plasma indicated that all the coumarin was absorbed from the gut Its volume of distribution during the beta phase was 1 7 times the body weight This indicates that coumarin is taken up by tissue Evidence indicates that coumarin is, in fact, widely distributed to both soft and deep tissues 7-OHCG is excreted by active tubular secretion, with approximately 90% of the dose of coumarin being excreted in this way This is different in other animals - In rats 32-38% is excreted in the faeces, while in rabbits 80-90% is excreted in the urine (see fig V 3) (Cohen, 1979)

To explain the rapid metabolism of coumarin into 7-OHC, coumarin is often considered to be a pro-drug, which is converted into an

active form, 7-OHC Work on high protein lymphoedema (HPLO) showed that 7-OHC had no effect on macrophages, while coumarin was active This cast doubt on the pro-drug theory But, the short half-life of coumarin, and its low bioavailability would suggest that it was unlikely to be the active compound This was also supported by research on the metabolism of coumarin Since the major metabolic route in rats is via 3-OHC, not 7-OHC, and since coumarin is effective against HPLO in rats, then either 3-OHC is an active form of the drug as well as 7-OHC, or else coumarin is the active substance Further studies on HPLO showed that 7-OHC is effective in many forms of HPLO, indicating that possibly the pro-drug theory is correct Casley-Smith (1985) showed that the effects of coumarin on HPLO remained 2 weeks after its administration This was thought to indicate that cells can store coumarin, but pharmacokinetic data shows that coumarin is rapidly excreted, 80 % of the dose was eliminated within 24 hr, and thus, it is unlikely to be stored It is more likely that coumarin acts by initiating a sequence of events eg altering cellular metabolism, or activating a second messenger via a receptor system This sequence then continue after the coumarin has been excreted

V 1 5 TOXICITY

Over the last thirty years coumarin has been in the centre of a dispute over toxicity This arose from a ban on the use of coumarin in foodstuffs by the FDA in 1954, after preliminary data showed that coumarin was hepatotoxic in the rat Since then there have been conflicting claims over its carcinogenicity, mutagenicity, and hepatotoxicity Unfortunately it is easier to have a drug declared toxic than it is to have it declared safe again Part of the problem is that chemically, coumarin, as a benzopyrone, is sometimes confused with the benzopyrenes The benzopyrenes consist of 5 fused benzene rings, and are potent carcinogens Another group of chemicals, which are often confused with coumarin, are the dicoumarols These are formed from two 4-hydroxycoumarin molecules Coumarin, unlike the dicoumarols, has no anticoagulant properties (see below)

In acute studies the LD_{50} of coumarin in the rat ranged from 290 mg/kg (Hazleton et al , 1956) to 680 mg/kg (Jenner et al , 1964) In mice the LD_{50} ranged from 196 mg/kg in Hakka mice (Kitaguwa and Iwaki, 1963) to 780 mg/kg in DBA/2J mice (Endel and Seidel,

1978) The LD_{50} for CH3/HeJ mice was 420 mg/kg (Endel and Seidel, 1978) (see Fig V 4) Fontaine et al ,(1967) found that the LD_{50} of 3-OHC in mice was 1 8 g/kg, while 3 2 g/kg of 7-OHC in a similar study had no effect This shows that 7-OHC, the major metabolite in humans, is a lot less toxic than 3-OHC, the major metabolite in rodents This was further supported by Feuer et al , (1966) who showed that OHPAA (a product of ring fission of 3-OHC) causes liver damage at doses, which are lower than the dose of coumarin needed to cause damage It also takes coumarin 3 days longer to cause these effects

In a 2 year study Bar and Griepentrog (1967), and Griepentrog (1973) found liver damage and cholangiocarcinoma with 5,400 ppm in the diet of rats With 2,500 ppm there was delayed growth and with 1,000 ppm there was no effect In a similar study Hagan et al , (1967) found that with 5,000 ppm in their diet, rats had liver damage with cholangiofibrosis after 2 years With 2,500 ppm in their diet they had slightly fatty livers, with a reduction in growth Brune and Deutschwenzel (1985) found similar liver disorders with 5,400 ppm in rats' diet after 2 years They also found biochemical changes in the dose range 1,800-5,400 ppm They rated 600 ppm as the no effect level (see Fig V 5) Feuer et al, (1966) found that coumarin (20mg/kg/day, for 7 days) inhibited hepatic glucose-6-phosphatase Ueno and Hirono (1981) found that 0 5% coumarin in the diet of golden hamsters had no effect after 2 years Seidel and Kreuser (1979) found that after 32 weeks, 1 8 g/kg/day had no effect on DBA/2j mice, and caused only slight changes in liver enzyme levels in CH3/Hej mice Evans et al , (1979) found that after 2 years, 22 5 mg/kg/day had no effect on baboons, and 67 5 mg/kg/day had only a slight effect on the liver (dilation of hepatocytes) (see Fig V 5)

There is a certain amount of contradiction between the data on both chronic and acute toxicity Part of the discrepancy lies in the dose regimens used Doses that cause death during gastric lavage tend to be lower than those that cause death in food This can be due, in part, to the actual gastric lavage procedure itself, as well as the carrier used Thus, Hazleton et al , (1956) report an oral LD_{50} for coumarin, in rats, of 290 mg, using propylene glycol as the carrier and the LD_{50}, using corn oil as the carrier, was 520 mg (see Fig V 4) Another problem is that using a compound such as coumarin, which has a strong odour, in

food, may effect the eating pattern of the animals - hence it is difficult to estimate accurately the dose consumed When higher concentrations are used in the food the animals may well eat less, and thus they consume a lower dose of coumarin This is apparent in some of the chronic studies where there is a reduction in growth in the animals at high dose levels, often accompanied by a reduction in food consumption during the first few months of the study Thus, Endel and Seidel (1978) found that the oral LD_{50} for coumarin, in corn oil, in DBA/2J mice, was 780 mg/kg, while 2 g/kg/day in their food, for 32 weeks, had no effect (Seidel and Kreuser, 1979) This latter study should be classed as a sub-chronic study, rather than as an acute study Another problem is in converting dietary doses to doses of mg/kg/day Casley-Smith (1986) uses the ratio 1,000 ppm, equivalent to 100 mg/kg/day Evans et al , (1979) uses 1,000 ppm, equivalent to 50 mg/kg/day Schafer and Brummer use 1,000 ppm equivalent to 20 mg/animal/day in their literature review Allowing for an adult rat to be between 300 g to 500 g, this latter conversion gives 40-60 mg/kg/day Thus, the method used by Evans and that used by Schafer and Brummer give similar figures, which are half those of Casley-Smith

The relevance of LD_{50} data in acute toxicity studies is questionable Sodium chloride has an oral LD_{50} in rats, of 3 7 g/kg, while 7-OHC coumarin has an LD_{50} of 3 2 g/kg, and yet salt is non-toxic At best acute toxicity studies can give an estimate of the therapeutic ratio - the ratio of the ED_{50} (effective dose in 50% of the test animals) to the LD_{50} (lethal dose in 50% of the animals) There is a 10 fold difference between the highest dose used clinically (1,200 mg/day, ie 20 mg/kg - for a "normal" 60 kg male) and the lowest dose that caused death (196 mg/kg in mice) It must be remembered that this figure only applies to massive, single exposure to coumarin in mice This therapeutic ratio gives an idea of the safety margin between the dose that is used clinically and the amount that would have to be ingested accidentally to cause death With a conservative estimate, 12 g of coumarin has to be consumed as a single dose (in a 60 kg male), before death would be remotely likely This makes coumarin relatively safe

Of greater benefit in clinical applications are the chronic toxicity studies In the baboon 22 5 mg/kg had no effect on the

liver, after 2 years (Evans et al , 1979) This is comparable to the maximum dose used to treat humans, and even then they would only be kept on this dose for a short period of time In the same study, baboons showed signs of liver enlargement (due to dilation of the endoplasmic reticulum) at 67 5 mg/kg, which Evans et al , considered to be the start of hepatotoxicity This dose is lower than the dose that caused toxicity in rats - 1,800 ppm for 2 years (equivalent to 90 mg/kg/day) Both these doses cause slight changes in the liver and are probably just below the dose that causes more serious liver damage In baboons the no effect dose was 22 5 mg/kg/day, while in rats it was 30 mg/kg/day

Some authors discount the changes seen at the 67 5 mg/kg level, eg Cohen (1979), and maintain that this is not evidence of frank hepatotoxicity, as is seen in rats While the toxicity is different, Zimmerman (1978) considers dilation of the endoplasmic reticulum to be the ultrastructure equivalent of ballooning degeneration These changes are also the first signs of carbontetrachloride poisoning While Zimmerman does not consider these changes to be of clinical relevance, they must not be ignored The fact that the chronic toxicity studies are different than the acute studies is not unusual, since the toxic effects seen in both are often caused by different mechanisms Cohen also points out that the changes seen in the rat and the baboon are different This could be due to 2 factors The first possibility is that the toxicity seen is different Since the metabolism is different in each animal it is quite likely that the toxicity observed will be different, especially since the metabolites are suspected to be the toxic agents Thus, the depression of glucose-6-phosphatase (G-6-P) activity in rat livers could be the toxic effect due to 3-OHC (and/or its ring fission products), and the ultrastructure changes seen in baboons could be due to the toxic effect of 7-OHC Hence, while rats are not good models for the toxicity of coumarin in humans, neither are they good models for the toxicity of coumarin in baboons A second possibility is that the effect seen is the same, and that if higher doses were given to the baboon changes in G-6-P activity would be seen This is possible since biochemical changes did not occur in the rat below a dose of 1,800 ppm (equivalent to 90 mg/kg/day) (Brune and Deutschwenzel, 1985) which is above the highest dose given to baboons However, Feuer et al , (1966) found that this effect on G-6-P in rats occured at low doses in a short period of time (20mg/kg/day, for 7 days), and

thus, it would have been expected to occur in baboons at the dose levels they received The data from the baboon study is the most appropriate to use for extrapolation to human therapy, as the pharmacokinetic profile of baboons is very similar to that in humans

Bar and Ggriepentrog (1967) and Griepentrog (1973) found evidence of biliary carcinomas in rats given coumarin in their food at a dose of 5,000 and 6,000 ppm, for 2 years This resulted in the NIOSH registry of toxic effects of chemical substances citing coumarin as carcinogenic Hagan et al , (1967) found that rats given 5,000 ppm in their diet for 2 years developed cholangiofibrosis (proliferation of the bile ducts) Since Bar and Griepentrog were the only workers to find these carcinomas the question is whether their diagnosis of cholangiocarcinoma was correct, or would cholangiofibrosis be a more accurate diagnosis? Another important question is whether cholangiofibrosis is a neoplastic or pre-neoplastic lesion The original slides were examined by Evans and Conning and they found no evidence of carcinoma (Cohen, 1979) There was no evidence of metastases either It is generally considered that these lesions were cholangiofibrosis, misdiagnosed as cholangiocarcinoma Evans et al , went on to show that cholangiofibrosis is not a prequisite for hepatocellular carcinoma and that it is not part of a carcinogenic process (personal communication) The NIOSH registry also cites coumarin as being neoplastic, ie producing tumours not clearly defined as carcinogenic They cite the work of Roe and Salmon (1955) in this However, this is erroneous In their paper Roe and Salmon state that coumarin showed no evidence of initiating tumours

Ames tests performed on coumarin and its metabolites indicated that they were not mutagenic (HRC, 1978) Other tests for mutagenisis gave similar results, eg the micronucleus test (Sterner and Korn, 1981) In fact coumarin has been shown to have anti-mutagenic effects (Ohta et al , 1983) Grigg (1979) found that coumarin inhibited excision repair in bacteria Coumarin was found to be non-teratogenic (Preuss-Ueberschar et al , 1984) and also it had no phototoxic effects (Kaidbey and Kligman, 1981, Prosser et al , 1988)

A number of human studies have been performed using coumarin (Cerlek, 1977, Zanker et al , 1984, Marshall et al , 1987a) and all showed no adverse effects or evidence of liver dysfunction Faurshou (1982) reported one patient who developed an unpredictable hepatotoxicity to coumarin In section VI, there is a report on a clinical trial of coumarin The trial consisted of 2,163 patients with an incidence of 0 23% definite and 0 13% possible/probable hepatotoxicity This was an idiosyncratic type of hepatotoxicity and did not show up in other clinical trials due to the low incidence This toxicity is different to that found in rats

The LD_{50}s in all species of animals is well above the clinically used dose in man The dose used clinically has been found to be non-toxic in 2 year chronic studies in both rats and baboons There is no evidence of teratogenic, mutagenic or carcinogenic activity by coumarin There is a very low incidence of idiosyncratic hepatotoxicity in human studies, but these are well within acceptable limits for clinical use Thus, despite early fears about the safety of coumarin all the current evidence indicates that coumarin is safe for clinical use

V 1 6 PHARMACOLOGY

In 1936 Szent-Gyorgyi and Rusznyak showed that an extract of lemons, which they called "citrin", had vitamin C like activity, and it also increased the permeability and fragility of capillaries Further work found no vitamin C activity in the extract, which was probably due to a contamination of the sample This activity was also found in paprika, and thus, the active ingredient was called vitamin P Subsequently, the active agents from these extracts were identified as flavones, and the name vitamin P was dropped in favour of bioflavenoids Pharmacologically the benzopyrones can be classed as bioflavenoids, along with other compounds such as rutin, eriodictyol, quercetin, and hesperetin

The role of the bioflavenoids in normal physiology is controversial It is estimated that the average western diet contains approximately 1 g of flavenoids (Middleton, 1984) This is probably sufficient to exert a physiological effect It is postulated that the flavenoids play a role in maintaining the

capillary bed They have been shown to have a weak vaso-constrictor action on the capillaries and also to decrease capillary permeability and fragility (Casley-Smith et al , 1975) The existence of a capillary defect that responds to bioflavenoids is questionable but it is thought that diabetic retinopathy may be one such defect

Coumarin has been shown to reduce oedema by activating macrophages (see below), as well as acting on the rest of the immune system (see below), and it also has effects on the growth of tumour cells (see below) Coumarin inhibits the release of histamine from the mast cells and also has a mild adrenergic activity (Middleton, 1984), probably due to its inhibition of cathecol-o-methyltransferase This enzyme metabolises noradrenaline and thereby acts to terminate the adrenergic signal This may possibly account for coumarin's reported spasmolytic activity (Kitagawa and Iwaki, 1963) Parente et al , (1979) and Koh (1979) showed that coumarin had anti-inflammatory activity Ritschel et al , (1982, 1984) and Kitagawa and Iwaki,(1963) showed that coumarin had anti-pyretic effects on endotoxin-induced fever in rabbits Coumarin itself has little effect as an antibacterial or antifungal agent (Feuer, 1974), but some of its derivatives have eg dicoumarol and novobiocin

Coumarins are perhaps most famous for their anticoagulant activity This was first noticed by Schofield in sweet clover disease in cattle This was later found to be due to the formation of dicoumarol in hay when it spoiled Dicoumarol is a potent anticoagulant and it was this effect which resulted in the death of the cattle These drugs inhibit synthesis of prothrombin and other coagulation proteins, by antagonising vitamin K Coumarin has no anticoagulant activity itself (Feuer, 1974)

V 1 7 HIGH PROTEIN LYMPHOEDEMA

Oedema is the swelling of tissue due to an increase in interstitial fluid The lymphatic system drains the interstitial fluid, but during oedema the system cannot cope with the excess fluid, resulting in oedema This failure to drain the interstitial fluid can be due to a blockage in the lymphatic vessels, or too much fluid entering the tissues The main role of the lymphatics is to remove proteins and other macromolecules from the tissues These macromolecules cannot enter the blood, because

their concentration is usually higher in the blood than in the tissue Also, being so large, they cannot easily enter through the close junctions

In low protein oedemas there is an increase in fluid in the tissues, which cannot be removed quickly enough by the lymphatics This can easily be treated by diuretics, which increase fluid loss through the urine (diuresis) The second type of oedema is high protein lymphoedema, and it is due to an accumulation of protein in the tissues Diuretics will remove the fluid, and decrease the oedema But, when they are stopped, the osmotic pressure of the protein draws the fluid back into the tissue, causing oedema This increase in macromolecules in the tissues can be due to an increase in permeability of the capillaries (due to inflammation, or trauma), a deficiency in the lymphatics, or a loss in tone in the tissues, resulting in less fluid being carried to the lymphatics

Foldi-Borcsok et al , (1971) found that coumarin was effective in treating oedemas due to thermal damage, surgically-produced lymphostatic oedema, and oedema due to intraperitoneal and subcutaneous injection of protein Clodius and Piller (1978) found that 82% of patients, with postmastectomy lymphoedema of the arm responded to treatment with coumarin The response was slow - 0 5 cm reduction in circumference in 10 months (as against 1 cm increase in circumference per year in untreated patients), but better than can be obtained with other drugs

Coumarin exerts this reduction in lymphoedema by a number of different effects Coumarin has been shown to increase lymph flow (Pflug and Taylor, 1975) which would help drain the tissue The relevance of this is uncertain, since in oedema the lymphatics would usually be maximally stimulated, and thus further stimulation would be ineffective Piller (1976c) showed that coumarin had no effect on thermally-induced oedema if the rats were pretreated with silica, which selectively kills macrophages Piller (1978) showed that coumarin increased the number of macrophages migrating to subcutaneously implanted coverslips, but also increased the percentage that were stimulated (Piller and Clodius, 1979) Piller (1977) showed that coumarin increased proteolysis in rats with high protein lymphoedema Bolton and Casley-Smith (1975) showed that coumarin increased macrophage

proteolysis in vitro Piller and Casley-Smith (1975) and Piller (1976a) showed that coumarin increases phagocytosis by the macrophages, ie it stimulates the reticuloendothelial system (RES) A number of authors have shown that coumarin increases vascular permeability, and thus, the leakage of protein, eg Casley-Smith and Window, (1976)

During oedema, eg after a burn, there is leakage of protein out of the vascular system, which draws fluid with it, causing the swelling When coumarin is administered a large percentage of it is protein-bound, especially to globulins and albumin (O'Reilly, 1971, Garten and Wosilait, 1971) Its initial effect is to increase the leakage of protein from the blood to the tissue This leakage of protein allows the bound coumarin to get to the site of oedema Coumarin also increases the number of macrophages at the site of oedema and acts to activate them The coumarin, which is bound to the proteins, may act as a hapten making the proteins more antigenic, and thereby facilitating phagocytosis It is also thought that altered proteins, are more chemotactic for macrophages than native proteins This activation of macrophages results in an increase in proteolysis, and the resulting protein fragments leave the cells by diffusion, or reverse phagocytosis These peptides are then removed by the vascular system because their size, and their concentration gradient (which, unlike that of proteins, is directed from the tissue) allow diffusion into the blood Once the protein is cleared from the tissue, the associated fluid will be quickly absorbed As well as the increase in intracellular proteolysis, there is an increase in extracellular proteolysis due to the leakage of acid lysosomal proteases during phagocytosis Coumarin also increases the activity of neutral proteases (Piller, 1976b) In the case of pre-existing lymphoedema there is usually a substantial amount of fibrosis at the site of the oedema Coumarin has been found to stimulate the breakdown of fibrotic tissue (Piller and Clodius, 1976)

Thus, by controlled proteolysis coumarin can slowly reduce the protein levels at the site of injury, and thereby reduce the oedema The effects of coumarin are limited to the site of the injury, since that is where the drug goes when it is protein-bound and that is where the macrophages are Because coumarin increases the natural protease activity of cells, and since its effects are limited to the site of oedema, its actions are termed "controlled

proteolysis".

V.1.8 IMMUNE SYSTEM

As well as its effect on macrophages (see above), coumarin also has other effects on the immune system. Marshall and Hollingsworth (1987) found that coumarin augmented the response of peripheral blood lymphocytes to phytohaemagglutinin *in vitro*, but not to Concanavalin-A, nor to pokeweed mitogen. Zanker *et al.*, (1984) found that coumarin moderately increased natural killer activity *in vitro* when the effector:target ratio was 1:30, but it decreased NK activity when the ratio was 1:10. Marshall *et al.*, (1987b) found no change in the peripheral blood leucocyte count or the differential count in patients treated with coumarin. They used monoclonal antibodies to B-cells, T-cells, helper and suppressor T-cells and NK cells for their enumeration by fluoresence activated cell sorting (FACS).

There is some evidence that coumarin can activate the cellular immune system if it is suppressed. In particular coumarin has been found to increase the helper T-cells relative to the suppressor T-cells (Thornes 1983a; 1983b). Because of the actions of coumarin on the immune system it is classed as a biological response modifier.

V.1.9 CANCER

Feuer *et al.*, (1976) found that coumarin inhibited the formation of 7,12-dimethylbenz(a)anthracene (DMBA) induced tumours in mice when given prior to tumour induction. There was no effect when coumarin was given after the DMBA. Wattenberg *et al.*, (1979) found similar results in mice with DMBA and benzo(a)pyrene induced tumours. They also found that 7-OHC had no effect on tumour occurence. In both cases there was a dose-dependent response in relation to tumour inhibition by coumarin.

These results, and earlier work done on the effect of oral coumarin anticoagulants on fibrinolysis, and its role in cancer therapy (Thornes, 1975), resulted in pilot studies on the the use of coumarin in the treatment of melanoma. Melanoma was the main cancer under investigation since it is easily diagnosed at an early stage, and there is no adequate chemotherapy available for it. Thus, there are no ethical reasons for not treating with coumarin. Coumarin therapy is normally used after surgical ex-

cision of the tumour Thornes et al ,(1982) used coumarin in combination with cimetidine, while Zanker et al , (1984) used coumarin alone in treating melanoma Thornes found a decrease in the recurrence rate during coumarin therapy, and a stabilisation of the disease in some patients Zanker found similar effects in his study Thornes (1982, 1983b) found that the histamine H2 receptor antagonist, cimetidene (1g/day), when used to supplement preexisting coumarin therapy (100mg/day) was more effective with melanoma Cimetidine is itself effective at activating the immune system At present multicentre trials are in progress to determine the effectiveness of coumarin in chemotherapy of melanoma, and to determine the appropriate dose regimen as well as the stages of the disease which are most susceptible to coumarin A report from a multicentre trial in Ireland indicated that the recurrence rate in untreated patients was 10 in 14 patients after 2 years The rate in patients treated with 50mg coumarin per day was 2 in 13

Marshall et al , (1987a) used coumarin and cimetidine to treat patients with renal cell carcinoma in a pilot study In a total of 45 patients they reported 3 patients with a complete response, 11 with a partial response, and 12 with static disease All the patients that responded, and 75% of the static patients had nephrectomies

Conley et al ,(1987) found that coumarin inhibited the growth of 3 cell lines (2 renal cell carcinomas and 1 erythroleukaemia) in vitro Tseng et al (1986) reported that coumarin inhibited tumourogenises in a cell line containing the ras oncogene

Thus, it would appear that coumarin acts at 3 levels Firstly, it inhibits the growth of the tumour cells Secondly, it activates the immune system which prevents metastases, and possibly it is tumouricidal Thirdly, it is also anti-mutagenic It is probably most effective in combination with surgery, and with cimetidine therapy

V 1 10 CHRONIC INFECTIONS

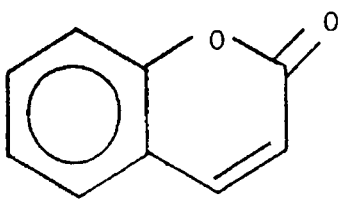
Brucella abortus is a major cause of brucellosis in humans It is estimated that 20% of acute cases become chronic During the chronic phase of the disease the organism infects macrophages, protecting it from the immune system The main symptoms of chronic brucellosis are chronic tiredness, intermittent fever and

night sweats, often accompanied by depression and anxiety There is also an associated anergy, also known as chronic fatigue syndrome, and Myalgic Myeloencepholopathy (ME) with the chronic infection (Thornes, 1977) This immune suppression is diagnosed by delayed hypersensitivity to 2,4-dinitrochlorobenzene and is characterised by a reduced helper/suppressor T-cell ratio

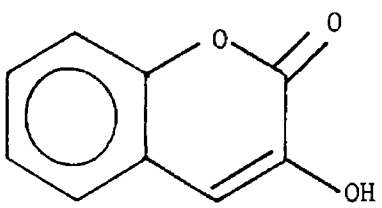
When immune stimulatory drugs such as coumarin and levamisole are administered the symptoms of the chronic infection disappear, often accompanied by an acute bout of brucellosis This indicates that the symptoms are caused by the immune suppression rather than by the infection (Thornes, 1977) Coumarin was found to be most effective, and the least toxic immune stimulant The sympathomimetic effects of coumarin would also be of benefit as they would be similar to the antidepressant effects of monoamine oxidase inhibitors

These results have encouraged the use of coumarin in the treatment of other chronic infections, including mononucleosis, mycoplasmosis, toxoplasmosis, Q fever and psittacosis Coumarin is also undergoing trial as an anti-leprotic and anti-tubercular drug There is also a possible role for coumarin in preventing the progression of AIDS in HIV positive patients

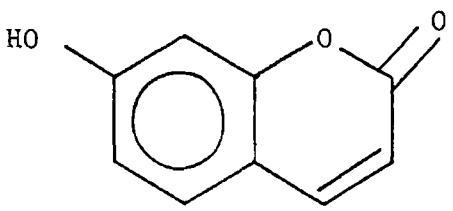
There are a number of unanswered questions about coumarin This project deals with 3 of these The first is whether coumarin has any effect on tumour cell growth The second is whether coumarin, or 7-hydroxycoumarin, its major metabolite in humans, is the active form of the drug Finally, the question of toxicity is also dealt with Does coumarin have an effect on liver function in humans, or is it a drug condemned due to experiments performed on inappropriate animal models?



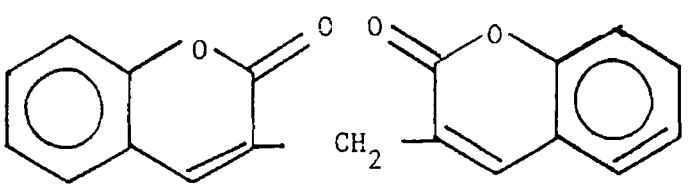
Coumarin



3-Hydroxycoumarin
(3-OHC)



7-Hydroxycoumarin
(7-OHC)



Dicoumarol

FIG V 1 Showing the structures of coumarin and some of its derivatives

Fig V 2 METABOLISM OF COUMARIN

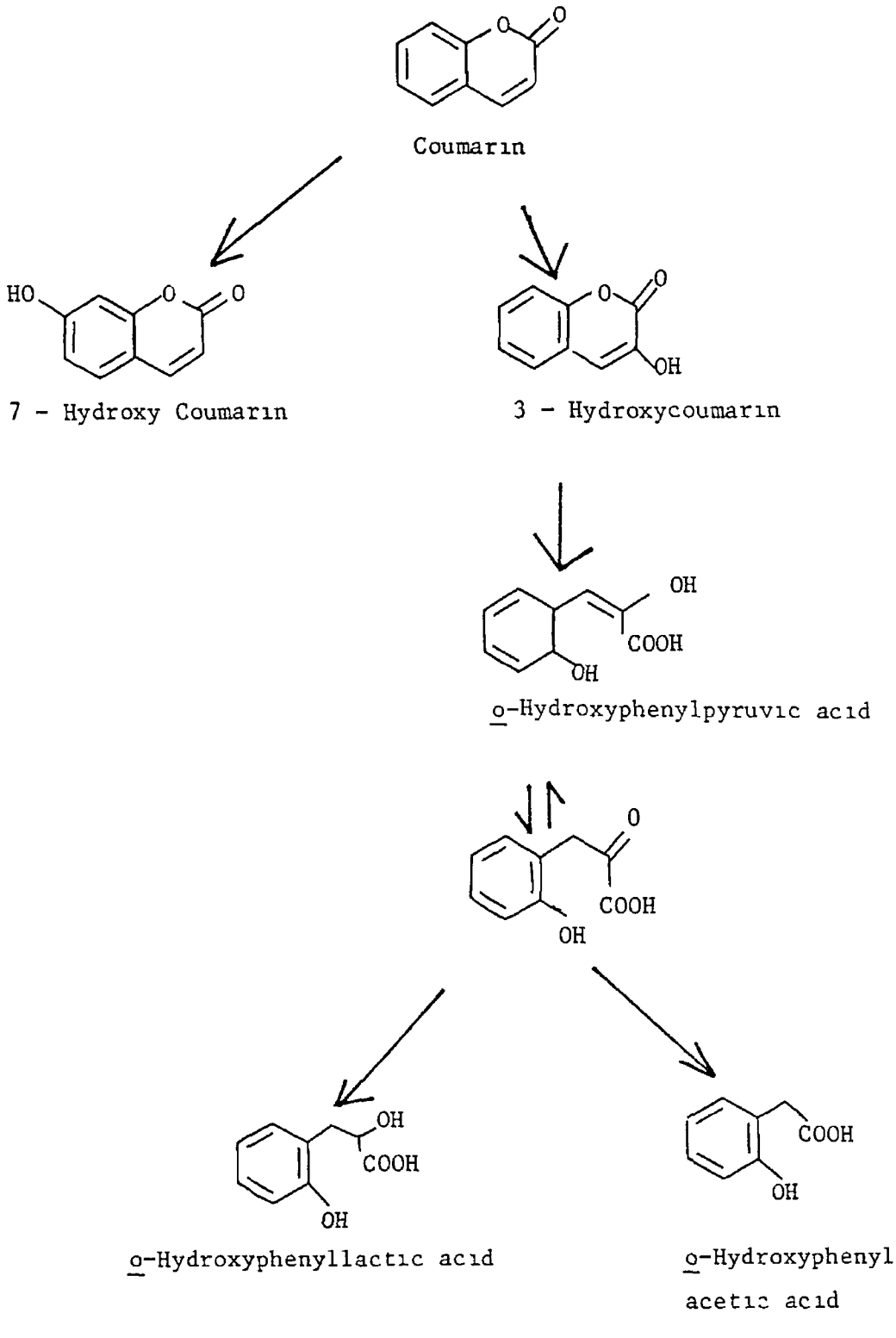


Fig V 3 Showing the percentage of the administered dose of coumarin excreted in the urine in man, rabbits, and rats Also shown are the major urinary metabolites expressed as a percentage of the administered dose

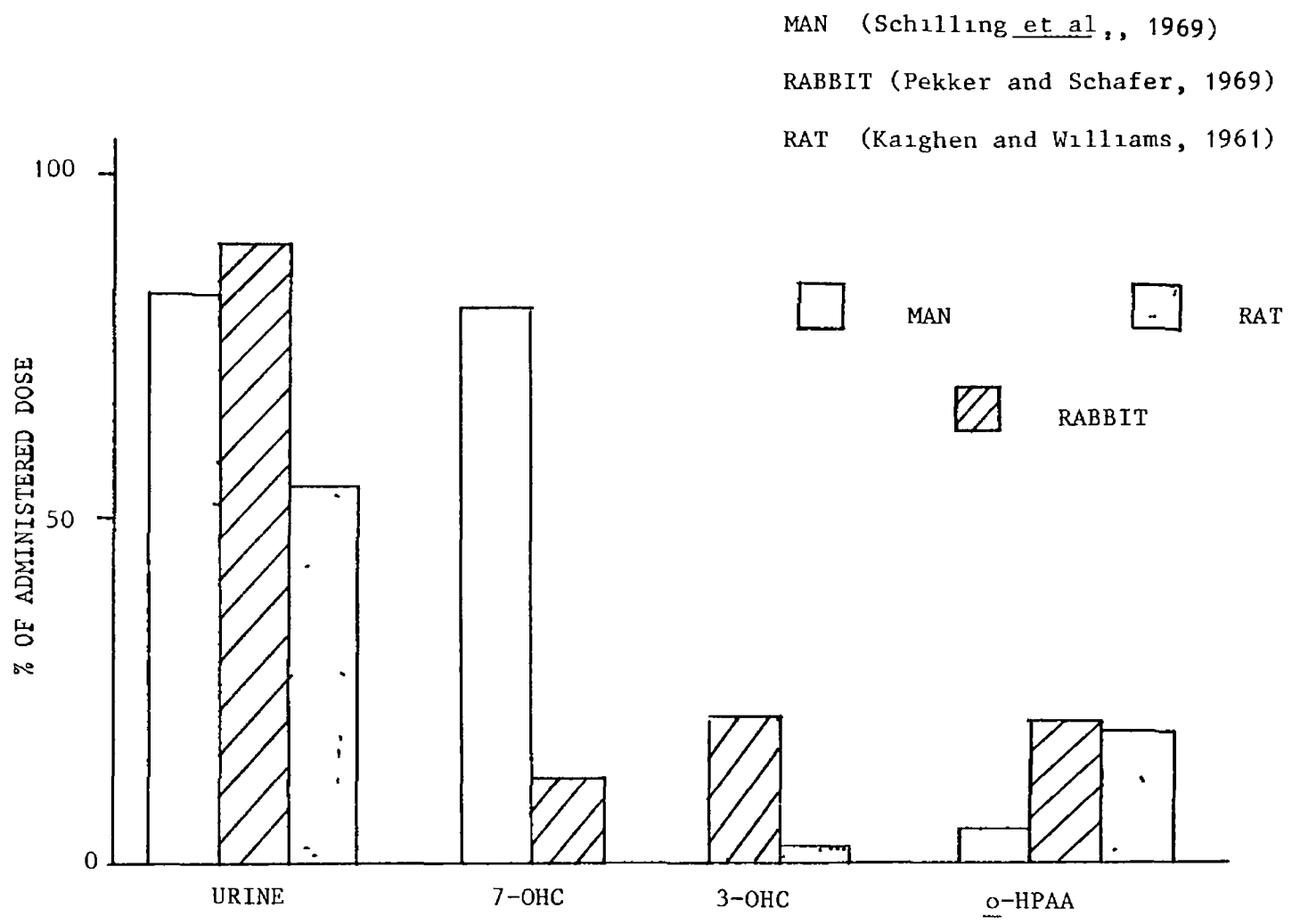
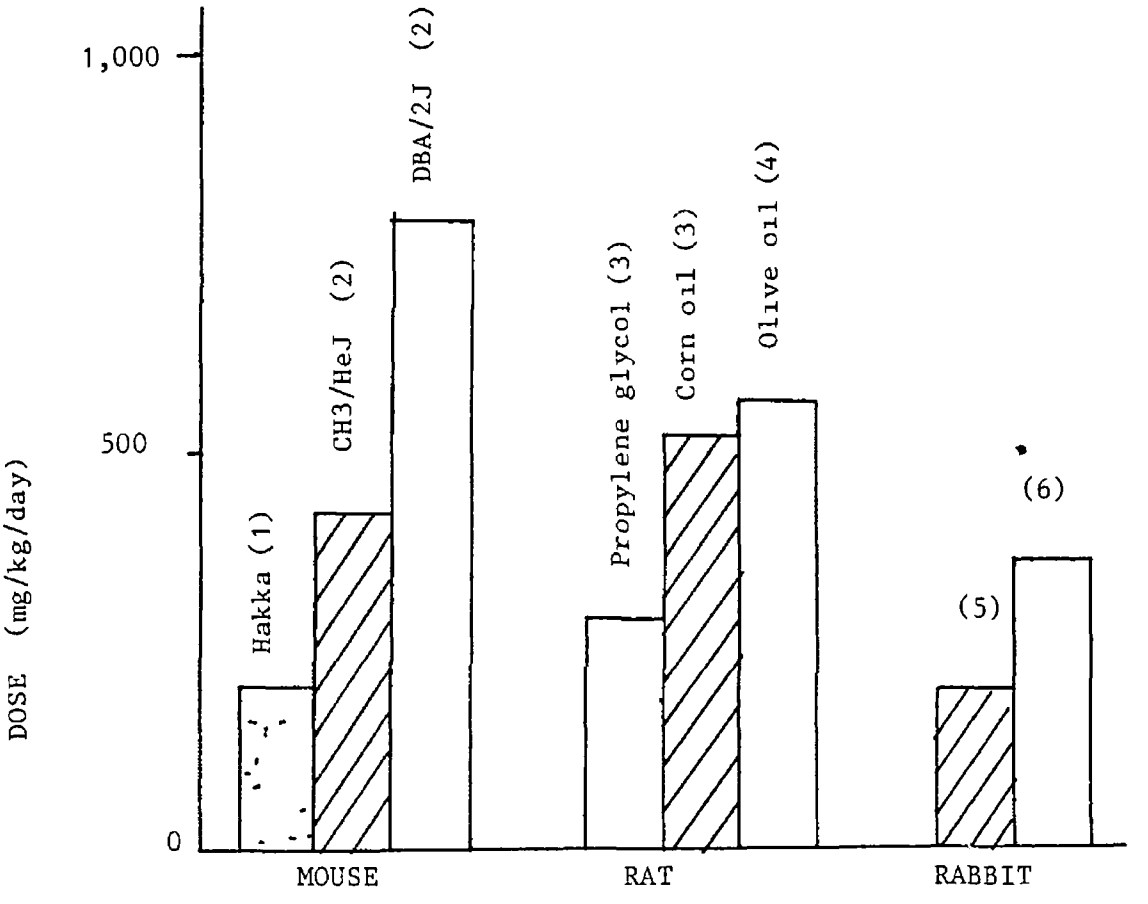
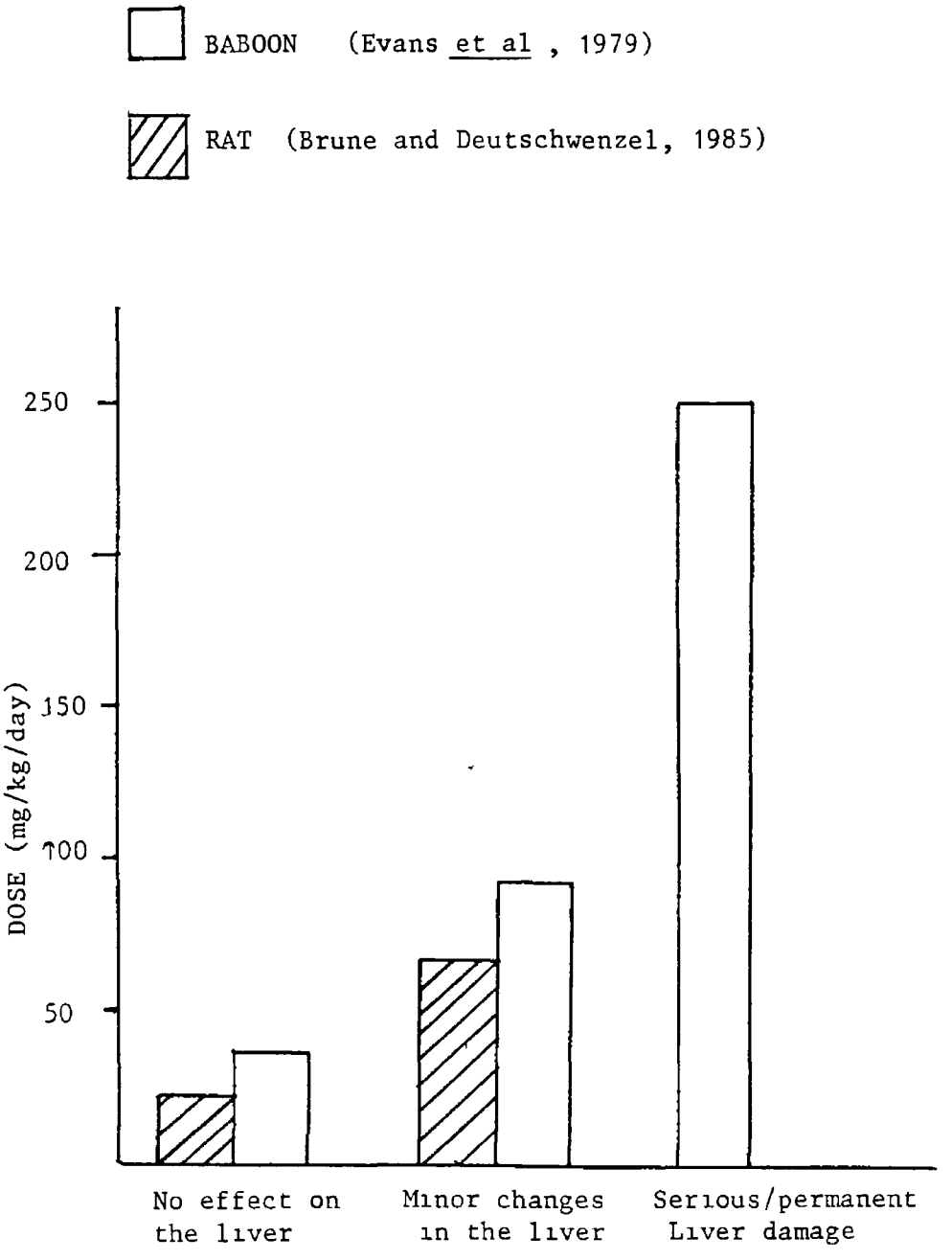


Fig V 4 Showing the LD_{50} of coumarin for different strains of mice Also shown are the LD_{50}s for different vehicles used in gastric lavage in rats The LD_{50} for rabbits from different laboratories are also shown



(1) Kitagawa and Iwaki, (1963)
(2) Endell and Seidel, (1978)
(3) Hazelton et al ,(1956)
(4) Foldi-Borcsok
(5) Feuer, (1974)
(6) Ellinger, (1980)

Fig V 5 Showing the dose of coumarin needed to cause an effect on the liver in 2 year studies in rats and baboons



V 2 1 STATISTICAL ANALYSIS

Table V 1 to V 10 summarise the effects of coumarin and 7-OHC on cell growth A t-test was used to test the hypothesis that the mean of the number of cells growing in drug 1 (1) was equal to the mean of the number of cells growing in drug 2 (2) The t test was performed using Minitab on a VAX 780 computer, and it was assumed that the variances of the means were unequal Figures in parentheses are the standard error of the means (SEM) P1 is the probability that 1 (coumarin control) was equal to 2 (coumarin test dose) P3 gives the similar value for 7-hydroxycoumarin P2 is the probability that 1 (coumarin test dose) equals 2 (7-OHC at the same dose) The level of significance selected was 0 05 (ie 95%) NS indicates that the value was not significant The smaller the value of P, the less likely that the two means were equal

A second test was used to compare the effects of each drug with its control This was performed using Dunnett's test (Steel and Torrie, 1980) This test produces a value d' given by the equation

$$d' = t(\text{Dunnett}) \times \sqrt{\frac{2xs^2}{r}}$$

where r is the number of replicates of each test and s is the error mean square (obtained from analysis of variance using Minitab) The t value in this equation was obtained from Dunnett's t table where p was the number of test doses excluding the control and df was the degree of freedom for the error mean square

A third test used was the least significant difference (lsd) (Steel and Torrie, 1980) This was calculated from the equation

$$lsd = t_{\alpha/2} \times \sqrt{\frac{2xs^2}{r}}$$

This was similar to Dunnett's test except that the t value was obtained from a t test The lsd results are expressed as line graphs The mean values from the relevant table are ranked in order of magnitude (to simplify the results, the power of ten is left out), with the drug and concentration used printed above them Sometimes more than one drug, or concentration will yield the same mean number of cells Any values underscored by the same line are not significantly different from each other by the lsd test

Both Dunnett's test and the lsd give a value that represents the minimum difference between samples that is significant Thus, any two samples that differ by either the lsd or the d' value are significantly different at the selected probability level Dunnett's test is more conservative than lsd or the t test, and is therefore less likely to detect a difference

V 2 2 TO COMPARE THE EFFECTS OF COUMARIN AND 7-HYDROXYCOUMARIN ON LAT CELLS

Table V 1 shows the results of an 8 day incubation of $5x10^4$ cells/well in 6 well plates (see also Fig V 6) There was a significant reduction in cell numbers when coumarin or 7-OHC at a concentration of 500 µM were used, compared with the controls There was also a significant reduction in the number of cells in wells containing 500 µM 7-OHC compared to the wells containing 500 µM coumarin (see also Table V 6) The effect of 7-OHC at a concentration of 500 µM was significant by Dunnett's test at the 1 percentile level, while the effect of coumarin at the same concentration was significant at the 5 percentile level The line graph below indicates the values that are significantly different by the lsd method at the 5 percentile level for the data in Table V 1

C	C	7HC	7HC	C	7HC	7HC	C	C	7HC
50	100	100	0	0	50	200	200	500	500

2 9 2 8 2 6 2 5 2 4 2 1 2 0 1 9 1 6 0 75

Table V 2 shows a similar experiment after 7 days at 37°C This showed no significant difference in cell numbers at any dose levels The reason for this was the high standard deviation in the 7-OHC control The line graph below shows that there is a significant difference between 500 μM 7-OHC and its control by the lsd method

7HC	7HC	7HC 100	C	7HC	7HC 500	C
0	50	C 50	0/500	200	C 200	100
3 2	3 1	2 8	2 6	2 4	2 1	2 0

V 2 3 TO COMPARE THE EFFECTS OF COUMARIN AND 7-HYDROXYCOUMARIN ON SP2 CELLS

Table V 3 shows the results of incubation of $5x10^4$ SP2 cells in 6 well plates for 6 days, with coumarin and 7-OHC At no dose level did coumarin have a significant effect on cell number compared to the control The reduction in cell numbers by 7-OHC was significant at a concentration of 500 μM by both the t test and Dunnett's test (5 percentile level) 7-OHC also had a significantly greater effect than coumarin on cell numbers at a concentration of 500 μM Below is the line graph representation of the data for the lsd test

C	C	C	7HC 0	7HC	7HC	C	7HC	7HC
50	200	0	C 100	50	100	500	200	500
3 8	3 4	3 1	3 0	2 9	2 8	2 5	2 1	2 0

Table V 4 shows a similar experiment after a 9 day incubation period Coumarin had a significant effect on cell number at both the 200 μM and 500 μM dose levels 7-OHC only had an effect at the 500 μM concentration The reduction in cell number by 7-OHC at 500 μM was significantly greater than that of 500 μM coumarin Dunnett's test indicated that the effect of both coumarin and 7-OHC at a concentration of 500 μM were significant at the 1

percentile level, while the effect of coumarin at 200 M was significant at the 5 percentile level Below is the line graph for the lsd test for this data

C	C	7HC	7HC	C	7HC	7HC 200	7HC
0	50/100	50	0	200	100	C 500	500
2 8	2 6	2 3	2 1	1 9	1 8	1 6	0 42

Table V 9 shows the effects of coumarin and 7-OHC on $1x10^6$ cells in 25 cm^2 flasks after 9 days Both coumarin, at concentrations of 200 and 500 µM, and 7-OHC, at all concentrations, reduced the numbers of cells compared to their respective controls 7-OHC at all concentrations were significant by Dunnett's test at the one percentile level Coumarin at a concentration of 200 µM was significant at the 5 percentile level, while at a concentration of 500 µM it was significant at the 1 percentile level by Dunnett's test The only significant difference between the effects of coumarin and 7-OHC were found at a concentration of 500 µM Figure V 7 shows the dose-response curve for this data, and the line graph representation for the lsd test on this data is given below

7HC	C	7HC	C	7HC	C	7HC	C	7HC
0	0	100	100	50	200	200	500	500
5 4	5 0	4 4	4 3	4 1	3 9	3 3	2 6	1 1

Table V 5 shows the growth of $1.05x10^5$ SP2 cells in 24 well plates in the presence of coumarin and 7-OHC Coumarin significantly reduced cell numbers at both the 200 and 500 µM concentrations, while 7-OHC reduced cell numbers at 500 µM concentration These effects were significant by Dunnett's test at the 1 percentile level While the mean cell numbers at 500 µM 7-OHC were lower than that at 500 µM coumarin, this difference was not significant at the 5 percentile level 7-OHC at a concentration of 50 µM and 100 µM produced a significant increase in cell number (t test), but this increase was not significant by Dunnett's test Below is

the line graph representation of the lsd test for this data

7HC	7HC	C	7HC 0	7HC/C	C	7HC
50	100	0	C 100	200	500	500
14	13	11	10	8	3	2

V 2 4 COMPARING THE EFFECTS OF INCUBATION PERIOD AND CELL NUMBER ON THE RESPONSE TO COUMARIN AND 7-HYDROXYCOUMARIN

Table V 7 shows the effects of coumarin and 7-OHC on the growth of LAT cells in a 24 well plate 1 $13x10^6$ cells were incubated in 24 well plates for 18 hr There were no differences in the cell numbers between the controls and any concentration of either drug used by either the t test or Dunnett's test There was a significant difference in cell number between 7-OHC and coumarin at 100 µM concentration Table V 8 shows the same experiment except the incubation period was increased to 72 hr There was no significant difference in cell number between any of the wells by either the t test or Dunnett's test

Table V 6 shows the results obtained when $5x10^5$ cells were incubated for 9 days with both coumarin and 7-OHC in the wells of 24-well plates The only significant effect found was a reduction in cell numbers with 100 µM 7-OHC compared with the control This was significant by Dunnett's test at the 5 percentile level

V 2 5 COMPARING THE EFFECTS OF COUMARIN AND 7-HYDROXYCOUMARIN ON CELL DEATH

Table V 10 shows the number of dead cells found after the incubation of $1x10^6$ cells in 25 cm^2 flasks for 9 days, in the presence of coumarin and 7-OHC As can be seen, the only significant effect was the reduction in the number of dead cells in the wells containing 500 µM 7-OHC compared to both the control flasks and the flasks containing 500 µM coumarin

V 2 6 DOSE RESPONSE CURVES

Table V 11 shows the parameters for the dose response curve for both coumarin and 7-OHC (see also Fig V 7) The data used was from table VI 9, ie SP2s in 25 cm^2 flasks The linear correl-

ation coefficient for 7-OHC dose-response curve was -0 952, while the linear correlation coefficient for the log-dose response curve was -0 89 The linear correlation coefficient for the dose-response curve for coumarin was -0 925, while for the log dose-response curve it was -0 857 This indicates that the data is linear on a dose-response curve The data from this experiment fitted a straight line better than the data from any other experiment In all cases log transformation of the data did not improve the linear fit

TABLE V 1

This table shows the effects of coumarin, and 7-hydroxy-coumarin, on the growth of LAT cells after an 8 day incubation of 5 10^4 cells/well, in a 6 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) 7-OHC	P3
0	---	2 37 (0 05)	NS	2 45 (0 05)	---
50	NS	2 88 (0 25)	0 09	2 08 (0 3)	NS
100	NS	2 80 (0 3)	NS	2 55 (0 4)	NS
200	NS	1 85 (0 2)	NS	2 03 (0 2)	NS
500	0 03	1 58 (0 2)	0 03	0 75 (0 15)	0 001

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 2

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of LAT cells after a 7 day incubation of 5 10^4 cells/well, in a 6 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) 7-OHC	P3
0	---	2 58 (0 2)	NS	3 18 (0 55)	---
50	NS	2 83 (0 3)	NS	3 13 (0 35)	NS
100	NS	1 98 (0 35)	NS	2 78 (0 25)	NS
200	NS	2 05 (0 25)	NS	2 35 (0 3)	NS
500	NS	2 63 (0 3)	NS	2 10 (0 35)	NS

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 3

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of SP2 cells after a 7 day incubation of 5 10^4 cells/well, in 6 well plates

CONC (μM)	P1	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) 7-OHC	P3
0	---	3 05 (0 25)	NS	2 98 (0 25)	---
50	NS	3 73 (0 4)	NS	2 88 (0 35)	NS
100	NS	3 03 (0 2)	NS	2 80 (0 4)	NS
200	NS	3 38 (0 45)	NS	2 13 (0 25)	NS
500	NS	2 48 (0 25)	0 017	2 03 (0 03)	0 036

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 4

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of SP2 cells after a 9 day incubation of 5 10^4 cells/well in a 6 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) 7-OHC	P3
0	---	2 75 (0 15)	0 062	2 13 (0 2)	---
50	NS	2 58 (0 25)	NS	2 28 (0 3)	NS
100	NS	2 60 (0 3)	0 094	1 75 (0 25)	NS
200	0 014	1 85 (0 2)	NS	1 63 (0 3)	NS
500	0 017	1 58 (0 25)	0 022	0 42 (0 03)	0 004

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 5

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of SP2 cells after a 7 days incubation of 1 05 10^5 cells/well in a 24 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) 7-OHC	P3
0	---	11 1 (0 7)	NS	9 5 (1 2)	---
50	NS	10 8 (1 0)	NS	13 5 (1 1)	0 029
100	NS	9 7 (1 2)	0 038	13 1 (0 8)	0 028
200	0 0048	7 9 (0 3)	NS	7 5 (0 6)	NS
500	0 0	3 3 (0 5)	NS	2 0 (0 5)	0 0009

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 6

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of LAT cells after a 9 day incubation of 5 10^5 cells/well in a 24 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) 7-OHC	P3
0	---	10 2 (0 5)	0 047	12 6 (0 5)	---
50	ND	ND	ND	10 4 (0 7)	NS
100	NS	10 6 (2 0)	NS	9 8 (0 3)	0 017
150	ND	ND	ND	10 7 (0 5)	NS
200	NS	9 9 (0 6)	NS	11 1 (0 6)	NS

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

ND Not determined

TABLE V 7

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of LAT cells after an 18hr incubation of 1 13x10^6 cells/well in a 24 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^5) MEAN (±SEM) 7-OHC	P3
0	---	17 2 (0 7)	NS	14 1 (2 7)	---
100	NS	16 2 (0 8)	0 012	19 8 (0 9)	NS
200	NS	16 6 (0 9)	NS	18 1 (1 1)	NS

TABLE V 8

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of LAT cells after a 72hr incubation of 1 13x10^6 cells/well in a 24 well plate

CONC (μM)	P1	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL (x10^6) MEAN (±SEM) 7-OHC	P3
0	---	1 44 (0 1)	NS	1 43 (0 1)	---
100	NS	1 59 (0 1)	NS	1 51 (0 1)	NS
200	NS	1 39 (0 1)	NS	1 26 (0 1)	NS

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 9

This table shows the effects of coumarin and 7-hydroxy-coumarin on the growth of SP2 cells after a 9 day incubation of $1x10^6$ cells per 25 cm^2 flask

CONC (μM)	P1	VIABLE CELLS /WELL ($x10^6$) MEAN (±SEM) COUMARIN	P2	VIABLE CELLS /WELL ($x10^6$) MEAN (±SEM) 7-OHC	P3
0	---	4 96 (0 15)	NS	5 38 (0 25)	---
50	ND	ND	ND	4 12 (0 15)	0 01
100	NS	4 25 (0 05)	NS	4 36 (0 1)	0 03
200	0 009	3 93 (0 15)	NS	3 34 (0 25)	0 002
500	0 004	2 61 (0 3)	0 027	1 41 (0 05)	0 0005

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

TABLE V 10

This table shows the effects of coumarin and 7-hydroxy-coumarin on the death of SP2 cells after a 9 day incubation of $1x10^6$ cells per 25 cm^2 flask

CONC (μM)	P1	DEAD CELLS /WELL ($x10^5$) MEAN ($\pm$SEM) COUMARIN	P2	DEAD CELLS /WELL ($x10^5$) MEAN ($\pm$SEM) 7-OHC	P3
0	---	10 8 (0 5)	NS	6 50 (0 5)	---
50	ND	ND	ND	6 70 (0 25)	NS
100	NS	5 30 (1 0)	NS	4 55 (1 0)	NS
200	NS	6 45 (1 0)	NS	7 30 (0 7)	NS
500	NS	4 75 (0 2)	0 03	3 05 (0 45)	0 003

P1 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of coumarin

P2 The probability that the mean no cells in the wells with a given dose of coumarin is equal to the mean no of cells in the wells containing the same dose of 7-OHC

P3 The probability that the mean no cells in the control wells, are equal to the mean no of cells in the test wells containing a given dose of 7-OHC

NS Not significant, ie a probability of greater than 0 05 (less than 95%)

ND Not Determined

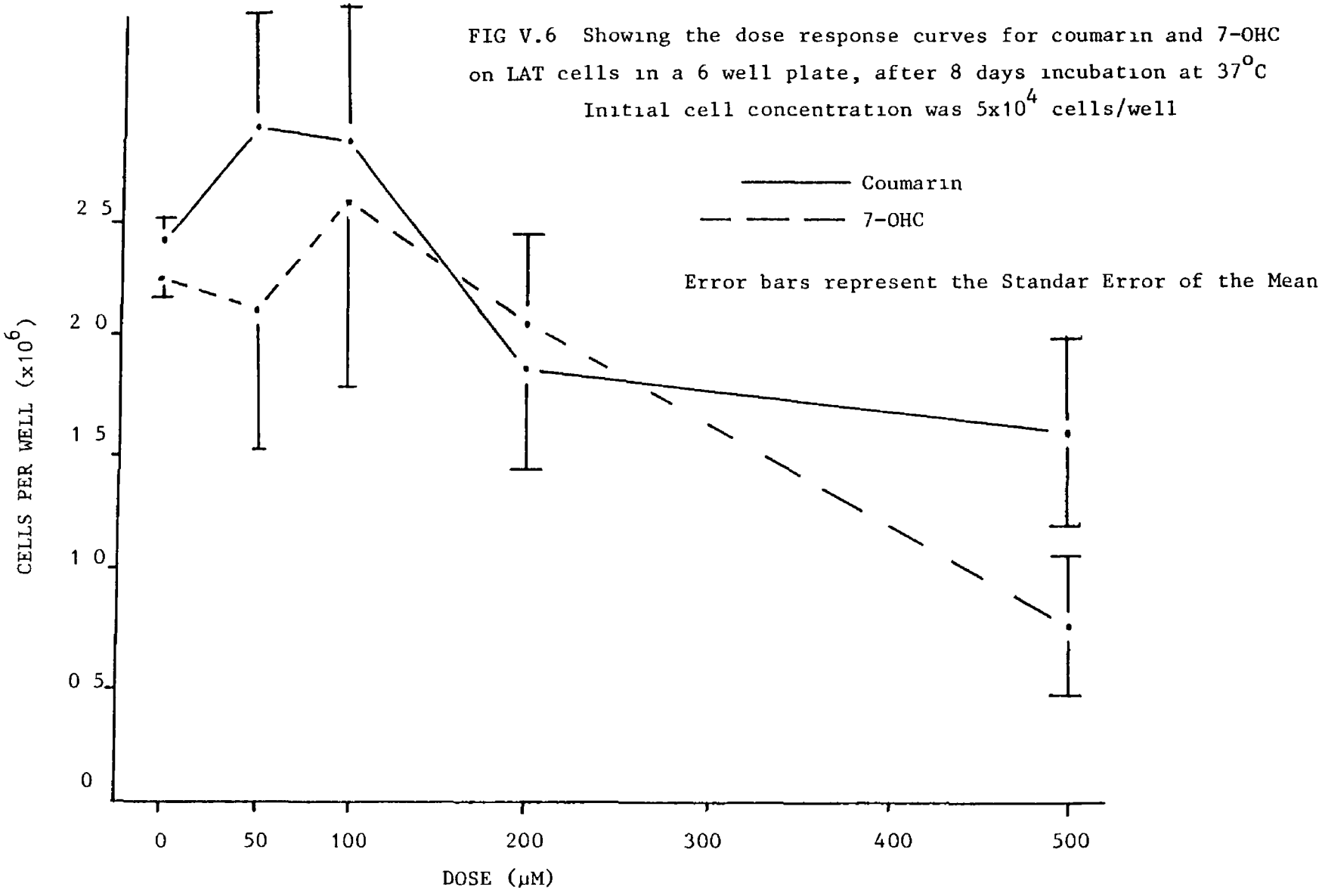
TABLE V 11

This table shows the parameters for the least squares line (Y = mX + C) calculated for the data from Table V 9

DRUG	CORR COEF (r)	SLOPE (m)	INTERCEPT (C)
7-OHC	-0 952	-0 727	4 96
COUMARIN	-0 925	-0 449	4 84

r the correlation coefficient

FIG V.6 Showing the dose response curves for coumarin and 7-OHC on LAT cells in a 6 well plate, after 8 days incubation at $37^{o}C$ Initial cell concentration was $5x10^{4}$ cells/well



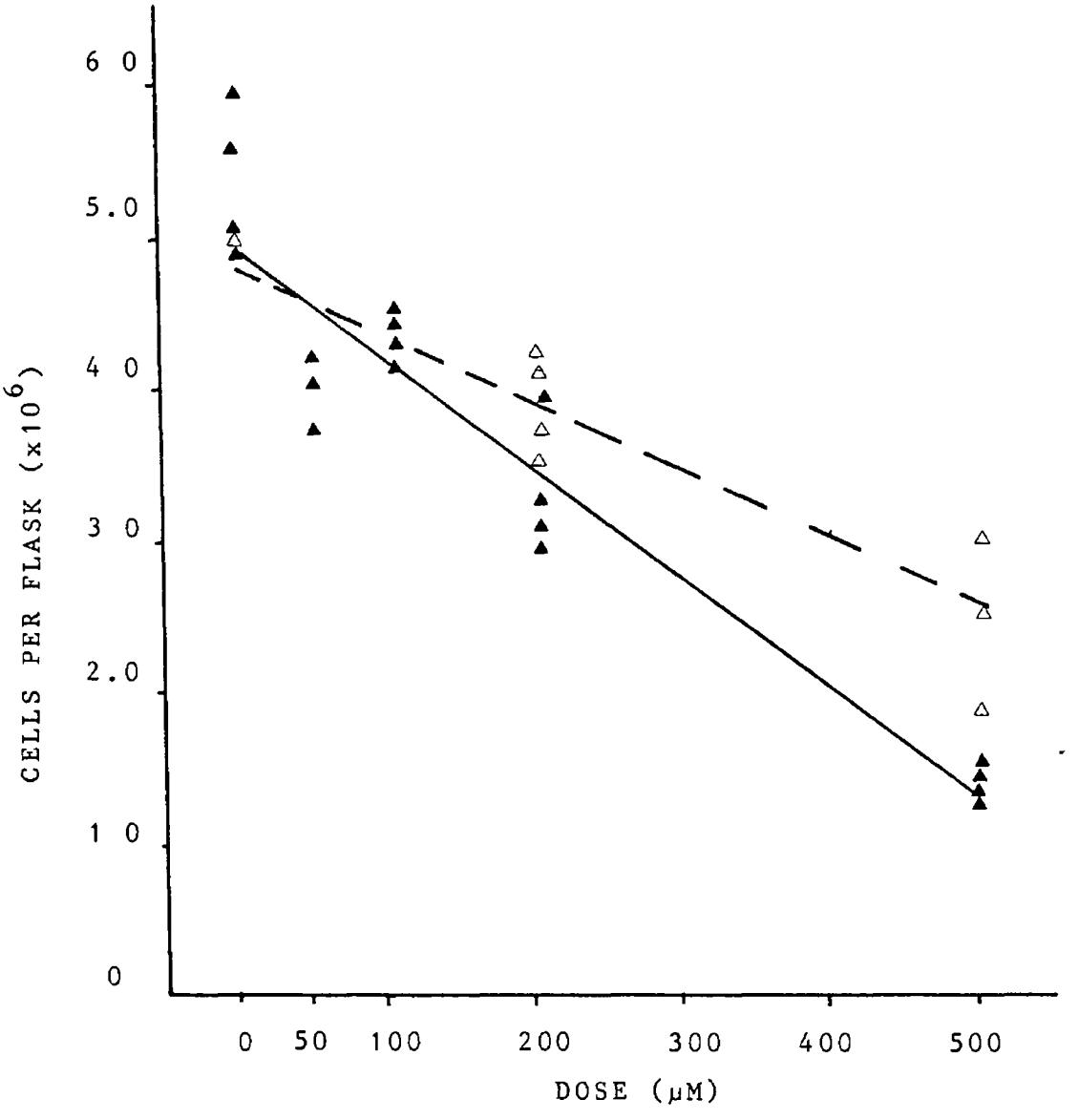


FIG V.7 This figure shows the dose-response curves for coumarin(- - -△—) and 7-OHC (——▲——) on SP_2 cells incubated in 25 cm^2 flasks for 9 days at 37°C The initial cell concentration was $1x10^5$ cells per flask The lines represent the least squares line that best fits the data

It was found that both 7-OHC and coumarin had effects on the growth of SP2 and LAT cells in culture The main effect was a reduction in cell numbers at a concentration of 500 μM At this concentration the effect of 7-OHC was greater than the effect of coumarin At lower concentrations the effects are not so obvious This was found to be due to the test system Thus, when 25 cm^2 flasks were used an effect was seen with 50 μM 7-OHC, while in 24-well plates an effect was only seen with 500 μM 7-OHC

The reason for the variation in responses in the different test systems is due to the difference in cell numbers Thus, to see a minor change in cell number, such as with a concentration of 50 μM, a large number of cells are necessary However, with gross effects, such as with a concentration of 500 μM of either drug, a reduction in cell number could be seen, even with a low cell number Another limiting factor was in the standard deviations of the counts When a low cell number was used, eg 24-well plates, a small variation in the counts produced a large standard deviation, resulting in an increase in statistical errors When larger cell numbers were used, ie a 25 cm^2 flask, the errors in counting produced a smaller standard deviation, and a therefore statistical errors were reduced Thus, in the 24-well and 6-well systems, there was often a difference in the means, which was not significant due to the large standard deviation

There are a number of conditions necessary for the reduction in cell numbers to be detected The most important variable is cell number The optimum response occured with $1x10^5$ cells in a 25 cm flask, or $5x10^4$ cells/well in a 6 well plate A second factor was the incubation period The cells should be counted before the control well becomes confluent If cells were not counted until after the control wells became confluent, the effect could be masked This was because the cells in the control well stopped growing due lack of growth surface, a depletion of the media, and a drop in pH However, cells in the test wells would continue to grow until they reached confluence, thus masking the effects of the drugs The time needed for cells to grow to confluence depended on the initial number of cells in each well, on the batch

of cells and the batch of media/serum Different batches of cells grow at different rates, and therefore different incubation periods would be required There is also a lot of variation in the growth promoting ability of different batches of serum This means that the incubation period has to be adjusted for each experiment

The decrease in the number of viable SP2s was not reflected in an increase in the number of dead SP2s In fact, there was a decrease in the number of dead cells following incubation in 500 μM 7-OHC This decrease could be explained if the dead cells were due to natural cell death If there were fewer viable cells present, there would be less dead cells This would indicate that neither drug had a cytotoxic effect on the cells, but rather inhibited the growth of the cells This was found with both SP2 and LAT cells

Both drugs failed to reduce the number of LAT cells when they were incubated at high cell concentration (1 $x10^6$ cells/well) for 18 hr or 72 hr The absence of an effect could be explained if the drugs inhibit cell growth The high cell concentration would prevent growth due to the lack of available surface area for growth The short incubation period would also mask any effects since the cells would only undergo one or two cell cycles and divisions

There was also a second effect seen, with both coumarin and 7-OHC At low concentrations of either drug there was often an increase in the mean number of cells, compared with the control (see Tables V 1, V 2, V 3, V 5, V 7, and V 8) The relevance of this increase in the mean number of cells is uncertain In only one instance, Table V 5, was the increase significant, although it was not significant by Dunnett's test It is not known whether this represents a mild growth stimulatory effect of coumarin, and 7-OHC, or else it reflects an error in counting

It would appear that both drugs act by slowing the growth rate of the cells rather than by killing them Thus, a low cell number and long incubation period is necessary to detect the effect The LAT cells are not a good model system to study the effects of coumarin because they were passaged as an ascitic tumour in mice, and therefore, were not adapted for growth in culture Thus,

their growth rate was poor after the initial transfer to in vitro growth in tissue culture This problem can be overcome by growing the cells in culture for several passages prior to the assay, to enable them to adapt to in vitro growth

Inhibition of in vitro cell growth by coumarin was also found by Conley et al , (1987) They used 3 human malignant cell lines an erythroleukaemia (K562), and two renal carcinomas, (ACHN, and Caki-2) in their studies Coumarin at 200 µg/ml (approx 100 µ M) reduced the growth of all 3 cell types They analysed the cells after incubation with the coumarin, using a fluorescent activated cell sorter From this they concluded that coumarin caused an accumulation of cells in phase G_0/G_1 of the cell cycle, and thus prevented entry of cells into the S phase

Pharmacokinetic studies in humans have found that coumarin has a very short half-life This led to speculation that coumarin was a pro-drug, and that its major metabolite, 7-OHC, was the active form However, the lack of data indicating that 7-OHC was as effective as coumarin caused problems with this The results presented here show that 7-OHC is much more effective than coumarin at slowing the growth of tumour cells in vitro This would suggest that in the treatment of cancer coumarin is acting as a pro-drug The growth reducing effects of coumarin could be due to weak activity in coumarin, or else due to the formation of 7-OHC from the coumarin due to metabolism by the test cells

When coumarin is used to treat patients with cancer it appears to have two effects Firstly, coumarin stimulates the immune system via the macrophages A second effect is due to its ability to prevent the growth of tumour cells In particular, this cytostatic activity is enhanced after coumarin is metabolised to 7-OHC which has a longer half-life than coumarin This activation of the immune system, along with its cytostatic effects would be expected to reduce metastases Since the cytostatic activity of coumarin occurs at high concentrations, it explains the improvement in patients on high dose coumarin (up to 2 g/day)

It would appear that coumarin has a role to play in the treatment of cancer It is especially effective in the prevention of recurrence, which explains why Marshall et al , (1987) found that coumarin is more effective in patients with nephroma, if they had

a nephrectomy Thus, therapy with coumarin would appear to be of benefit after removal of the tumour by surgery By activating the immune system and preventing cell growth it can prevent the tumour spreading Whether it has any benefit in treating the primary tumour without prior surgery is uncertain It is also very safe for the long term therapy (2 years or more) that is necessary for preventing recurrence In section VI the results of a clinical trial indicate that there is a toxicity of only 0 2-0 3%, and that it is well tolerated at doses up to 2g/day

SECTION VI

COUMARIN A CLINICAL STUDY

VI 1 1 THE LIVER STRUCTURE AND FUNCTION

The liver is the largest single organ in the body (approximately 2% of the body weight) The liver consists of 4 major components the parenchymal cells or hepatocytes, which constitute 60% of the liver mass, the reticuloendothelial cells or Kupffer cells, the biliary tracts, and the blood vessels

The hepatic blood supply, which is approximately 1 5 litres per minute, comes from the hepatic portal vein (80%) and the hepatic artery (20%) The hepatic artery provides oxygenated blood and nutrients to the liver The hepatic portal vein drains the gastrointestinal tract

The classic functional units of the liver are called lobules Each lobule is hexagonal in shape, with a cross sectional area of about 2 mm^2 Portal triads, which contain the hepatic portal vein, the hepatic artery, and the bile duct, run in the spaces between the lobules Within each lobule the hepatocytes are arranged in sheets that are orientated towards the central vein Biliary canaliculi run within these sheets, and carry secretions from the cells to the periphery of the lobule, where they connect with cholangioles, which then drain into the bile ducts The spaces between the sheets of hepatocytes are occupied by sinusoids The sinusoidal walls consist of endothelial cells and Kupffer cells The sinusoids receive blood from the hepatic portal venules and hepatic arterioles The sinusoids drain into the central vein which in turn drains into the hepatic vein

Rapporort and coworkers (1969, 1976) suggested that the classic lobular structure does not represent the functional unit of the liver They defined the functional unit in terms of liver acini A liver ascinus consists of a mass of hepatocytes of irregular shape and size, arranged around an axis consisting of a portal triad, along with lymph vessels, and nerves The ascinus lies between 2 or more central veins (terminal hepatic venules) The ascini can be divided into 3 zones based on their distance from the terminal afferent vessel The periportal region, which surrounds the hepatic and portal vessels, is zone 1 This zone has the highest oxygen tension and respiratory enzyme activity

The centrilobular region, which surrounds the central vein, is zone 3 The oxygen tension is lowest here, and the activity of the microsomal enzymes is highest here The midzonal region between the two is zone 2

The liver has a major role to play in carbohydrate, lipid and protein metabolism It is also the major site of metabolism of drugs and other foreign compounds Metabolism by the microsomal enzymes, converts foreign substances into more water soluble forms, which enables them to be excreted into the urine There are 2 phases involved Phase I consists of oxidation of the compound by cytochrome P_{450}, which uses molecular oxygen and NADPH This results in the introduction of polar groups into the molecule This step is often called detoxification, since the end result is usually a less potent/toxic compound This is a misnomer because metabolism can sometimes yield a more potent/ toxic compound Phase II involves a conjugation reaction, usually to a glucuronide This uses the polar group, mainly hydroxy groups, introduced in phase I

The liver is highly susceptible to damage for 3 reasons Firstly, it has a high blood flow through it, and thus it is exposed to more of a circulating compound than other organs Secondly, its role in metabolism means that it has proteins which bind the compound, eg cytochrome P_{450} This binding results in an accumulation of the compound in the liver cells Thirdly, the liver, being the site of metabolism, has a higher concentration of metabolites than any other organ, which can leave the liver open to damage if these metabolites are toxic

VI 1 2 TYPES OF HEPATIC TOXICITY

Many attempts have been made to classify hepatic injury on the basis of mechanism and type of injury The system proposed by Zimmerman (1972, 1978) is probably the most useful, and the most used There are 2 major types of hepatotoxicity, acute and chronic Each have their own pattern of liver damage In each case the damage can be cytotoxic (hepatocellular) if the damage is mainly to the hepatocytes, cholestatic if bile flow is interrupted, and mixed if elements of both are present

There are 3 types of cytotoxic injury necrosis, degeneration and steatosis

(i)Necrosis is due to the death of cells, as a result of leakage of the contents of cells, after damage to the cell membrane Necrosis can be zonal, massive or diffuse Zonal necrosis can be peripheral (zone 1), central (zone 3) or midzonal (zone 2) Necrosis in zone 3 is characteristic of carbontetrachloride and chloroform Necrosis of zone 1 is characteristic of phosphorous Necrosis of zone 2 can be caused by ngaione The zonal nature of necrosis may reflect the distribution of enzymes that produce toxic metabolites Instead of producing necrosis in a localised zone, some drugs produce a massive necrosis throughout the lobule/ascinus, eg aflatoxins, while others produce a diffuse necrosis throughout the lobule/ascinus, eg galactosamine In general, zonal necrosis is due to intrinsic hepatotoxins, while idiosyncratic hepatotoxicity produces massive, or diffuse necrosis Viral hepatitis also produces diffuse necrosis

(ii)Degeneration, which is a form of sub-necrotic damage, is also produced by agents which cause necrosis This is seen prior to the development of necrosis, or concomitantly in non-necrotic cells Ballooning degeneration produces large vacuole filled hepatocytes, while eosinophilic degeneration forms the acidophilic bodies

(iii)Steatosis is caused by interference in lipid metabolism, resulting in accumulation of lipid in the cells It is uncertain if there is a zonal distribution in steatosis There are 2 types of steatosis micro- and macro-vesicular In microvesicular steatosis small droplets of fat, which do not displace the nucleus, form in the hepatocytes This type of fatty liver is caused by tetracycline, among other drugs In macrovesicular steatosis, a large droplet of fat, which displaces the nucleus, forms in the hepatocyte This type of steatosis can be caused by alcohol

In cytotoxic injury to the liver, any combination of the above may be present

Cholestatic injury occurs when the bile flow is halted There are 2 types of cholestasis canalicular jaundice, which is characterised by little or no injury to the hepatocytes, and no evidence of portal inflammation, and hepatocanalicular jaundice,

which is usually accompanied by some damage to the hepatocytes, along with portal inflammation Bile casts and jaundice are present in both types Anabolic and contraceptive steroids can cause canalicular jaundice, while chlorpromazine can cause hepatocanalicular jaundice

Some drugs produce liver damage that is characteristic of both cytotoxic and cholestatic damage Sulphonamides can produce a mixed hepatocellular toxicity, while chlorpromazine can produce a mixed hepatocanalicular toxicity

Repeated exposure to drugs can lead to various types of chronic liver damage There are 2 main characteristic lesions in chronic liver disease cirrhosis and fibrosis Necrosis, degeneration and steatosis can also be present Fibrosis is due to an increase in fibrous tissue, which can lead to cirrhosis Cirrhosis is characterised by a loss of hepatocytes, and their replacement with fibrotic tissue There is also a resulting distortion of the normal hepatic architecture Cirrhosis can be micro- or macro-nodular Chronic exposure to ethanol can cause cirrhosis

A second type of chronic liver damage is chronic active hepatitis (CAH), also called chronic persistent/aggressive hepatitis This is characterised by necrosis, lymphocyte infiltration and mild fibrosis, and can lead to cirrhosis It can be caused by chronic administration of methyldopa

VI 1 3 MECHANISMS OF HEPATIC INJURY

Zimmerman (1972, 1978) classifies hepatotoxins as being either intrinsic, or idiosyncratic Intrinsic hepatotoxicity is characterised by high incidence of toxicity, reproducibility of toxicity in animals, short latent period and dose-dependence The toxicity is thought to be due to a direct effect by the drug Idiosyncratric hepatotoxicity is characterised by a very low incidence, it is not experimentally reproducible, it has a long latent period and it is not dose-dependent This is thought to be due to a host-dependent response, rather than any intrinsic property of the drug

Zimmerman (1972, 1978) subdivides intrinsic hepatotoxins into 2 types The first are direct hepatotoxins These cause widespread damage to the liver, and also, to other tissues They are char-

acterised by a very short interval between exposure and evidence of liver damage They produce characteristic lesions, and are highly reproducible An example of a direct-acting hepatotoxin is carbontetrachloride Within hours of exposure to carbontetrachloride there is evidence of liver damage The toxic components are free radical metabolites, which damage the cell membranes

The second type of intrinsic hepatotoxin are the indirect hepatotoxins There is a longer interval between exposure to these agents and the onset of liver damage Their dose-dependence, and reproducibility, are not as obvious as that with direct-acting hepatotoxins Indirect hepatotoxins act by interfering with metabolic pathways within the cell Their effect is very specific, and localised, in comparison to that of the direct acting hepatotoxins There are 2 main types The first acts on the hepatocytes, interfering with essential metabolic pathways The other interferes with the secretion of bile

Zimmerman (1972, 1978) proposes 2 mechanisms of idiosyncratic reactions The first involves hypersensitivity to the drug This involves the drug, or a reactive metabolite, binding to a protein, which would make the protein antigenic Subsequently, antibodies produced against this antigen would be able to recognise the native protein, and on binding to it, would interfere with the cells function A long latent period would be required to allow antibody production to occur Desensitisation can also occur

The second type of idiosyncratic hepatotoxicity is caused by the production of "novel" metabolites This involves the use of an alternative metabolic pathway to metabolise the drug in certain patients The metabolites produced by this pathway then exert their toxic action on the liver The delay in onset of the toxicity would be due to the time required for the accumulation of the toxic metabolite

It is very difficult to determine the mechanism involved in most cases of idiosyncratic hepatotoxicity, since the number of patients involved is usually very low, and there are no animal models available to obtain experimental data Evidence for a role for novel metabolites in hepatotoxicity, is difficult to obtain, since they are difficult to detect and identify Usually, the

only evidence of an immunological basis in a case of hepatotoxicity, is the presence of extrahepatic symptoms which are suggestive of an allergy, eg a rash Drug specific antibodies in a patients serum, cannot be used to establish an immunological basis for hepatotoxicity, as their presence does not necessarily imply a role for them in the toxicity Thus, many cases of idiosyncratic hepatotoxicity are considered to be due to an allergic, or immune reaction if there are extrahepatic signs If these are absent the mechanism is often considered to be that of a novel metabolite

VI 1 4 DIAGNOSIS OF HEPATIC INJURY

Hepatic injury can be diagnosed biochemically, and histologically Histological examination of a biopsy preparation gives information about the type of lesion, and location Biopsy is an invasive technique, and is not routinely performed, but this technique provides the best information on type and cause of the injury

The tests that are routinely used to establish the presence, and nature of hepatitis are serum levels of certain enzymes The most important indicator of hepatic injury are the serum levels of transaminases The 2 transaminases used are Aspartate aminotransferase (GOT/AST) and Alanine aminotransferase (GPT/ALT) These are strictly intracelluar enzymes, found mainly in the liver There is usually a low level of these enzymes in the serum, but an increase above the normal level indicates probable cell damage, as this is the only source of these enzymes Another very important indicator of liver damage is serum bilirubin Its level rises during cholestasis, and high levels result in deposits in the skin, resulting in a yellow pigmentation Other enzymes used in diagnosis are Alkaline phosphatase (AP),γ-Glutamyl transferase (GGT), Lactate dehydrogenase (LDH), Glutamate dehydrogenase (GLDH), Creatine kinase (CK) and cholinesterase (ChE)

The serum enzyme pattern is very useful in determining the type and cause of hepatic injury Elevated levels of the transaminases indicates cytotoxic damage to the hepatocytes Elevated levels of bilirubin are indicative of cholestasis In alcoholic cirrhosis, the levels of GGT are elevated more than any other enzyme GLDH is a marker for chronic active hepatitis and acute occlusion of the biliary tract AP is a marker of cholestasis and also of tumours

The relative levels of enzymes in the serum does not always reflect the distribution in tissue This distortion of the the enzyme pattern can be of diagnostic use (Schmidt, 1986) In the hepatocytes GOT activity is slightly higher than GPT activity, but 40% of the GOT is mitochondrial, thus in the cytoplasm GPT activity is higher than GOT In acute hepatitis there is mainly a loss of cytoplasmic enzymes, thus, GPT levels are greater than GOT in the serum In necrosis there is leakage of all intracellular enzymes, regardless of their location, and serum levels reflect that in the hepatocytes, ie GOT activity is higher than that of GPT This distortion is due to the nature of the injury In acute hepatitis a large number of cells are involved, but the damage is only minimal, thus only the soluble enzymes leak out During necrosis, there is severe damage done to the cells, but this is usually localised This allows all enzymes to leak out

The levels of serum enzymes are not independent of each other Their levels vary along with the other enzymes Thus, the relationships between certain enzymes, measured as quotients, is also useful in diagnosis One of these is the deRitis ratio (DR), which is the ratio of GOT to GPT (deRitis et al , 1955) This has 4 different levels around 0 7, below 0 7, above 0 7 and far above 0 7 Levels far above 0 7 usually indicate either primary, or secondary carcinoma of the liver While the DR cannot neccessarily distinguish between viral hepatitis, and drug-induced hepatitis, since different types of each can have different DR values, it can help establish a clinicopathological (CP) pattern for a given hepatotoxin Other ratios can also be used, such as the ratio of the total transaminase levels (GOT + GPT) to that of GLDH (useful for obstructive jaundice), and the ratio of GGT levels to those of GPT (Schmidt, 1986) These are especially useful in differential diagnosis when used together, and in combination with the absolute levels of the serum enzymes

VI 1 5 ETIOLOGY OF HEPATITIS

The 4 major causes of hepatitis are viral infection, drug toxicity, alcohol abuse and exposure to chemicals, especially organic solvents The clinicopathalogical pattern (ie the patients symptoms, serum enzyme levels, biopsy studies etc), can sometimes indicate the cause of the hepatitis, eg alcoholic cirrhosis has a distinctive CP pattern The etiology of most

cases of hepatitis is not easily elucidated, thus in most cases, the elimination of other possible causes plays a major role in determining the etiology of a case of hepatitis Most of the liver disorders seen in patients, can be caused by either drugs, or non drug related factors (Sherlock, 1986) Thus, in any case of hepatitis, an iatrogenic cause should be considered until evidence to the contrary is obtained This is especially so in high risk patients, eg elderly patients

Stricker and Spoelstra (1985) recommend a 3 step process for determining the role of a suspect drug in hepatitis (i) the identification of the CP pattern, (ii) the establishment of a temporal relationship between exposure to drug/chemical, and the hepatitis, (iii) the exclusion of other possible causes

If the CP pattern is highly specific, ie it has features that are only found after exposure to the suspect agent, this can be used as confirmation of the etiology Sometimes, a specific CP pattern is observed, ie it is similar to that found in other cases of exposure to drugs, and these can be a strong indicator of the etiology

A strong temporal relationship between exposure to the suspect chemical, and occurrence of the hepatitis, especially if a specific CP pattern is present, is a strong indicator of hepatotoxicity Even if there is no specific CP pattern, the absence of other possible causes is a strong indicator of hepatotoxicity, when associated with a temporal relationship The establishment of a temporal relationship between drug administration, and the hepatitis, is best achieved by re-treating the patient with the suspect drug, but this is not always ethically possible This can also be hampered by the fact that the doctor treating the hepatitis is sometimes not the doctor that prescribed the medication Patient compliance, and the use of other non-prescribed medication, also has to be examined The failure to establish a temporal relationship between exposure to a suspect agent, and occurrence of hepatitis, does not necessarily imply that the drug was not responsible as desensitization may have occured, the dose could be too low, or the challenge period too short

The third step involves screening for possible viral infection, in particular hepatitis A and B antigen, infectious mononucleosis and cytomegaly virus Liver biopsy cannot distinguish between viral hepatitis and hepatocellular toxicity (Scheuer, 1968) Thus, the levels of viral antigens in the blood have to be used Non-A, non-B hepatitis can be excluded, if blood transfusion/surgery, intravenous drug abuse, and a promiscuous life style can be eliminated Other causes of hepatitis can be excluded, if the patients liver scan is normal Exposure to organic solvents, especially in work, should also be excluded, but this can be difficult, as many patients may not suspect that they are exposed to these solvents

In many cases there is a correlation between the type of drug, and the type of hepatotoxicity Anesthetic agents cause cytotoxic injury only Among the neuroleptics, antidepressants usually cause cytotoxic hepatitis, tranquilizers mainly cause cholestatic injury and tricyclic antidepressant drugs can cause both Most of the anti-inflammatory drugs cause hepatocellular, or mixed hepatocellular toxicity, eg ibuprofen, indomethacin, etc , but, gold compounds and phenylbutazone can cause either cytotoxic, or cholestatic injury C-17 alkylated steroid hormones cause canalicular hepatitis Many antimicrobial agents produce cytotoxic damage eg tetracyclines, sulphonamides etc, while others, eg erythromycin, produce cholestatic injury The fact that so many drugs from the same class, produce the same lesions, would suggest that the mechanism of action of some drugs, and the mechanism by which they produce injury, are related However, many drugs cause hepatic injury that does not appear to be related to their therapeutic action

VI 1 6 PROGNOSIS WITH HEPATITIS

The prognosis with hepatitis depends on its etiology, the time taken to diagnosis, and the type of hepatitis

For viral hepatitis, the time taken to diagnosis is irrelevant, as there is no treatment for it If drug related hepatitis is diagnosed early, and therapy with the suspect drug stopped, the patient will often suffer no permanent damage To facilitate early diagnosis, patients undergoing therapy with drugs that are

suspected of causing hepatitis, should be routinely monitored for any signs of abnormal liver function

The major factor in the prognosis is the type of hepatitis In particular, drug-induced hepatocellular toxicity, if undetected, can have a high mortality rate, ranging from 10 to 50% of patients affected (Zimmerman, 1978) Patients, who survive acute hepatocellular disease, usually make a full recovery and their necrosis does not usually lead to chronic disease The prognosis for patients with other types of hepatitis is usually very good, with a very low mortality rate Thus, it is essential to diagnose any cases of hepatocellular toxicity as early as possible

The purpose of the work presented here was to determine if coumarin had any toxic effects on liver function If any toxic effects were detected it was hoped that they could be characterised, and a safe dose (if any) determined

VI.2.1 COUMARIN CLINICAL TRIAL

2,163 patients in a sequential trial, were treated with coumarin at doses ranging from 25mg every two days to 2,000 mg daily. 38 patients had elevated serum transaminase levels during therapy with coumarin. These were divided into two groups. The first showed an increase of less than twice the normal maximum levels of serum transaminases, which was accompanied by an exacerbation of their symptoms. This was usually followed by a period, in which their condition was much improved. The second group had a much higher elevation in serum transaminase levels, without an exacerbation of their condition, nor an improvement. Hepatotoxicity was only considered in patients with a rise of at least twice the normal maximum levels of serum transaminases, without an exacerbation, nor improvement in their condition. 17 of the 38 patients fitted this classification, and were examined for evidence of abnormal liver function. Four of these patients had chronic brucellosis, 4 had carcinoma, 4 had melanoma, and 1 had a nephroma. All patients with elevated LFTs were screened for hepatitis A and B infection.

VI.2.2 CASE HISTORIES

Brief case histories of the 17 patients suspected of having hepatotoxicity to coumarin are given below. See also Tables VI.1 to VI.6. The different levels of hepatotoxicity, mentioned below, are described in Section III.12.4.

CW: male, aged 41 with chronic brucellosis; received two courses of coumarin (50mg/day). On the first occasion his transaminase levels rose to 248 IU/l (SGOT) and 500 IU/l (SGPT), with a DR of 0.50, after 6 months. These returned to normal when coumarin was stopped. The second course, two years later, resulted in a rise in SGOT to 136 IU/l, an SGPT to 326 IU/l and a DR of 0.42, after 4 months. These also returned to normal when coumarin was stopped.

COMMENT: There were no obvious reasons for the coincidence of coumarin therapy and abnormal LFTs other than the administration of coumarin. There was a strong temporal relationship between the toxicity and the administration of coumarin and thus, this patient

was definite in level B

ML female, aged 38 with chronic infectious mononucleosis, received two courses of coumarin (50mg/day) After 5 months on her first course of coumarin her SGOT rose to 440 IU/l, her SGPT rose to 696 IU/l, her AP rose to 253 IU/l, and her DR was 0 63 These levels all returned to normal when coumarin was stopped After 1 month on her second course of coumarin, her SGOT rose to 125 IU/l and her SGPT rose to 296 IU/l Her DR was 0 42 These also returned to normal when coumarin was stopped

COMMENT There was no clinical reason for the coincidence of abnormal LFTs and coumarin therapy Thus, this patient was definite in level B

DM female, aged 64 with carcinoma of the stomach, received one course of coumarin (100mg/day) and cimetidine (400mg/day) She was admitted to hospital 5 weeks later with suspected hepatitis Her serum level of bilirubin were 227 μM/l, her SGPT was 467 IU/l, her AP levels were 136 IU/l, and her serum albumin levels were 34 g/l (normal is 37-50 g/l) Coumarin therapy was stopped, and 2 weeks later her LFTs were repeated Her bilirubin level had risen further to 440 μM/l, her SGPT had fallen to 410 IU/l, her AP levels had fallen to 85 IU/l, her albumin levels had fallen further to 32 g/l, her globulin levels had risen to 35 g/l (normal is 23-32 g/l), and her prothrombin time had risen to 17 sec (normal is 14 sec) By the following week, all enzyme levels started to return towards the normal values The patient had a white blood cell count of 3 5 (normal 4-11) and she had no smooth muscle nor nuclear autoantibodies She was negative for leptospirosis, cytomeglia virus, hepatitis A and B and infectious mononucleosis Her liver ultrasound was normal A needle biopsy was performed on the liver This showed ballooning degeneration and liver cell regeneration Zonal hepatic cell necrosis, hyaline bodies and mild diffuse lymphocytic infiltration were present There were bile thrombi in the canaliculi There was no evidence of metastatic cells 7 months later the patient was retreated with coumarin (100 mg/day) and within 1 month her ALT level had risen to 944 IU/l, her AP was 149 IU/l and her bilirubin level was 227 μM/l Coumarin treatment was stopped and her LFTs returned to normal within 5 weeks An abdominal ultrasound scan showed no abnormalities and the patient was

clinically recurrence-free (see Fig VI 1)

COMMENT Her liver biopsy was consistent with viral hepatitis, but since she was negative for both hepatitis A, and B, and had no history of drug abuse (thus, non-A, non-B hepatitis was unlikely) a cause other than viral infection was probably involved Since neither ultrasound, nor biopsy showed metastatic cells, and the levels returned to normal, cancer could be ruled out Due to the strong temporal relationship between abnormal LFTs and coumarin therapy this patient was definite in level B

ER female, aged 77 with melanoma, received four courses of coumarin Her serum transaminase levels were raised prior to treatment (her SGOT was 65 IU/l and her SGPT was 116 IU/l) By the fifth month on coumarin (100 mg/day) her SGOT was 360 IU/l and her SGPT was 625 IU/l, with a DR of 0 58 These returned to normal when coumarin was stopped After 1 month on coumarin the second time, her SGOT was 124 IU/l and her SGPT was 190 IU/l, with a DR of 0 65 These returned to normal again when coumarin was stopped She was treated a third time with coumarin (50 mg/day), and after 1 month her SGOT was 61 IU/l, her SGPT was 96 IU/l, and her DR was 0 63 Again, these returned to normal when coumarin was stopped The patient was treated a fourth time, and after 1 month her SGOT was 74 IU/l and her SGPT was 103 IU/l with a DR of 0 72 These returned to normal within 2 months when coumarin was stopped The patients isotope liver scans were normal (see Fig VI 2)

COMMENT This patient had slightly elevated serum transaminase levels prior to treatment, but her liver scans were all normal Her levels returned to normal when not on coumarin There were no clinical reasons for her abnormal LFTs other than treatment with coumarin The very strong temporal relationship between coumarin therapy, and elevated LFTs, would indicate that the patient was definite in level B

NM female, aged 40 with melanoma, received two courses of coumarin After 4 months on coumarin (25mg per day) there was an increase in SGOT to 512 IU/l, in SGPT to 648 IU/l and in bilirubin to 29 μM/l Her DR was 0 79 These returned to normal when coumarin was stopped The patient had brain metastases removed, and was retreated with coumarin (300mg per day), cimetidine

(1000mg per day) and dexamethasone (10mg/day) Enzyme levels remained normal (SGPT rose very slightly) until the dexamethasone course was completed Coumarin was lowered to 100mg per day and the cimetidine stopped The serum transaminase levels started to rise after 1 month, and had reached 220 IU/l (SGOT) and 260 IU/l (SGPT) by the second month, and her DR was 0 85 These returned to normal when coumarin was stopped All liver scans were normal The patient died a short while later (see Fig VI 3)

COMMENT This patient had advanced melanoma, with brain metastases, and thus, liver secondaries would be an obvious source of liver problems This possibility can be ruled out for a number of reasons Firstly, her LFTs returned to normal when coumarin therapy was stopped, and remained normal until her death, a few months later This would not be expected in a patient with cancer of the liver Secondly, the lack of involvement of AP in the abnormal LFT, and the low level of bilirubin are not characteristic of liver cancer Thirdly, the patient had liver scans, which were all negative This would indicate that, if present, any liver tumour would have been very small, and thus unlikely to cause any change in LFTs There was no other obvious reason for abnormal LFTs with this patient, thus, she probably had hepatotoxicity to coumarin The return to normal of her LFTs while on dexamethasone, which is a powerful immunosuppressant/anti-inflammatory drug, would indicate that it suppressed an inflammation in the liver, or immune reaction, induced by coumarin This patient was definite in level B

AH female, aged 60 with breast carcinoma, stage IV, received one course of coumarin (25 mg alternate days, increased to 25 mg/day after 8 months) Her SGOT and SGPT had risen after 3 months, and had reached 340 IU/l and 380 IU/l respectively, after 7 months, with a DR of 0 89 Her dose of coumarin was then increased to 25mg/day and she was put on prednisone (60 mg) and cyclophosphamide (50 mg) on alternate days Her enzyme levels returned to normal, even though coumarin treatment was continued Her liver scan was normal (Fig VI 4)

COMMENT Since the patient had breast carcinoma, stage IV, a likely cause of the rise in serum transaminases was liver secondaries The patient had a liver scan, which showed no evidence of metastases Her LFTs further support this The small

rise in serum transaminases is not consistent with liver secondaries, especially since it disappeared so quickly Since cyclophosphamide and prednisone are potent anti-inflammatory and immunosuppressive agents, it is possible that they are reducing an inflammatory/immune response induced by coumarin This patient was possible in level D

CD Male, aged 70 with hypernephroma Patient had a nephrectomy prior to treatment with coumarin After 1 month on 100mg coumarin and 1,000mg cimetidine per day his LFTs started to rise, and within 4 months his SGPT level was 273 IU/l and his SGOT was 175 IU/l Cimetidine was then stopped and prednisone (60mg), and cyclophosphamide(50mg), 3 days per week, 1 week in 4,were started Within 1 month his LFTs had returned to near normal Coumarin was then increased to 1,600mg/day and the patient was continued on cyclophosphamide and prednisone His LFTs then started to rise again and reached a peak 3 months later with SGOT levels of 427 IU/l and SGPT levels of 626 IU/l 3 weeks later these had started to drop but his AP level had risen to 307 IU/l A liver scan showed no abnormalities (Fig VI 5)

COMMENT Since coumarin therapy was never stopped, as the patients condition was too serious for therapy to be stopped, it is very difficult to confirm the role of coumarin in the elevated transaminases One possibility for this rise in LFTs would be liver secondaries, especially since the patient had lung secondaries But, a liver scan showed no abnormalities This patient probably has an adverse reaction to coumarin Immunosuppressive therapy seemed to reduce the LFTs with the lower dose of coumarin, but not at the higher dose Possibly continuous immunosuppressive therapy may have a stronger effect in lowering LFTs

DN male, aged 51 with chronic brucellosis, received two courses of coumarin The first course (100mg, 3 days per week) resulted in a rise in serum transaminases to 104 IU/l (SGOT) and 208 IU/l (SGPT), after 5 months, with a DR of 0 5 The second course of coumarin (50mg/day) resulted in a rise in serum transaminase levels to 46 IU/l (SGOT) and 66 IU/l (SGPT) after 1 month, with a DR of 0 7 Four months later, these levels were much the same, and they eventually returned to normal while the patient was still on coumarin (not shown)

COMMENT His LFTs returned to normal while still on coumarin, and thus the role of coumarin in the elevated LFTs is uncertain Possibly the elevation is not due to a toxic effect, but reflects some therapeutic response to the drug It could also be due to a mild, transient toxic effect Thus, the role of coumarin is uncertain

JG male, aged 60 with melanoma, received three courses of coumarin (50mg per day) During the first course his LFT's started to rise after 3 months and by month 6 his SGPT had reached 170 IU/l, his SGOT was normal and his DR was 0 1 These returned to normal when coumarin was stopped After 2 months on coumarin, the second time, his SGPT was 140 IU/l, his SGOT was 68 IU/l, and his DR was 0 48 Again, these returned to normal when coumarin was stopped On the third occasion (not shown on table), the increase was only slight, and the levels returned to normal during treatment with coumarin (see Fig VI 6)

COMMENT His LFTs returned to normal while still on coumarin, and thus the role of coumarin in the elevated LFTs is uncertain Possibly the elevation is not due to a toxic effect, but reflects some therapeutic response to the drug It could also be due to a mild, transient toxic effect Thus, the role of coumarin is uncertain

PW male, aged 59 with melanoma, received two courses of coumarin (50mg/day) After 4 months on the first course his SGOT was 64 IU/l, his SGPT was 138 IU/l and his DR was 0 46 These returned to normal when coumarin was stopped During the second course of coumarin, the serum transaminase levels started to rise after 1 month, and reached a peak after 3 months, when, his SGOT was 47 IU/l, his SGPT was 110 IU/l and his DR was 0 43 These returned to normal, even though coumarin therapy was continued

COMMENT His LFTs returned to normal while still on coumarin, and thus the role of coumarin in the elevated LFTs is uncertain Possibly the elevation is not due to a toxic effect, but reflects some therapeutic response to the drug It could also be due to a mild, transient toxic effect Thus, the role of coumarin is uncertain

MN female, aged 54 with immune suppression, received three courses of coumarin (only two shown) During the first course (25mg/day), her SGOT rose to 87 IU/l and her SGPT rose to 115 IU/l, with a DR of 0 74, after 10 months on coumarin During the second course on coumarin (25mg/day), her SGOT rose to 76 IU/l and her SGPT rose to 96 IU/l, with a DR of 0 79, after 3 months of treatment During her third course of coumarin (50mg/day), her SGOT rose to 51 IU/l, and her SGPT rose to 65 IU/l, with a DR of 0 78, after 2 months of treatment In each case her LFT's returned to normal when coumarin was stopped

COMMENT Her LFTs returned to normal while still on coumarin, and thus the role of coumarin in the elevated LFTs is uncertain Possibly the elevation is not due to a toxic effect, but reflects some therapeutic response to the drug It could also be due to a mild, transient toxic effect Thus, the role of coumarin is uncertain

TB male, aged 36 with chronic brucellosis, received one course of coumarin (50mg/day) His transaminase levels rose to 271 IU/l (SGOT) and 360 IU/l (SGPT), his AP levels rose to 278 IU/l and his DR was 0 75 His LFT's had started to rise after 8 months on coumarin Coumarin was stopped but the patient never returned to the clinic for further treatment

COMMENT There were no clinical reasons for the rise in his LFTs other than the administration of coumarin Thus, hepatotoxicity to coumarin was a probable cause of the hepatitis, but without being retreated, it is impossible to be certain This patient was probable in level D

LS female, aged 60 with immune suppression, received one course of coumarin (50mg/day), and her serum transaminases levels rose to 129 IU/l (SGOT) and 196 IU/l (SGPT), and her DR was 0 66 Her LFT's started to rise after 1 month on coumarin, but they returned to normal while still on coumarin

COMMENT Her LFTs returned to normal while still on coumarin, and thus the role of coumarin in the elevated LFTs was uncertain Possibly the elevation is not due to a toxic effect, but reflects some therapeutic response to the drug It could also be due to a

mild, transient toxic effect Thus, the role of coumarin is uncertain

JF female, aged 60 with breast carcinoma, stage II, was treated with coumarin (25 mg/day, increased to 300 mg/day after 5 months) After 7 months she was started on ibuprofen 2 months later her SGOT rose to 141 IU/l and her SGPT rose to 190 IU/l and her DR was 0 74 These returned to normal when coumarin and ibuprofen were stopped Her liver scan was normal and she was not retreated with coumarin

COMMENT Since the rise in transaminases only occured after 9 months it would seem unlikely to be due to coumarin In most cases the rise came by the sixth month of therapy The coincidence of treatment with ibuprofen 2 months prior to the rise in transaminases suggests a possible interaction between coumarin and ibuprofen But, without being retreated it is impossible to be certain

EL male, aged 70 with oesophageal carcinoma, received one course of coumarin (50mg/day) After the first month his SGOT had started to rise Coumarin was then increased to 200 mg/day, and by the second month his SGOT had risen to 330 IU/l, and his SGPT had risen to 254 IU/l His DR was 1 3 These started to fall when coumarin was stopped and prednisone (60mg/day) therapy was commenced, but within 2 months these had started to rise again His LFT's continued to rise, even though coumarin therapy was not restarted The patient died soon afterwards

COMMENT This patient was 70 years of age with oesophageal carcinoma and died soon after coumarin was stopped While coumarin could possibly have had a role to play in the rise in LFTs, the major cause was probably liver secondaries, especially since his LFTs continued to rise after coumarin was stopped Thus, the patient was classed as unlikely since there was no temporal relationship between the elevated LFTs and coumarin therapy

ED male, aged 65 with chronic brucellosis, received one course of coumarin (50mg per day) His LFTs rose while on coumarin, after 7 months, but they continued to rise when coumarin was stopped Bilirubin levels rose to 38 µM/l, serum transaminases

rose to 680 IU/l (GOT) and 960 IU/l (GPT), serum AP levels rose to 109 IU/l and his DR was 0 71 AP levels were elevated before coumarin treatment commenced, and fluctuated with coumarin therapy All levels returned to normal by month 17 The patient was hepatitis A and B negative, but there was infectious hepatitis in his family

COMMENT Coumarin may have had a role to play in this patients hepatitis, but this could not be confirmed, as the patient was not retreated While the patient was hepatitis A and B negative, the incidence of infectious hepatitis in his family would indicate a possibility of non-A, non-B hepatitis infection The delay in the LFT returning to normal after coumarin therapy was stopped, would also indicate that coumarin was unlikely to be involved

EN male, aged 59 with toxoplasmosis, received two courses of coumarin treatment During the first course (100mg/day) his serum transaminase levels started to rise after 3 months and continued to rise for a further 4 months, at which stage his SGOT was 192 IU/l, his SGPT was 380 IU/l, and his DR was 0 5 These levels returned to normal even though coumarin was continued One year later the patient was retreated with coumarin (50mg/day) Three months after treatment, the patient, while on holidays in Spain, felt unwell and doubled his dose of coumarin to 100mg/day On returning to the hospital (5 months after the course began), his SGOT was 370 IU/l, his SGPT was 824 IU/l, and his DR was 0 45 Infectious hepatitis was suspected and his LFTs were repeated His SGOT and SGPT had fallen, even though coumarin treatment had not been stopped The patient was kept on coumarin, and his LFTs returned to normal

COMMENT This second rise occured while the patient was holidaying in Spain, so a viral infection could be possible The patient was found to be hepatitis A negative, and had no history of drug abuse or recent blood transfusion Possibly a non-A, non-B hepatitis was involved Other liver infections are unlikely, since the patient did not suffer from jaundice, and only his serum transaminases were affected (and these were affected to a small degree compared to patients with hepatitis A) The patient had toxoplasmosis, which does not usually cause abnormal LFTs, especially of such a limited nature Since in each case his LFTs returned to normal while still on coumarin it was unlikely that

coumarin was involved in the rise in LFTs

TABLE VI 1

This table shows the liver functon test results of the patients with definite or possible/probable hepatoxicity, excluding those on immunosuppressive therapy

NAME	PARA-METERS	PRE	MONTHS ON DRUG 1	2	3	4	5	6	7	8	9	10	11	12	13	14
	DOSE	0	5	5	3	3	3	3	0	0						
CW	SGOT	17	19		19			248		30						
	SGPT	25	21		20			500		66						
	DOSE	0				3	0									
CW	SGOT	18				136	27									
*	SGPT	19				326	57									
	DOSE	0	5	3	3	3	3	0	0	3	0	0				
ML	SGOT	17	21				440	57	23	125	32	21				
	SGPT	28	30				696	118	39	296	49	27				
	AP	19	19				253	145	128	128	109	92				
	DOSE	0	3	3	3	3	3	3	5	3	3	3	3			
EN	SGOT	21			64			144	192				31			
	SGPT	23			120			200	380				34			
	DOSE	3	3	3	5	5	5	5	5	5	5	5	0	0	3	
EN	SGOT	20					370	53	56			30			23	
*	SGPT	17					824	94	60			28			18	
	DOSE	0	5	5	5	0	0	0	0	0	0	0	0	5	0	0
DM	SGPT	7			467	410	16	10		10			11	944	178	16
	Blrbn	<17			227	440	83	<17		<17			<17	149	85	19
	AP	44			136	85	47	51		51			48	227	55	47
	DOSE	0	5	5	5	5	5	0	5	0	0	3	0	3	0	0
ER	SGOT	65	58				360	34	124	44	20	61	34	74		30
	SGPT	102	116				625	46	190	66	27	96	40	103		33

NORMAL LEVELS SGOT (AST) < 40 IU/L Bilirubin < 17 uMol/L
SGPT (ALT) < 35 IU/L AP < 50 IU/L (or < 230 IU/L)

Dose regimens 0= 0mg/day 1= 25mg alternate days 2= 25mg/day
3= 50mg/day 4= 100mg 3 days per week 5= 100mg/day
7= 300mg/day 8= 1,600 mg/day

* Indicates a second course of drug, given much later than the first course

TABLE VI 2

This table shows the liver function test results and coumarin dosage regimens for patients on immunosuppressive therapy

NAME	PARA-METERS	PRE	MONTHS ON DRUG 1	2	3	4	5	6	7	8	9	10	11	12	13	14
	DOSE	0	2	2	2	2	0	7	7	7	7	7	5	5	0	0
NM	SGOT	20	12			512	18	28		16	31		90	220	19	10
	SGPT	21	10			648	18	74		28	50		90	260	28	10
	Blrbn	4	6			29	8	6		6	6		11	9	11	9
	DOSE	0	1	1	1	1	1	1	1	2	2	2	2	2	2	
	SGOT	14			120				340		28				18	
AH	SGPT	16			274				380		27				18	
	Blrbn	5			10				17		12				11	
	AP	33			38				61		38				32	
	DOSE	0	5	5	5	5	5	5	5	8	8	8				
	SGOT	29	118			175		165	40	92	427	233				
CD	SGPT	39	162			273		216	63	120	626	309				
	AP	159	161			245		187	127	164	242	307				

NORMAL LEVELS SGOT (AST) < 40 IU/L Bilirubin < 17 uMol/L
SGPT (ALT) < 35 IU/L AP < 50 IU/L (or < 230 IU/L)

Dose regimens 0= 0mg/day 1= 25mg alternate days 2= 25mg/day
3= 50mg/day 4= 100mg 3 days per week 5= 100mg/day
7= 300mg/day 8= 1,600 mg/day

* Indicates a second course of drug, given much later than the first course

TABLE VI 3

This table shows the liver function test results of the patients not shown in table VI 1 or VI 2

NAME	PARA-METERS	PRE	MONTHS ON DRUG 1	2	3	4	5	6	7	8	9	10	11	12	13	14
	DOSE	0	5	5	7	7	7	0	0	0	3	3	3	3	3	
DN	SGOT	30		34			104		27	24	46				41	
	SGPT	36		52			208		41	25	66				79	
	DOSE	0	3	3	3	3	3	3	0	0	0	5	5	0		
JG	SGOT	19			26			17			16		68	20		
	SGPT	20			39			170			17		140	22		
	DOSE	0	3	3	3	3	0	3	3	3	3	3	3	3	3	
PW	SGOT	16	18			64	23	38		47	26	23			31	
	SGPT	17	23			138	34	70		110	43	31			46	
	DOSE	0	5	3	3	3	3	3	3	3	3	3	0	3	3	3
MN	SGOT	18	25			19			19			87	20			76
	SGPT	14	22			20			16			115	15			96
	DOSE	0	5	5	5	5	5	5	5	5	5					
TB	SGOT	19		20		22				37	271					
	SGPT	18		20		25				65	360					
	AP	39		27		34				282	278					
	DOSE	0	5	3	3	3	3	3								
LS	SGOT	20	69			129		38								
	SGPT	19	103			196		60								
	DOSE	0	2	2	2	2	7	7	7	7	7	0	0	0	0	
JF	SGOT	17				21			26		141	31			37	
	SGPT	19				19			33		190	60			25	
	DOSE	0	3	6	0	0	0	0								
EL	SGOT	36	47	330	27	46		52								
	SGPT	18	22	254	35	28		120								
	AP	95	90	152	114	150		192								
	DOSE	0	3	3	3	3	3	3	3	0	0	0				
	SGOT	18	28		26				172		680	150				
ED	SGPT	20	41		33				240		960	192				
	BLRBn	6	8		6				6		38	6				

NORMAL LEVELS SGOT (AST) < 40 IU/L Bilirubin < 17 uMol/L
SGPT (ALT) < 35 IU/L AP < 50 IU/L (or < 230 IU/L)

Dose regimens 0= 0mg/day 1= 25mg alternate days 2= 25mg/day
3= 50mg/day 4= 100mg 3 days per week 5= 100mg/day
7= 300mg/day 8= 1,600 mg/day

TABLE VI 4

This table shows the deRitis ratio (SGOT/SGPT) for the initial (DR1) and subsequent elevations in serum transaminase levels for all the patients

PATIENT NAME	DR1	DR2	DR3	DR4
CW	0 50	0 42	---	---
ML	0 63	0 42	---	---
EN	0 50	0 45	---	---
ER	0 58	0 65	---	---
AH	0 89	---	0 63	0 72
NM	0 79	0 85	---	---
ED	0 71	---	---	---
DN	0 50	0 70	---	---
TB	0 75	---	---	---
LS	0 67	---	---	---
MN	0 74	---	---	---
JF	0 74	0 79	0 79	---
EL	1 30	---	---	---
JG	0 10	0 48	---	---
PW	0 46	0 43	---	---
CD	0 64	----	---	---

TABLE VI 5

This table summarises the data on the number of courses of coumarin and the role this played in the hepatitis

PATIENT NAME	NO OF COURSES OF COUMARIN	LEVEL *	STATUS *
CW	2	B	def
ML	2	B	def
EN	2	B	unl
DM	2	B	def
ER	4	B	def
NM	2	B	def
DN	2	B	pos/unl
JG	3	B	pos/unl
PW	2	B	pos/unl
MN	2	B	pos/unl
CD	1	D	pos/prob
AH	1	D	pos/prob
TB	1	D	pos/prob
LS	1	D	pos/unl
JF	1	D	unl
EL	1	D	unl
ED	1	D	unl

* See section II 8 4 for definition of these terms

def= definite , prob= probable, pos= possible, unl= unlikely

TABLE VI 6 Summarising the data on all patients

PATIENT	DOSE (mg/day)	DIAGNOSIS	PEAK ALT LEVELS (IU/l)	TIME TO 1st ALT RISE (MONTHS)	TIME TO 2nd ALT RISE (MONTHS)
CW	50	Brucellosis	500	6	4
ML	50	Mononucleosis	696	5	1
DM	100	Carcinoma stomach	467	3	1
ER	100/50	Melanoma	625	1	1
CD	100/1,600	Hypernephroma	626	4	--
NM	25/300	Melanoma	648	4	6
AH	25	Brucellosis	380	3	--
TB	50	Brucellosis	360	8	--
EN	300/50	Brucellosis	208	2	1
JG	50	Melanoma	170	3	2
PW	50	Melanoma	138	4	1
MN	50	Chronic Fatigue Synd	115	4	1
LS	50	Chronic Fatigue Synd.	196	1	--
JF	25/300	Carcinoma Breast	190	9	--
EL	200	Carcinoma Oesophagus	254	1	--
ED	50	Brucellosis	960	7	--
EW	50/100	Toxoplasmosis	380	3	5

FIG VI 1 Showing the effects of coumarin on serum GPT (ALT) and bilirubin levels for patient DM C1 and C2 are 100 mg of coumarin per day

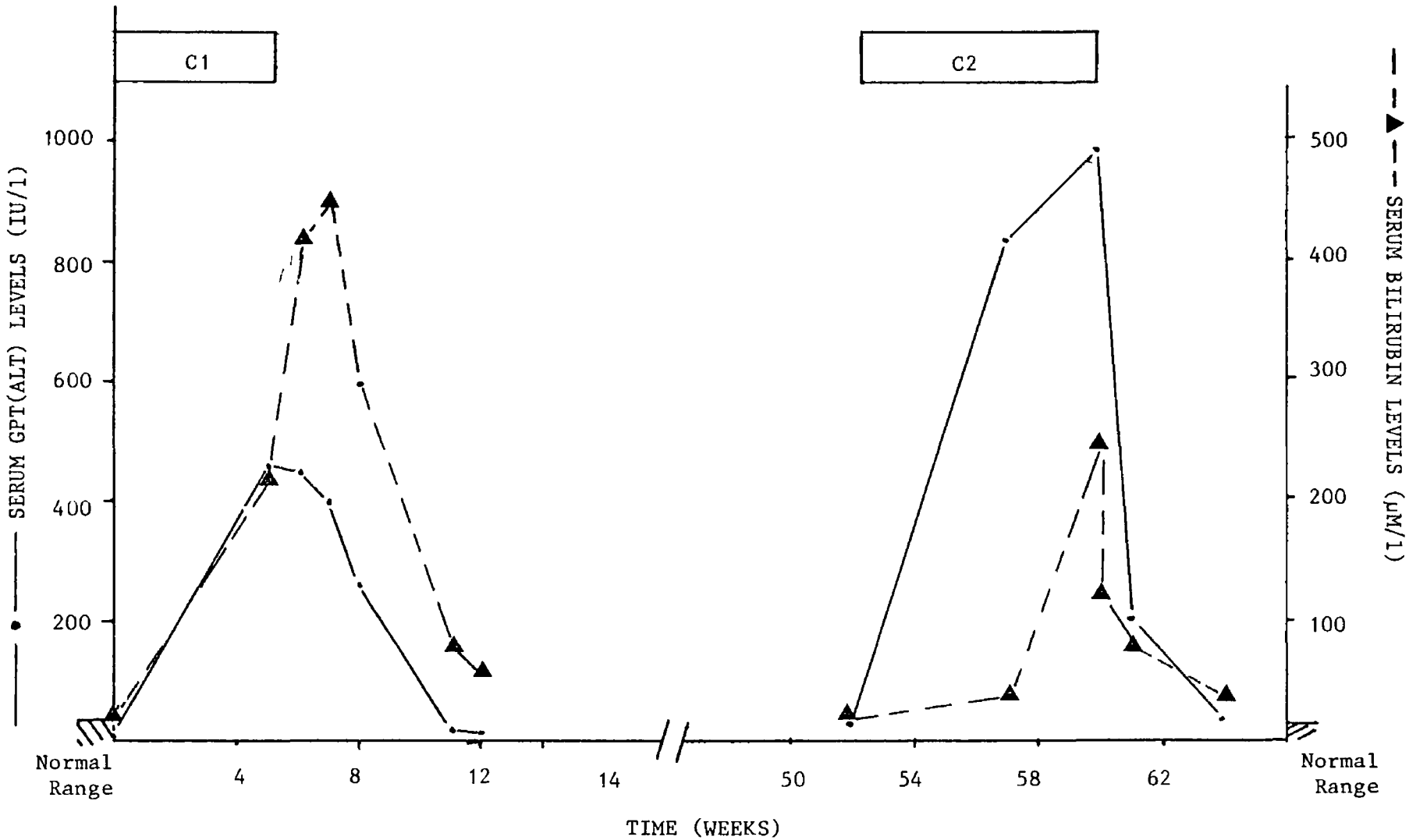
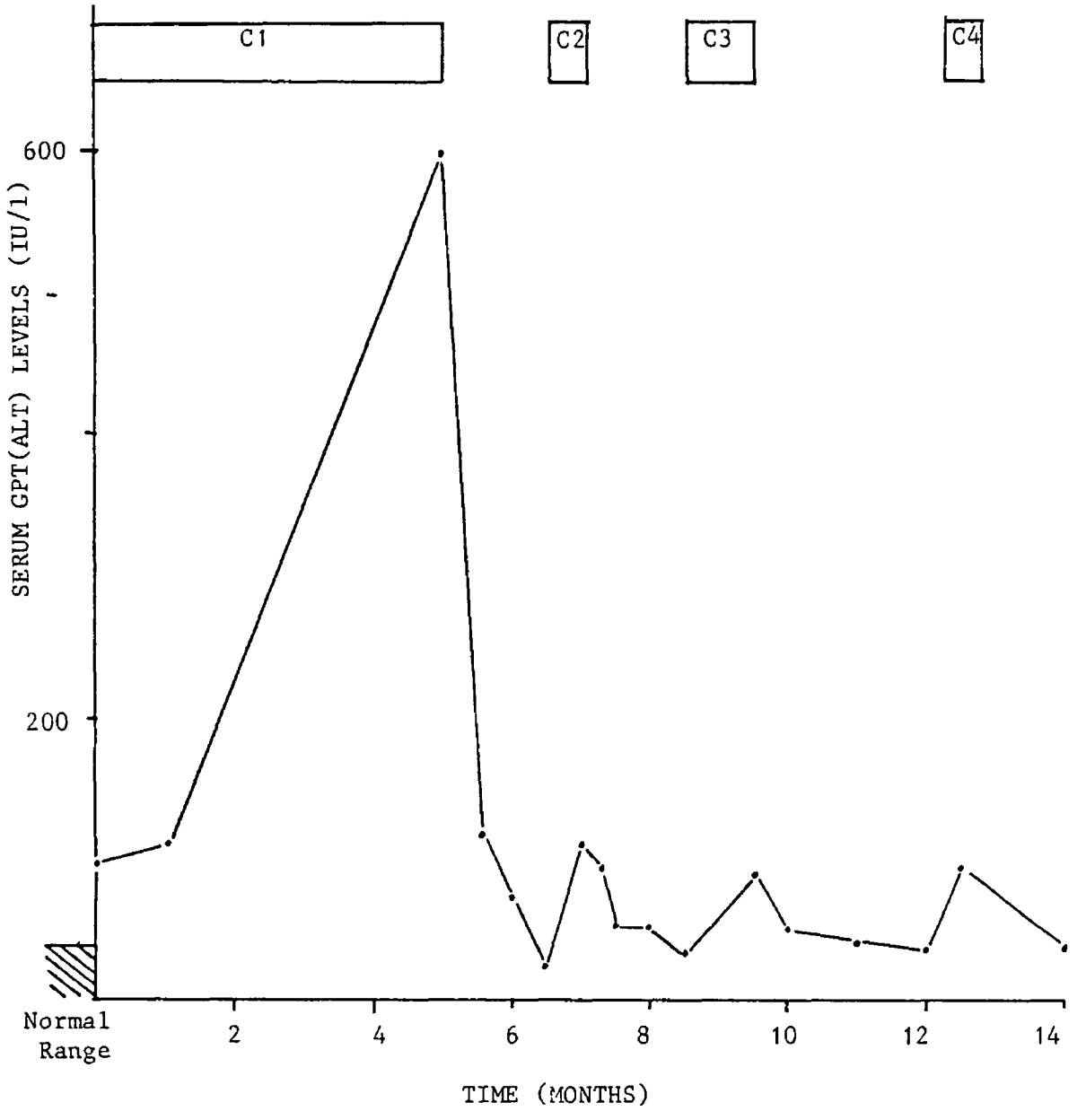


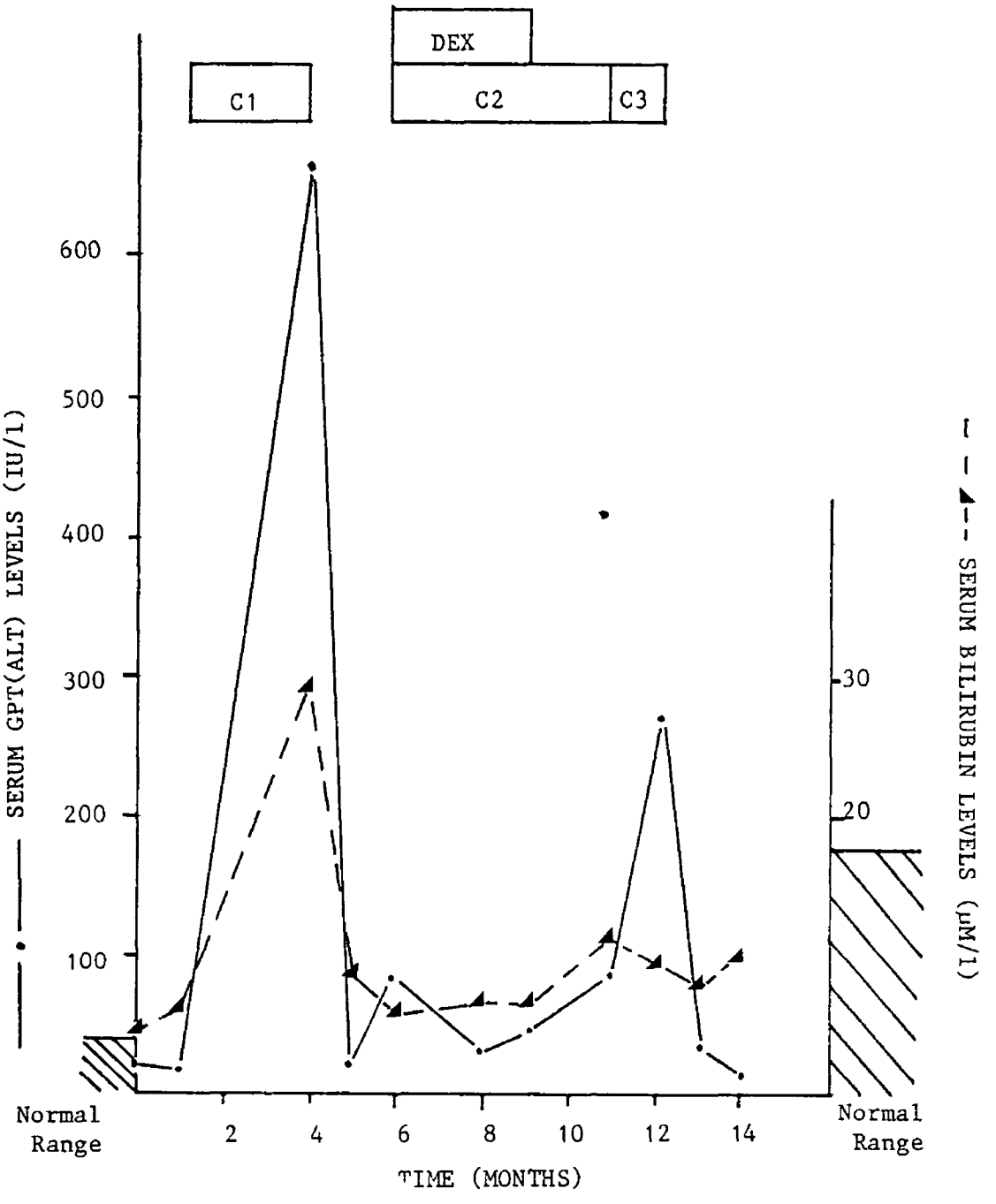
FIG VI.2 Showing the effects of coumarin on serum GPT(ALT) levels in patient ER



C1, C2 = 100 mg/day

C3, C4 = 50 mg/day

FIG VI.3 Showing the effects of coumarin on serum GPT(ALT) and bilirubin levels in patient NM



C1 = 25 mg coumarin per day

C2 = 300 mg coumarin per day

C3 = 100 mg coumarin per day

DEX = 10 mg dexamethasone per day

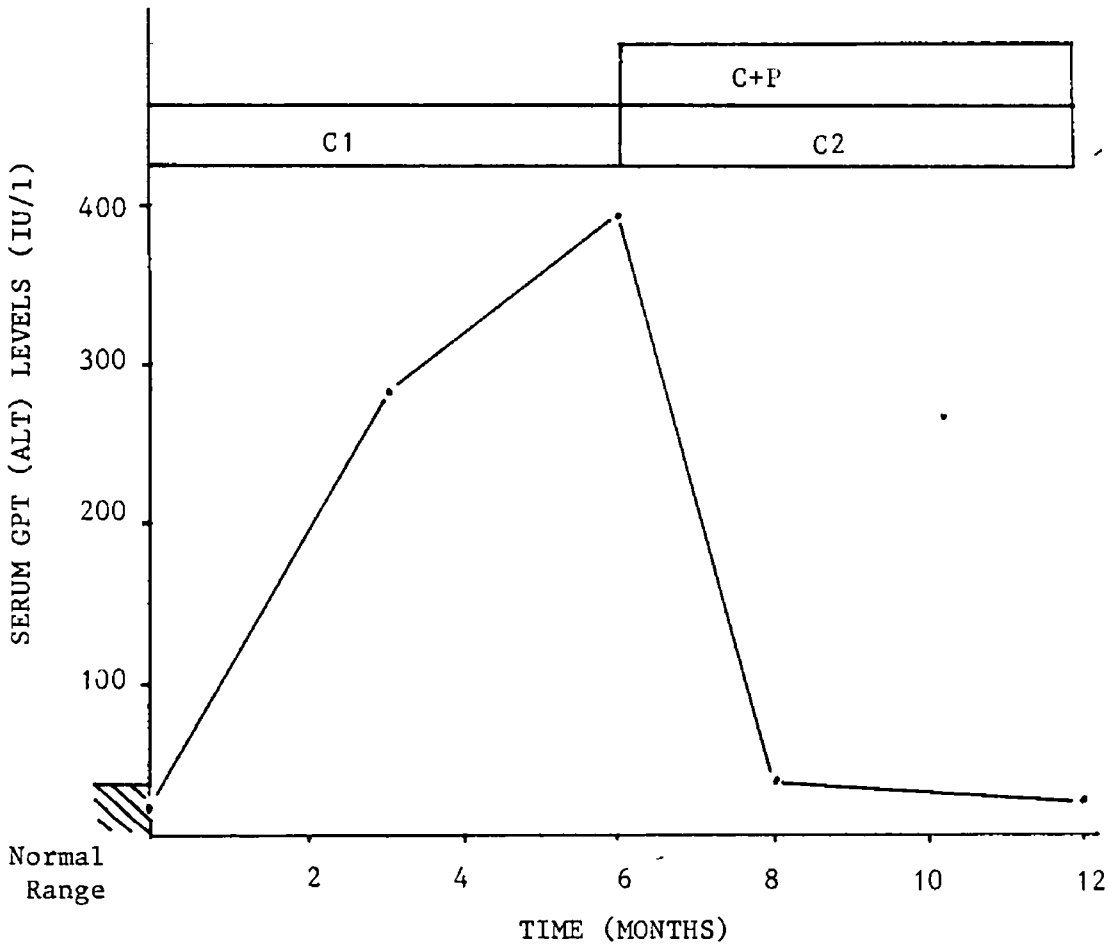
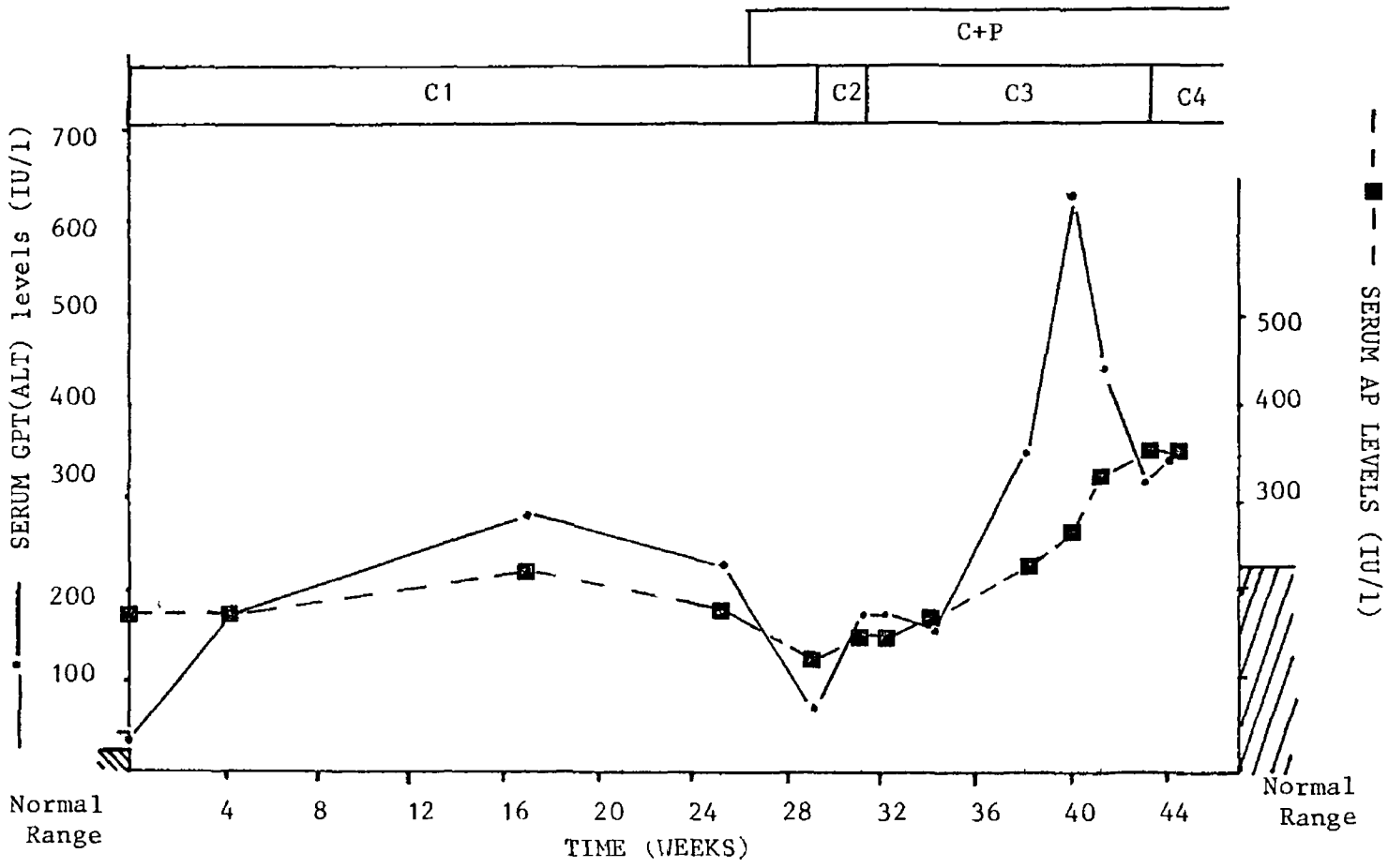


FIG VI.4 Showing the effects of coumarin on serum GPT (ALT) levels for patient AH

C1 = 25mg coumarin on alternate days
C2 = 25 mg coumarin per day

C+P = 50 mg cyclophosphamide and 60 mg prednisone on alternate days

Fig VI 5 Showing the effects of coumarin on serum GPT(ALT) and alkaline phosphatase levels for patient CD



C1 and C4 = 100 mg coumarin per day C2 = 600 mg coumarin per day

C3 = 1600 mg coumarin per day

C+P = 60 mg prednisone and 50 mg cyclophosphamide on alternate days (1 week in 4)

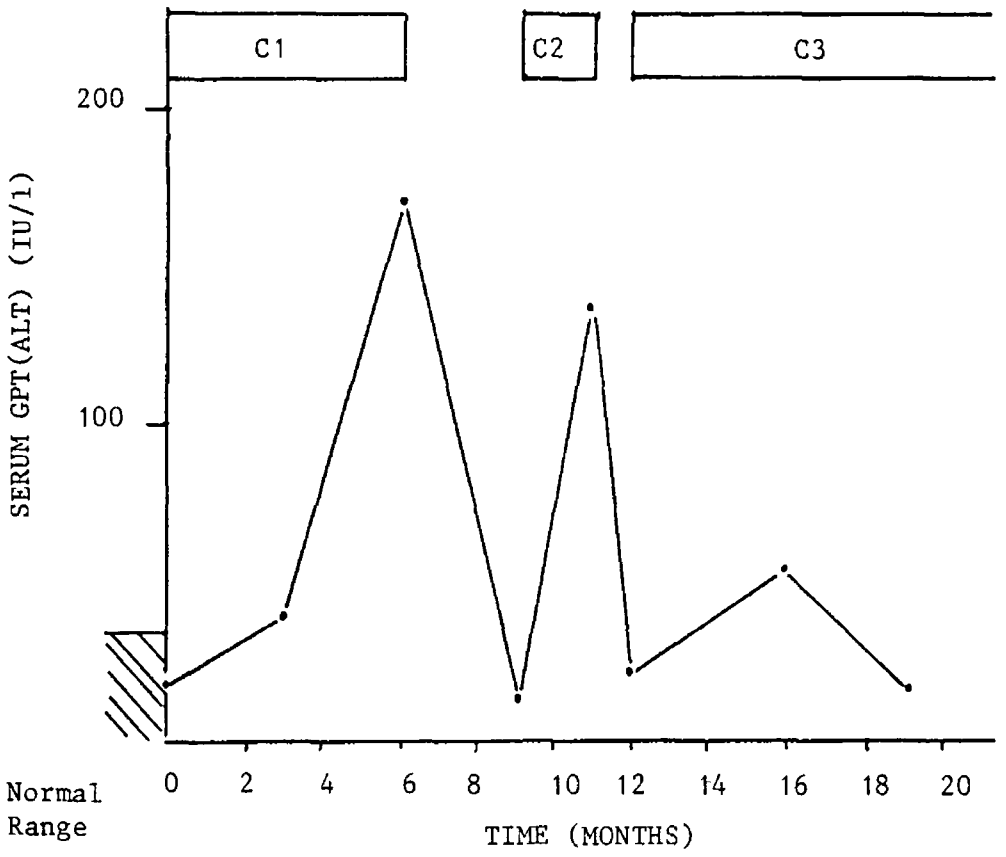


Fig VI 6 Showing the effects of coumarin on serum GPT(ALT) levels for patient JG

C1, C2, and C3 = 50mg coumarin per day

38 patients treated with coumarin had abnormal LFTs during therapy Only 16 of these had increases in their enzyme levels that were sufficiently large (ie twice normal value) to warrant further investigation This level was selected to eliminate patients with small rises in LFTs due to variation in assay procedure, or due to other stresses upon the liver Patients were classified according to the method of Stricker and Spoelstra (1985) They divide cases into different levels and also into the categories definite, probable, possible and unlikely (see Materials and Methods) Since there has only ever been 1 reported case of hepatotoxicity to coumarin (Faurschou, 1982), level A does not apply

VI 3 1 THE ROLE OF COUMARIN

17 patients in the study showed a sufficiently large rise in their LFTs to have further investigation for hepatotoxicity 4 of these (ED, EL, JF, and EN), were considered unlikely to be adverse reactions to coumarin, due to the nature of the hepatitis EL's hepatitis was probably due to liver secondaries, both ED's and EN's hepatitis were thought to be infectious in nature, and JF's hepatitis was possibly due to an interaction with ibuprofen 5 patients (DN, JG, PW, MN, and LS) developed elevated LFTs which returned to normal while still on coumarin The significance of this is uncertain, and whether it represents a mild hepatotoxic effect to which the patients became tolerant, or a therapeutic response by coumarin is unknown

8 patients (CW, ML, DM, ER, NM, AH, CD, and TB) developed elevated LFTs which had no apparent clinical cause other than as a result of the administration of coumarin 5 of these patients were retreated with coumarin, and were thus in level B These 5 had definite hepatotoxicity, due to the strong temporal relationship, and the lack of other possible causes TB never returned for treatment after coumarin therapy AH's LFTs returned to normal when immunosuppressive therapy began, so her coumarin therapy was never stopped CD's prognosis was very poor, so it was considered too risky to stop coumarin therapy as long as his LFTs did not rise to a dangerous level These 3 patients who were

not retreated, or who did not have a break in therapy were in level D, and their elevated LFTs were possible/probable, rather than definite hepatotoxicity drug Thus, in 8 of the 17 patients, there was a very strong association between administration of coumarin, and appearance of the hepatitis, in 5 of the patients there was a weak association while in 3 of the patients the association was probably coincidental

VI 3 2 THE NATURE OF THE ADVERSE REACTION TO COUMARIN

In 15 cases there was a distortion of the enzyme pattern, with SGPT levels higher than SGOT levels This indicates that the loss of enzymes was mainly cytoplasmic Thus, while the necrosis may have been extensive, it was not severe 5 patients had a slight rise in AP levels, and 3 had elevated bilirubin levels 8 patients had a deRitis ratio of less than 0 7

In all, 8 patients had a relationship between coumarin, and their hepatitis, which was predominantly hepatocellular This was indicated by the relatively large elevations in serum transaminases, and the limited rise in AP and bilirubin levels The presence of zonal necrosis, and balooning degeneration, in the needle biopsy, further confirms this The jaundice experienced by 3 of the patients, and the presence of bile casts in the needle biopsy, would indicate that it was possibly mixed hepatocellular hepatotoxicity

The liver toxicity seen in these patients was unpredictable in nature The dose that produced abnormal LFTs varied from patient to patient, the time taken to produce an elevated LFT varied from 3 to 8 months, subsequent elevations usually occured much quicker than the first elevation, and the number of patients affected was very small (0 3%) All these factors suggest an idiosyncratic hepatotoxicity (Zimmerman, 1972, 1982)

Three patients responded favourably to immunosuppressive therapy Since there was no evidence of inflammation from the biopsy, and none of the patients had a palpable (enlarged) liver, these drugs were unlikely to be controlling the toxicity via an anti-inflammatory effect They were more likely suppressing an immune reaction, induced by coumarin Shoham (1985) reports on a number of adverse effects to treatment with immunostimulatory drugs Many of these responses are directly due to stimulation of

the immune system In some cases an inappropriate response is created, while in other cases, eg interferon therapy, many of the side effects are due to the therapeutic effects of the treatment This can be seen in viral infections where many of the symptoms are absent when the immune system is suppressed However, when the immune system is restored these symptom reappear In this case the effect is not a toxic effect, but a therapeutic effect This could be the case with patients treated with coumarin, especially those with the transient elevated LFTs

The abnormal liver function seen here is different to the hepatotoxicity seen in animal studies Hepatotoxicity data from rats cannot be extrapolated to humans, as the metabolism of coumarin in rats is very different to that in humans (Cohen et al, 1979) The doses used clinically are much lower than the doses that caused hepatotoxicity in baboons (67 5 mg/kg/day) (Evans et al , 1979) The toxicity seen in baboons was dose dependent, and resulted in liver enlargement, unlike the dose independent toxicity seen in humans

There was one previously reported case of hepatotoxicity to coumarin in humans (Faurschou, 1982) This was in a 33 year old woman with Turner's syndrome She had elevated bilirubin, AP and SGOT levels Her biopsy showed portal tract inflammation with eosinophilia, multinucleated hepatocytes (a form of degeneration), and moderate centrilobular cholestase This could have been hepatocanilicular, but since her SGOT was very high (843 IU/l), it would indicate that there was extensive necrosis, and therefore, this was probably mixed hepatocanalicular Her jaundice cleared up when coumarin was stopped She was not retreated with coumarin

VI 3 3 CONCLUSION

This study shows that 0 23% of patients treated with coumarin had a definite, and 0 13% had a probable/possible, adverse reaction to coumarin This adverse reaction was expressed as hepatotoxicity, which was idiosyncratic and hepatocellular Since hepatocellular toxicity is potentially very serious, patients, undergoing treatment with coumarin, should have liver function tests every 3-6 months These LFTs should include the determination of serum bilirubin, GOT, GPT and AP levels If there is evidence of abnormal liver function in any patient, coumarin therapy should

be stopped When the patient's LFTs return to normal, coumarin therapy can be restarted, with more frequent LFTs (eg monthly) If the patient reacts again, coumarin therapy should be stopped completely In all 14 cases, liver function returned to normal when coumarin therapy was stopped, without any permanent liver damage There was some evidence to show that immunosuppressive therapy can moderate the toxicity Thus, in any patient who has a severe reaction to coumarin, immunosuppressive therapy (eg glucocorticoids) may help to control the toxicity

SECTION VII

CONCLUSIONS

VII CONCLUSION

The work presented here focuses on the production and characterization of antibodies, as well as a study of the *in vivo* and the *in vitro* effects of coumarin Monoclonal antibodies against FVIII CAg and fibrinogen were successfully produced and characterised While the monoclonal antibody technique proved successful in this case, it did not produce any results when a diazepam conjugate was used This highlights one of the problems with the technique - variability Part of the reason for this variability is a lack of data on the essential parameters for a successful fusion Thus, different operators have different methods for fusions, and no particular reason for using them other than that "they work" Two other sources of variability are batch-to-batch variation in foetal calf serum, and in the Polyethylene glycol Once fusions work, this variability is unimportant However, it is very difficult to determine the cause(s) for unsuccessful fusions when the essential factors are unknown

Despite the problems with monoclonal antibody production the technique has a very important role to play, especially in the production of antibodies to conjugates If 10% of the antibodies against a particular conjugate are directed against the hapten portion, then 90% of the antiserum from an immunised animal would be lost during purification of the antiserum This makes polyclonal antiserum against haptens very expensive This problem can be overcome by selecting a clone of cells that produces antibodies against the hapten only

While antibodies have a role to play *in vivo*, their major role is in analysis The monoclonal antibodies produced against the blood coagulation factors will all be used for development using voltammetry, bifunctional antibodies, and a novel method of immunoanalysis using iodinated Bolton Hunter-conjugated antibodies These antibodies have recently been developed in our laboratories, and those of our collaborators (Rodriguez Flores *et al* , 1988a, 1988b, O'Kennedy *et al* , 1988, Reading *et al*., 1988)

Blood coagulation factors have a role in tumour growth Brown *et al* , (1988) reported a role for fibrin in the growth of tumours

McCulloch and George, (1988) found that a complex of blood coagultaion factors had ablity to promote tumour metastasis idependently of fibrin Metastases can be inhibited by certain coumarins, such as the anticoagulant warfarin This association between coumarin anticoagulants and cancer, led to a study of the effects of other coumarins on tumour growth This resulted in research on coumarin, and more recently a synthetic coumarin-like compound, Flavone acetic acid (Urba et al , 1988), as anticancer agents Thus, a study on both blood coagulation factors and coumarin are very relevant to understanding tumour growth, and may have a role to play in cancer therapy

SECTION VIII

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