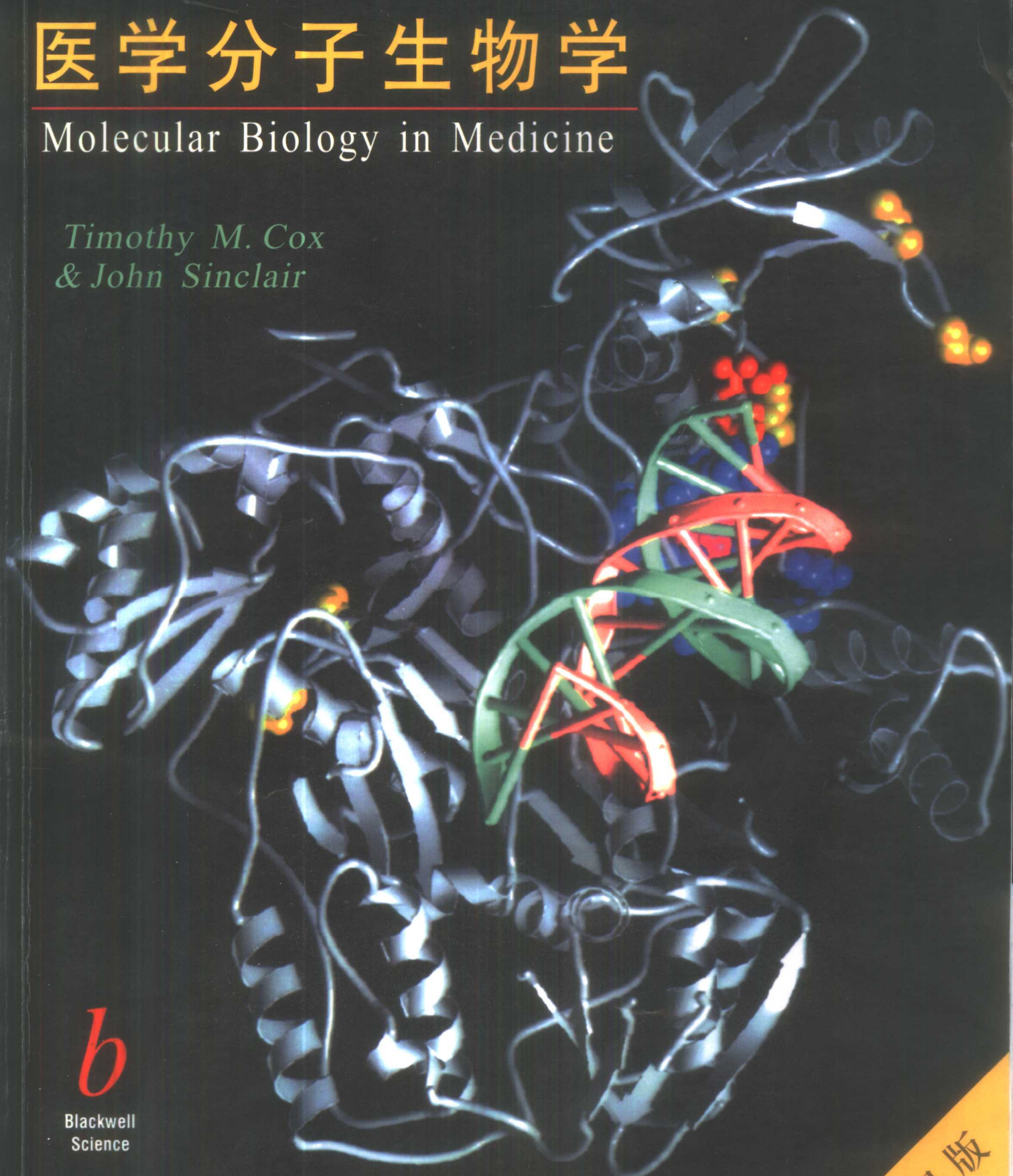
医学分子生物学

Molecular Biology in Medicine

Timothy M. Cox
& John Sinclair



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Molecular Biology in Medicine

Timothy M. Cox MA MSc MD FRCP
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内 容 简 介

本书由剑桥、牛津等名校的医学院资深教授和学者联合编著而成，图文并茂，彩图极具参考价值。内容涉及基因结构、基因表达、基因及药物治疗和人类疾病的遗传学缺陷，包括微生物感染、病毒感染、血友病、免疫系统紊乱及癌症等等。是一本有关分子生物学概念、原理和方法在医学中应用的指导性书籍，视角独特，思维清晰。

可作为医学院校及综合性大学生物类专业教师和学生的教学科研参考用书。

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Molecular Biology in Medicine

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Preface

This book has been produced during an intellectual revolution in medical science from which there can be no retreat. The revolution is not a conceptual one but amounts simply to the realization that human physiological processes and the full spectrum of human disorders, congenital and acquired, are rapidly becoming susceptible to analysis in molecular terms. The techniques of molecular biology have evolved because of the need to understand the genetics of primitive organisms such as bacteria and phages, and also eukaryotic cells. It is a time of revelation in biology but given the ethos of medicine, the distractions of its practice and its pastoral demands, this excitement has neither been generally felt nor appreciated by established physicians or those in training.

Molecular biology is beginning to attack problems beyond those posed by the study of viruses or isolated cells, whether they be prokaryotes or eukaryotes. Its methods are now being applied to questions concerning the control of development and differentiation of whole tissues and organs, as well as the function of neural networks. Tumour formation, embryology and neurobiology, once subjects for observation, are now susceptible to decisive experiment, a triumph of analytical reductionism. Clearly, these advances bring with them implications for ethics in therapy as well as diagnosis—issues which are complex and which are themselves in a state of evolution.

Although the conceptual framework and lexicon of molecular biology is based upon the genetics of phage, yeast and bacteria, this probably does not explain the failure of many medical minds to grasp it. Simply the pace of discovery and the expansion of knowledge has been too rapid for full integration into training programmes and practice. By now, 20 years since methods for sequencing cloned genes have been developed, we already have novel reagents for the analysis, diagnosis and definitive treatment of human disease. These practical benefits have followed, with unprecedented rapidity, the tail of discovery.

The practice of medicine itself is changing and with it a self-conscious realization of its limitations and failure to influence favourably the demography of human disease. In response to a questioning public and a political climate obsessed with 'cost-effective' therapies, thoughtful doctors are increasingly conscious of ethical issues raised by contemporary 'high-tech' practices. Application of molecular genetics, especially in relation to predictive DNA testing for predisposition to conditions such as neurodegenerative disease or cancer, will precipitate arguably the most important ethical crisis that doctors, and the communities they serve, have ever faced. Although these are societal issues, they will be felt by nearly all practitioners of medicine and have relevance to this textbook.

Given the pace of discovery, none of the sections can be complete but the book reflects our desire to show how molecular biology is impinging on medicine and to indicate what lies ahead. Our contributors have not comprehensively revisited the historical framework of DNA and protein structure, molecular evolution, replication and microbial genetics: this is covered in undergraduate texts and, we feel, unrelated to our practical brief. However, our net has been cast wide to show the extent of scientific integration that is possible in medicine, and we have also tried to be honest about Pandora's box of genetic gifts. After the evils had issued from this beguiling goddess's box, hope alone remained to assuage the lot of man. There is now a molecular substance to this hope and we have attempted to demonstrate the extent to which it is tangible in medicine. The aim of this book has been to make introductions: to provide the curious student or practitioner with a basis to explore the medical potential of molecular biology; and still we hope to convey a real sense of wonder.

Acknowledgements

We thank our contributors for their co-operation and the efforts that they should be able to recognize in this version of the book.

No work of this kind can be produced without the dedication of a few professional enthusiasts who believe in publishing and work way beyond ordinary duties to see the task through. Peter Saugman of Blackwell Science had the courage to take us on and has left his splendid imprint on the completed work. We are especially grateful to Dr Andy Robinson, commissioning editor, who guided us almost from the beginning and worked tirelessly to help us realize our aims; he has become a true friend. By the same token, we were delighted when Jane Fallows, an old friend, took on the art work—an essential aspect of the book—and produced such excellent illustrations, often miraculously originating from apalling sketches! Julie Jones, our production editor, has brought the completed manuscript together and we are sincerely grateful for her uncompromising attention to detail.

We would also like to thank a whole host of academic reviewers (who shall remain nameless) for constructive criticism of each chapter, which has much improved the whole manuscript. In addition, we thank Jason Millington of University College and Middlesex Hospital Medical School for giving us the 'student's perspective' and Dr Vinod Achan of St George's Hospital for commenting as the 'junior doctor'. We trust that the book hits the mark.

Finally we thank Joan Grantham for secretarial forebearance over 7 years and for typing the entire manuscript accurately.

Contents

Chapter 1 Haemophilia: molecular biology at the centre of human disease

Editors' introduction

This introductory chapter sets the stage. It is the story of haemophilia from its time as a fatal disease of boys, best known to the Rabbis of antiquity, to the present day. Haemophilia is the paradigm of a human disease where the application of molecular genetics has already had tangible effects in diagnosis and in therapy.

The author has conveyed much of the optimism and excitement of molecular genetics as applied to medicine through his first-hand association with developments in this disease. Haemophilia has long been implicated as a cause of disability and death (see Plates 1–4, facing p. 214). Latterly, successful treatment of the condition by the administration of blood products had been complicated by life-threatening infections with hepatitis viruses and the human immunodeficiency virus (HIV). The use of recombinant methods to manufacture a purified product for protein replacement has had a dramatic effect on the outcome of therapy for haemophilia, a therapy which is now free from the risk of viral transmission. This, and the diagnostic methods brought into clinical use only a few years after their initial discovery, is a landmark in the catalogue of benefits that have already accrued.

The following chapter is in the form of a light scientific narrative: it presumes some knowledge of the techniques and strategies used in molecular biology and the naive reader may not understand all the details on a first reading. We intend, nonetheless, that you will be swept along and that the tale will entice you to delve further: much of this book is about the issues encompassed in this chapter.

An ancient scourge solved by molecular biology

Zippori, Israel, second century

The Rabbi was puzzled. A young mother had come to him in great distress. Her first son had died after circumcision, performed with the usual practised skill at 8 days. Bleeding usually stopped after the operation in a few minutes, but her little boy had bled to death. The same sad end had befallen her second son. Here she was with her third boy child, now due to undergo the ritual that killed his older brothers. Rebbe Judah pondered: the law demanded circumcision, but in all circumstances saving life took precedence. He gave his verdict—dispensation from circumcision should be allowed in such cases. We know this because it is recorded in the Babylonian Talmud. Evidently, the Rabbis saw larger families with the problem, for the dispensation was later extended to maternal cousins of bleeders, showing remarkable insight into the pattern of sex-linked inheritance.

Cordoba, Moorish Spain, tenth century

The great surgical writer Khalaf Ibn Abbas, also known as Alsaharavius, visited a village where men were afflicted with uncontrollable bleeding from cuts and wounds. The problem was also evident in young boys of the village who sometimes bled to death after merely rubbing their gums. The sufferers generally died of haemorrhage but, Khalaf added, 'I have seen that the treatment of a cut is to quickly cauterize the place until

blood is held back. I have proof of this, for it was demonstrated in my presence'.

Philadelphia, New England, 1803

A Philadelphia physician, John Otto, heard about a family in which males bled for many days after trivial injury, becoming prostrated. He particularly noted that no females in the family were afflicted, although 'still capable of transmitting it to their male children'. Otto's report appeared in *The Medical Repository*, New York and was the first recognizable account of an inherited sex-linked bleeding syndrome in the modern medical literature. It aroused widespread interest and was soon followed by many similar family studies and case reports. The medical publishing bandwagon was rolling. By 1820, Nässe could compile a review and draw up a list of laws of inheritance. Significantly, he noted that women were never affected, that they could have affected or normal sons, and that their seemingly normal daughters could have affected sons. Despite available evidence from the Hay kindred (Fig. 1.1), he missed the fact that sufferers' daughters are always carriers, although correctly noting that their sons, if any, were normal. This error was propagated by subsequent authors for almost a century, demonstrating the difficulty of correcting a mistake once it has appeared in print.

Wurzburg, Germany, 1820

In his inaugural dissertation, Hopff referred to four brothers who had each bled to death after trivial injury or in one case after rupture of a tumour in the thigh (evidently a haemophilic pseudotumour). He applied the curious term 'Haemophilie', literally 'blood lover' to these patients, a name which has stuck ever since.

The nineteenth century—clinical observations accumulate

Many more cases were described throughout the nineteenth century, culminating in a monumental review by Bulloch and Fildes (1911) which summarized almost 1000 cases and family reports, abstracting from them 224 pedigrees. This was in the heyday of the **eugenics movement** and the review was published by the Galton's eugenics laboratory as parts V and VI of their treasury of human inheritance series. Still influenced by Nässe,

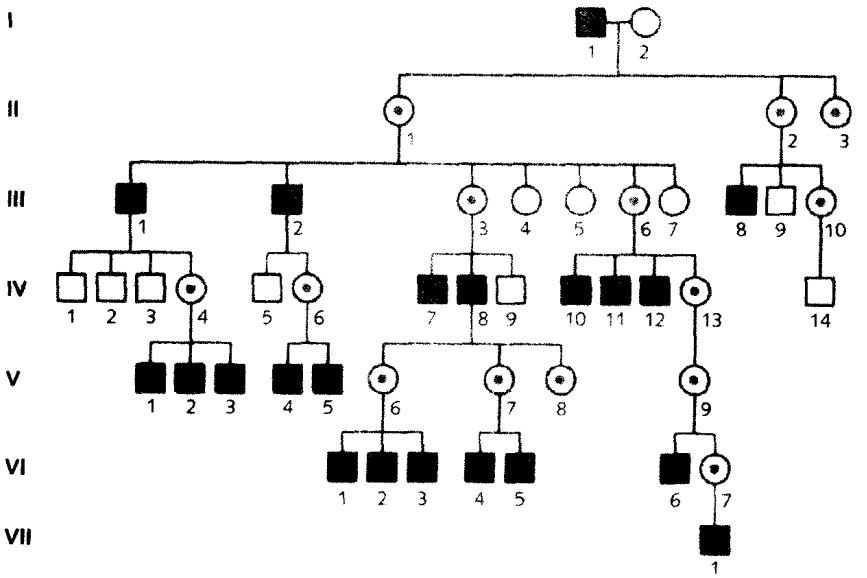


Fig. 1.1 Hay kindred of 'bleeders'. This family was first described in 1834 in the local guide book to a New England town (*History of Ipswich*) and later republished in the *Boston Medical and Surgical Journal* (1851). Osler reinvestigated the family in 1885. Strikingly, the affected males (filled-in squares) pass the bleeding tendency to their grandsons via carrier daughters (circles with inner dots). Despite this, the misconception that haemophiliacs could not pass on their illness to descendants persisted until 1911 (redrawn from pedigree 408 in Bulloch, W. and Fildes, *Eugenics Laboratory Memoir XII, Treasury of Human Inheritance parts V and VI, Haemophilia*). Hay, a physican who described the family, was married to one of the carrier females.

Bulloch and Fildes denied that a haemophiliac could pass the disorder to his descendants, despite the Hay family pedigree (Fig. 1.1) updated and illustrated in their review. However, they realized that the key to further progress in haemophilia lay in understanding the underlying blood disorder. Almroth Wright had shown in 1893 that haemophiliac blood was slow to clot, compared with normal blood, in a glass capillary. A case they studied together was a female haemophiliac, a possibility denied by Nässe and by Bulloch and Fildes. Later studies confirmed that this lady, who lived to be 90 and was restudied in the 1960s, actually had true haemophilia, being the offspring of a haemophiliac father and a carrier mother. This possibility was predicted and correctly explained by Bateson in 1909.

The twentieth century—modern genetics applied to haemophilia

Gregor Mendel's work had been rediscovered at the turn of the century and a new generation of experimental geneticists were enthusiastically applying Mendel's ideas to plant, animal and human heredity. Bateson, in his classic book *Mendel's Principles of Heredity* (1909), showed that colour blindness and haemophilia had similar sex-linked inheritance. He saw the importance of the normality of affected men's sons and the carrier status of their daughters and drew up a scheme for sex-linked inheritance, essentially as shown in Fig. 1.2, that we still use today.

The most famous haemophiliac family in history was busy spreading its gene through the royal households of Europe at this time (Fig. 1.3). Queen Victoria was an obligate carrier of haemophilia, passing the defective gene on her X-chromosome to Leopold and to at least two daughters, Alice and Beatrice. Leopold's daughter Alice, an obligate carrier, had an affected son who died in childhood. The other affected males in this family all died without issue from haemorrhage, or in the Tsarvitch's case, met a violent end. The last definite carrier of the Royal Haemophilia died in 1980, but as her blood was not tested we still do not know if the underlying disorder in this family was type A or B (see p. 5). Early death without issue is in fact the commonest fate for untreated severely affected haemophiliacs and this fact was used by the geneticist Haldane in 1935 to predict the rate of spontaneous mutation causing the disease. By a brilliant *reductio ad absurdum* argument, he showed that new mutations must arise continuously to maintain the frequency of haemophilia in the general

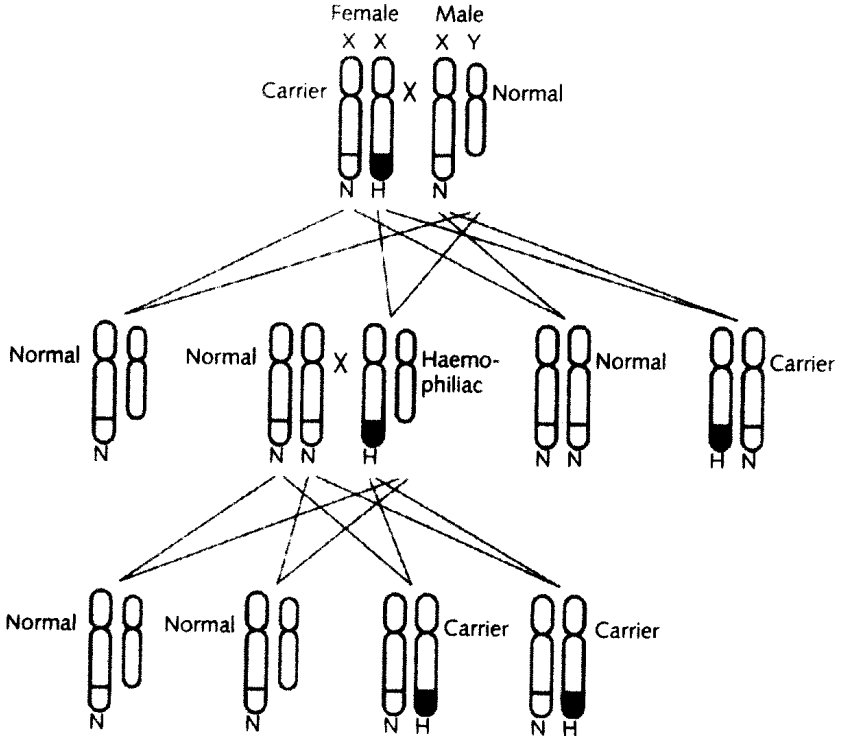


Fig. 1.2 X-linked inheritance. Three chromosomes are shown, a normal X (tip of long arm unshaded), an X bearing a mutant factor VIII gene (tip of long arm shaded) and a normal Y. A female carrier with a normal partner has four types of offspring with equal frequency: normal son, haemophiliac son, normal daughter and carrier daughter. A haemophiliac male has only two types of offspring: carrier daughters and normal sons.

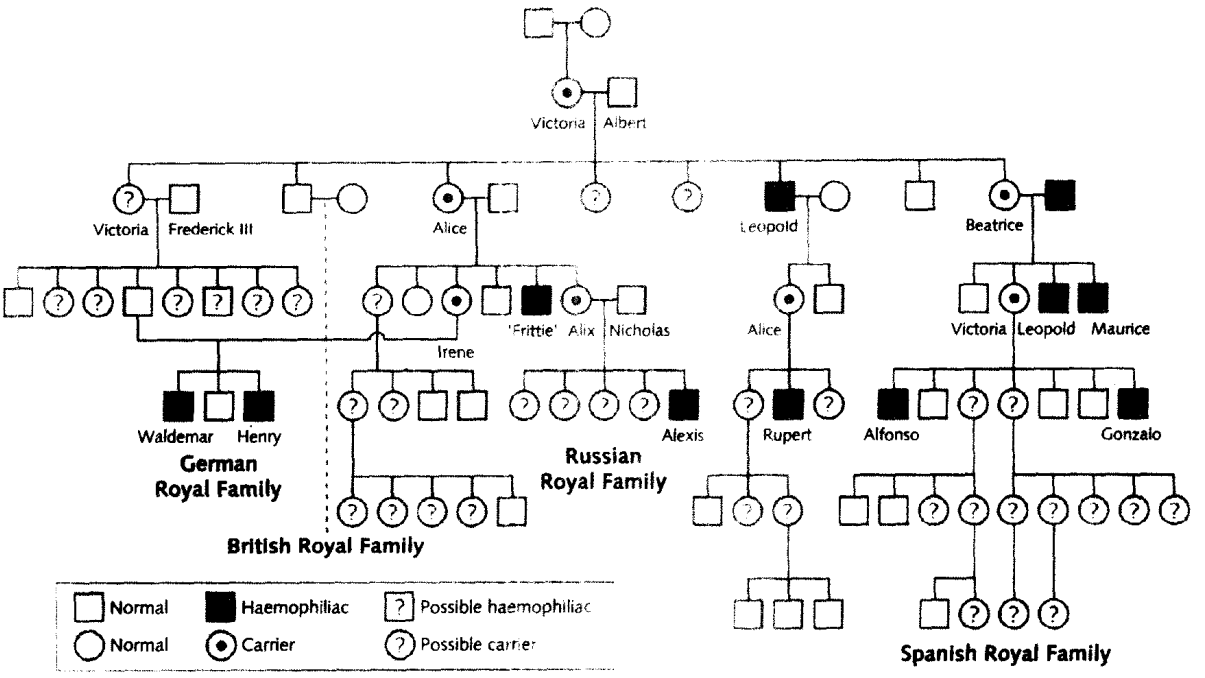


Fig. 1.3 The Royal Haemophilia. Queen Victoria was the first to carry haemophilia in this family having most likely received a mutant gamete from her normal father, Edward Duke of Kent. Her sixth child Leopold had severe haemophilia (it is still unknown whether it was A or B). He was the only bleeder male to live long enough to have children, including the obligate carrier Alice, whose son Rupert died of bleeding in childhood. At least two of Victoria's daughters were carriers, Alice and Beatrice, who passed the affliction to the German, Russian and Spanish Royal Families. The gene has almost certainly died out by now, due to the early death of affected males.

population. For if it were not so, given the rate of loss of mutant alleles, every male in the UK must have been a haemophiliac at the time of the Norman conquest. He concluded that about a third of mutations in any given generation must be of recent origin and likely to be heterogeneous. Both deductions, as we shall see, were to be triumphantly confirmed in the era of molecular genetics.

1911–1937 Early attempts to understand the coagulation defect in haemophilia

Back in 1911, however, attempts to understand the basic defect in haemophilic blood were running into difficulties. Working in Edinburgh, Addis took blood from normal and haemophiliac volunteers, anticoagulated it with sodium oxalate, so that the plasma could be separated, then allowed it to clot by adding calcium salts. The haemophilic plasma showed a much delayed clotting time, taking at least three times as long as normal plasma to form a clot after addition of calcium. He soon disproved Wright's idea that a deficiency of calcium caused the problem. With an extract of normal plasma made by dilution and acidification he managed to correct the delayed clotting of the haemophiliac plasma. The fibrinogen content of the haemophiliac blood was normal and the corpuscles seemed to have normal amounts of thromboplastin—whatever that was. The prevailing blood coagulation theory of the day was that propounded by Morawitz in 1905: until very recently, you could see versions of it in physiology textbooks for medical students! This 'classical theory' supposes that coagulation is initiated by a mysterious thromboplastin from white cells which, in the presence of calcium ions, activates prothrombin to thrombin, which in turn converts fibrinogen to fibrin—thus forming the clot. The only constituent of this theory not tested for by Addis was prothrombin, which he was forced to conclude must be deficient in haemophilia. In the 1930s Armand Quick developed his 'prothrombin time' and showed it to be normal in haemophiliac blood. Thus an impasse had been reached. This was overcome by resurrecting Addis's experiments and by a new attack on theory. In 1937, Patek and Taylor in Boston showed again that a simple extract of plasma similar to Addis's corrected the defect in haemophilic blood, and they simply outflanked the

Table 1.1 Clinical severity versus factor VIII level.

Residual factor VIII (AHG) level	Bleeding manifestations
Less than 1% of normal	Frequent spontaneous bleeding into any tissue or organ, especially load-bearing muscles and joints. Early death from exsanguination common
2–5% of normal	Occasional spontaneous bleeds, bleeding after minor trauma
More than 5% of normal	Bleeding only after major trauma or surgery

Morawitz theory by calling the correcting factor anti-haemophilic globulin (AHG for short; it did not acquire the name factor VIII until 1962).

1950–1965 Factor VIII defined and separated from factor IX

At Oxford in 1950 a highly important advance was made by Mersky and Macfarlane, who devised a sensitive specific assay for AHG. In retrospect this can be seen to be the essential prerequisite for efforts to purify the elusive factor missing from haemophiliacs' blood, that were to take another 30 years to reach a successful conclusion. Using the new quantitative AHG assay it was shown that the most severely affected patients had the lowest levels of or absent AHG, whilst milder cases had from 5% of normal upwards (Table 1.1).

In 1952, to everyone's surprise, it was found (by three groups independently in the USA and UK) that not all sex-linked haemophilia is due to AHG deficiency. About a sixth of such patients lack a different factor, so that the deficient plasmas cross-correct each other. The commoner type is haemophilia A or AHG deficiency, the rarer is haemophilia B or Christmas disease (named after one of the first patients to be described, who is still alive as it happens), now known as factor IX deficiency. Another surprise came the following year when French researchers found that AHG is deficient in the blood of patients with von Willebrand's disease, a bleeding disorder that affects males and females equally. How could a clearly X-linked defect also be part of an autosomal dominant disorder? The two conditions are also quite distinct clinically (Table 1.2). The solution to this puzzle would prove to be the key to the whole problem of haemophilia A, 20 years later.

In 1962 an international committee assigned Roman numerals to the clotting factors. AHG became factor VIII and the principle lacking in Christmas disease became factor IX (note that factor VI does not exist).

1950–1983 Development of plasma factor VIII concentrates for treatment

In the 1950s treatment for haemophilia, still a dreaded and usually fatal disorder, was developed, particularly by Macfarlane and colleagues at Oxford, where the first specialized centre for haemophilia treatment was set up. Supplies of human plasma were then very limited, and although plasma infusion was known to be effective for minor bleeding, its content of AHG was low preventing the attainment of high enough blood levels to treat major bleeding or to prepare the patient for surgery. Macfarlane therefore turned his attention to developing concentrates of AHG from animal blood, especially porcine and

Table 1.2 Comparison of haemophilia A with von Willebrand's disease.

	Haemophilia A	von Willebrand's disease
Inheritance	Sex-linked	Autosomal dominant or rarely recessive
Incidence	1:5000 males	1:1000 males and females
Clinical features	Joint bleeding Muscle bleeding Cerebral haemorrhage	Nose bleeds Menorrhagia Prolonged bleeding from cuts
Investigation	Factor VIII low or absent von Willebrand factor normal Bleeding time normal	Factor VIII low—never completely absent von Willebrand factor low Bleeding time prolonged
Molecular genetics	Mutations at factor VIII locus Xq 28	Mutations at von Willebrand factor locus chromosome 12p12

bovine blood, available then as now in large quantities from the abattoirs. Although the concentrates he developed had the drawback of eliciting reactions and antibodies after repeated infusions, they saved many lives.

Gradually during the 1960s the plasma fractionation industry developed along commercial lines, with thousands of paid donors recruited to supply an internationally traded commodity—human plasma. The fractions derived from this, using technology developed by Cohn in the 1940s, included albumen, immunoglobulins, fibrinogen and later factor IX and factor VIII. The new factor VIII concentrates of the 1970s, although highly potent and convenient to use, were derived from large numbers of donors. Soon complications became apparent, particularly the presence of viruses. Hepatitis B was frequently transmitted, and by the late 1970s it was clear that a second hepatitis virus, C, was present in all factor VIII concentrates. Between 1979 and 1983 most commercial factor VIII concentrate also became contaminated with HIV, with the well-known tragic consequences for haemophiliacs. Subsequently, heat or solvent detergent treatment was developed to inactivate these viruses, making the concentrates considerably safer.

1965 The coagulation cascade hypothesis

The effort to understand blood coagulation as a biochemical process was hugely advanced in 1965 when Macfarlane proposed his cascade hypothesis and Davie and Ratnoff put forward a similar concept as the waterfall theory. With modification, the idea of enzymatic amplification (analogous to a photomultiplier cascade), by which an initial enzymatic step converts a second zymogen to an active form which in turn converts a third and so on, has been supported by increasingly sophisticated experiments. Figure 1.4 shows a modern concept of the coagulation cascade. Notice that factor VIII is a cofactor, not itself a proteolytic enzyme, which enhances the action of factor IXa on factor X in the middle step of the clotting cascade. From this (and the X-chromosomal location of the factor VIII and IX genes) it becomes obvious why haemophilia A and B are clinically indistinguishable.

1972–1982 Factor VIII and von Willebrand's disease: the puzzle resolved

The next major step in understanding haemophilia was taken in 1972 when Zimmerman, Ratnoff and Powell attempted to raise antibodies in rabbits against purified factor VIII. To their surprise the rabbit antibodies detected an antigen that was reduced or absent in von Willebrand's disease, but always present in haemophilia A plasma. At first this entity was called factor VIII-related antigen. In 1975 Zimmerman and Edgington deduced that factor VIII coagulant activity and the new antigen co-purify, but are separate molecular entities. Because of its importance in von Willebrand's disease, the new antigen was soon redesignated von Willebrand factor antigen. Several groups (Hoyer in Connecticut, Bloom in Cardiff, Weiss in New York, amongst others) began to propose that factor VIII is an X-chromosome-encoded gene product and that the von Willebrand factor antigen is autosomally coded and somehow responsible both for maintaining the blood factor VIII level and for the bleeding time. Figure 1.5 illustrates this concept, in which factor VIII and von Willebrand factor circulate as a non-covalent complex. These clinically inspired ideas were to be completely vindicated as the tools of molecular biology became available to researchers in the 1980s and were applied to the problems of haemostasis.

In 1979, human factor VIII, completely free of von Willebrand factor, was purified by a group at the University of Connecticut (Tuddenham, Trabold, Collins and Hoyer).

1982–1984 Total purification and the race to clone factor VIII

In the early 1980s, the infant biotechnology industry was beginning to flex its muscles and look for projects with high potential earnings. The worldwide market for plasma-derived factor VIII concentrate, despite its increasingly manifest drawbacks, was worth several hundred million dollars annually. A recombinant factor VIII product, it was thought, could solve problems of quality and supply especially in relation to contamination with pathogenic viruses.

At least four industrial/academic partnerships were formed in 1982 to clone factor VIII, after a landmark conference in San Diego at which three groups announced total purification of factor VIII in sufficient quantity for biochemical characterization. The race was on. Figure 1.6 shows diagrammatically the procedure developed by the author's group at the Royal Free Hospital, London (Rotblat, O'Brien and Tuddenham). Very large quantities of starting plasma are first rather crudely reduced by cryoprecipitation to a solid chunk, retaining about half the factor VIII activity together with von Willebrand factor and huge amounts of other high molecular weight proteins such as fibrinogen and fibronectin. Indeed, for many years this crude prepara-

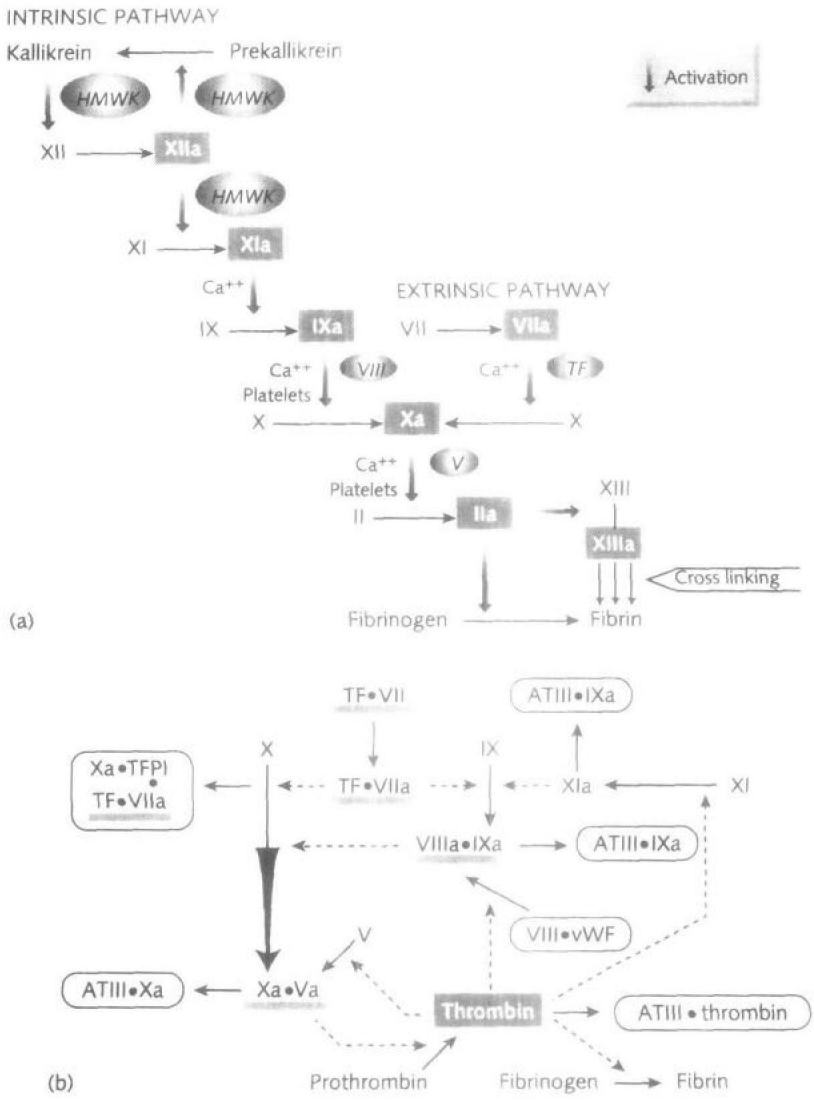
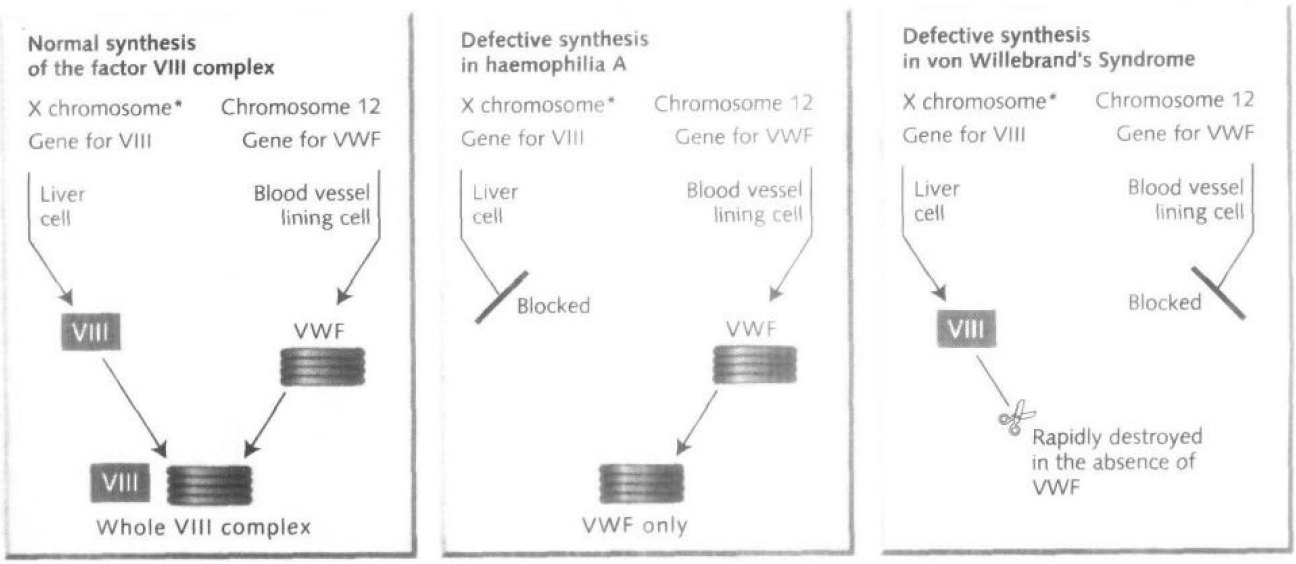


Fig. 1.4 The coagulation cascade. (a) A modified version of the scheme originally proposed by Macfarlane and by Davie and Ratnoff in 1964, in which the initiator is factor XII (top left). On binding to a surface, factor XII is reciprocally activated by kallikrein, then activates factor XI, which in turn activates factor IX and so on down to thrombin (IIa) which converts soluble fibrinogen to fibrin. Fibrin spontaneously polymerizes into a meshwork of fibres. Factor VIII is now known to be a cofactor for the activation of factor X by factor IXa. In this scheme, tissue factor initiation is added as an accessory or alternative pathway. Although the diagram is useful for interpreting coagulation screening tests performed *in vitro*, it has been realized more recently that tissue factor is in fact the physiological initiator, as shown in (b). (b) The current view of haemostasis. Coagulation is now seen as a network of interactions triggered by contact of blood with extravascular tissue factor. The initial conversion of factor X to Xa by the tissue factor/VIIa complex (TF·VIIa) leads to generation of small quantities of thrombin, which back-activate factor V and factor VIII. Rapid thrombin generation then proceeds with feedback to factor XI. Note that factor VIII bound to von Willebrand factor is inactive until proteolysed by thrombin.

tion, known as 'cryoprecipitate', was used to treat haemophilia—poor man's factor VIII concentrate maybe, but the only therapy available for more than a decade. The next step, a form of ion exchange chromatography, largely removes contaminating proteins but leaves factor VIII and von Willebrand factor with some hard-to-remove high molecular weight proteins. The next steps use immunoaffinity chromatography, first with monoclonal antibody to von Willebrand factor, then with a monoclonal antibody to remove fibronectin,



*Genes are distributed on 22 non-sex chromosome pairs and one sex chromosome pair. The X chromosome contains the genes encoding for VIII and IX.

Fig. 1.5 The factor VIII complex. The factor VIII and von Willebrand factor genes are on different chromosomes (X and 12) but the proteins produced naturally bind to each other in the blood and circulate as a high molecular weight complex (VIII.VWF). If either gene is non-functional factor VIII levels are low, but in von Willebrand's disease the whole complex is lacking, whereas in haemophilia A only factor VIII is lacking.

and finally a monoclonal antibody to factor VIII itself. The monoclonal antibodies were developed in collaboration with an immunologist at Royal Free Hospital, Dr Alison Goodall, and were the key to achieving total purity. Altogether, 10 000 litres of blood were processed batchwise through this procedure to yield about 20 mg of pure factor VIII, an odourless white powder. The specific activity of the final product was a staggering 5000 units/mg, implying that plasma contains only 200 ng/ml. This explains the long gap between Addis's experiments and our own results, since methods for isolating and manipulating such rare proteins were unavailable to earlier researchers. Zimmerman and Fulcher at the Scripps Clinic achieved a slightly lower level of purity — about 2000 units/mg — at the same time in 1982, and amazingly managed to obtain a patent on factor VIII of this and higher purity! With the backing of Armour Pharmaceuticals, this factor VIII patent survived several challenges and enabled Armour to settle out of court with the companies who successfully cloned factor VIII for a sum reputedly in excess of $200 million.

'A technical feat without parallel'

The factor VIII powder was freighted to Genentech Inc. in San Francisco where Gordon Vehar and his colleagues broke it down with the digestive enzyme trypsin and separated the fragments by reversed phase chromatography. The fragments were then subjected to automated Edman degradation to establish the sequence of amino acids of each peptide. One such fragment proved to have the sequence shown in Fig. 1.7. Dr Richard Lawn, a molecular biologist working at Genentech, was able to use this sequence to design an oligonucleotide probe by using the genetic code in reverse. Since more than one codon exists for all but two of the amino acids (the exceptions are methionine and tryptophan), a certain amount of guesswork and luck are needed to make a probe that closely matches the actual DNA sequence in the gene. The probe, a unique 36mer, was radiolabelled and applied to filters lifted from a gene library. The methods for making a human genomic library had been worked out by Lawn in Tom Maniatis' laboratory a few years earlier. Now they were applied in earnest to one of the most taxing gene cloning projects undertaken. The library was made using DNA from a cell line derived from an individual with four X-chromosomes, to enrich for the target sequences. The DNA was extracted, then partially digested with an enzyme that cuts rarely (the restriction endonuclease *Sau*3AI) and ligated into the vector phage λ charon 30.

Out of 500 000 recombinant clones from the charon phage library, 15 were selected by their ability to bind the radiolabelled synthetic oligonucleotide shown in Fig.