

## Historical Review

### COAGULATION HISTORY, OXFORD 1951–53

The purpose of this review is to record the history of coagulation studies in the Department of Clinical Pathology in Oxford, 1951–53, under the direction of R. G. Macfarlane and Rosemary Biggs. During that time the efficiency of the intrinsic pathway for prothrombin conversion was established, Christmas disease was characterized, and the thromboplastin generation test was developed. In order to understand the development of knowledge of coagulation prior to 1951 an introduction is given, based mainly on previous historical accounts.

R. G. Macfarlane in 1967 said 'Other people's reminiscences can be pretty tedious' and 'One has to remember what it was like not to know things which now seem self-evident'. This was a personal reminiscence of events in development of knowledge of blood coagulation in Oxford, 1951–53. The work was done in the Department of Clinical Pathology, at the Radcliffe Infirmary, Oxford, under the leadership of Gwyn Macfarlane (1907–1987) (later Professor R. G. Macfarlane, CBE, FRS) and Dr Rosemary Biggs. I was privileged to be in their Department and to make a contribution to its work during the tenure of an MRC Research Fellowship; moreover I had the great good fortune to arrive when earlier work was about to pay dividends.

In Oxford I worked on concepts provided by my supervisors; these came as a distillate of their scientific wisdom. If I had not gone to Oxford I would not have done the experiments described in our published work (Biggs *et al.*, 1952; Biggs & Douglas, 1953a, b; Biggs *et al.*, 1953a, b, c). After this academic training in blood coagulation in Oxford, I did experiments which followed my own ideas.

Professor Macfarlane died in 1987 and Dr Biggs lives in retirement near Oxford. In the later part of her career, Dr Biggs was Director of the Oxford Haemophilia Centre and made an enormous contribution to the field, bringing much of Professor Macfarlane's vision to fruition. To refer to surnames nowadays seems too formal, so I shall use Gwyn and Rosemary.

When I arrived in Oxford in October 1951 my technical skills were limited to performing the whole blood clotting time, the plasma recalcification time, and Quick's one-stage prothrombin time; I learned not only from my two senior supervisors but also from Dr Ethel Bidwell, who with great patience taught me the necessary biochemical methods.

My interest first in haemostasis and then in thrombosis was triggered by two patients. On my first day as house physician to the late Professor J. W. McNee in the Western

Infirmary, Glasgow, a teenage haemophiliac had a tooth extracted and I held a swab to the socket intermittently for 18 days. On discharge from the Army I held a second house-physician's post in Inverness, and one of the patients was a middle-aged severe haemophiliac who later died from myocardial infarction with a coronary thrombus found at autopsy.

To put the 1951–53 advances in perspective, I shall trace briefly the development of knowledge on blood clotting during the previous century and a half. Most of this work came from investigators on mainland Europe. These slow advances concerned the role of tissue in the mechanism. It was known that blood collected by clean venepuncture coagulated without tissue contamination, but this phenomenon had been much neglected.

There have been well-researched and detailed accounts on the history of blood coagulation. I have not re-researched this but have worked from these accounts (1909–97) and given them recognition (Mellanby, 1909; Howell, 1935; Owren, 1947; Biggs & Macfarlane, 1953; Douglas, 1962; Quick, 1966; Tullis, 1976; Macfarlane, 1976; Ratnoff, 1980, 1996; Forbes, 1997). The two reviews by Ratnoff (1980, 1996) are commended for extended reading. I have given Buchanan's work special mention because I had access to the *Proceedings of the Glasgow Philosophical Society*, and because none of the previous historical accounts have given enough detail on it (Buchanan, 1836, 1845).

#### *Early findings: pre-classical era*

*Fibrin and fibrinogen.* Although some observations were made in ancient times, I choose to start in the second half of the seventeenth century, when Malpighi (1686) separated fibres from clotted blood free of red cells and serum, and identified these using the single-lens microscope. When William Hewson, a young surgeon, was working in London in 1771 (Hewson, 1772, 1846), there was much inflammation and other gross pathology amongst his patients, and therefore red cell sedimentation would have been rapid. He observed that the coagulum of blood arose from the upper liquid part of the blood, above the red cells, which had previously been thought to be the source of the coagulum. The term fibrin was introduced by Fourcroy (1801) working in France. He demonstrated that plasma contained soluble substances, albumin and globulin, and that the precursor of fibrin was a soluble substance present in plasma but not in serum. The term globulin was used to describe the part of plasma precipitated when diluted in water, albumins remaining in solution. These observations led to the conclusions that there was a precursor of Malpighi's fibres and the term fibrinogen was applied to it (Babington, 1830).

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*Preparations of fibrinogen.* It was much later that crude preparations of fibrinogen were made. Denis (1859) made a preparation of fibrinogen by salt precipitation and later, using the same starting technique, Hammersten (1879/80) duplicated this, providing a purer preparation by repeated precipitation. Mellanby (1909) made an important precipitation containing fibrinogen by diluting plasma and acidifying with acetic acid following directions given by Schmidt (1892, 1895). We too used this technique in some of our work, because this precipitate also contains prothrombin freed from antithrombin.

*Thrombin.* Andrew Buchanan (1798–1882), a junior surgeon in Glasgow Royal Infirmary in 1836, reported to the Glasgow Philosophical Society on experiments he had conducted earlier in 1830–31 (Buchanan, 1836). He had taken a freshly formed blood clot and placed it in a linen cloth, squeezing the liquid into a separate container. He added this strained liquid to hydrocoele fluid and obtained a coagulum. Later in 1841, by then Professor of Physiology at the Institute of Medical Sciences in the University of Glasgow, he repeated those experiments and reported further. This more definitive paper was published in the *London Medical Gazette* (Buchanan, 1845) and because of its importance was reprinted in the *Journal of Physiology* in 1879. In these later experiments he diluted blood with water and allowed it to clot overnight. He then removed the coagulum, washed it in water, wrapped it in a linen cloth, and squeezed out the fluid. This extract also coagulated hydrocoele fluid. In the earlier experiments Buchanan had observed that fresh serum would coagulate hydrocoele fluid, which he believed was the same as the fluid part of the blood.

Buchanan (like Hewson) found that, when sedimented blood was allowed to clot, the upper part of the clot – the part with the ‘buffy coat’ of white corpuscles – produced most of his coagulant material, but that it was also present to some extent in the lower red-cell part. Buchanan made the additional observation that the coagulum in hydrocoele fluid did not retract in the same way as did whole blood clot (he did not know about platelets). In the *Proceedings of the Glasgow Philosophical Society* of February 1845, he wrote ‘It is scarcely necessary for me to add that I am now satisfied that the fibrin of animal fluids exists in them in solution’.

Buchanan also conducted experiments adding washed tissues to hydrocoele fluid and obtained a coagulum. These results were less convincing as the clotting time was very long, and despite the washing the tissue may have contained some of his coagulant material from serum; the role of tissue required subsequent work to substantiate its importance.

*Prothrombin.* Blood coagulation research in the second half of the nineteenth century was dominated by Alexander Schmidt (1831–1894) (Schmidt, 1872, 1892, 1895; Ratnoff, 1980, 1996). Schmidt worked in Germany and later at the University of Dorpat in Estonia from 1862 onwards. Schmidt repeated the experiments described by Buchanan and called the activity ‘fibrin ferment’ and this term was still in use in Mellanby’s papers (Mellanby, 1909); Schmidt found that ‘fibrin ferment’ could be precipitated by addition of alcohol to fresh serum. Virchow was the first to use the term thrombin (Tullis, 1976). When publishing

previously, Schmidt wrote two books (1892, 1895) recounting his observations. He had the rare gift of changing his hypotheses in the light of advancing experimental information. Since the presence of thrombin in the circulation did not allow blood to remain fluid, Schmidt realized that it could not exist as such in blood but must have a precursor, prothrombin. At first, therefore, prothrombin was hypothetical.

*Tissue factor.* Thackrah (1819, 1834) found that blood collected after flowing over tissues – say in an open wound – coagulated more quickly than if the blood had been drawn directly from the blood vessel. De Blainville (1834) demonstrated that if macerated brain tissue was injected into an animal’s vein the animal died rapidly, due to widespread coagulation. Following the lead given by Buchanan, the action of tissue was demonstrated more positively by Schmidt (1892, 1895), when he mixed blood and tissue in a container and found immediate coagulation; the role of tissue in converting the hypothetical prothrombin to thrombin was called zymoplastic. It was found that tissue which had been well washed and then macerated would accelerate whole-blood clotting, but would not coagulate Hammersten’s fibrinogen.

*Calcium.* Arthus & Pagès (1890) and Sabbatini (1902) observed that calcium precipitants inhibited coagulation, and that this effect was reversed when sufficient calcium was re-added. Calcium was not required for the reaction of thrombin with fibrinogen, but was required for the conversion of the hypothetical prothrombin to thrombin. Mellanby (1909) described the inhibition of coagulation by oxalate and citrate, these being immensely valuable for subsequent studies. Citrate was used for preservation of blood for transfusion.

To recapitulate (see also Howell, 1935), the central findings by the end of the nineteenth century were: (a) Alexander Schmidt (1872): Coagulation in shed blood is due to a ferment-like substance (thrombin) which exists in circulating blood as an inactive precursor (prothrombin). (b) Hammersten (1879/80): Fibrin is the consequence of a reaction between thrombin and fibrinogen. (c) Arthus & Pagès (1890): Calcium is essential in the earlier stages of the clotting process, but not in the clotting of fibrinogen by thrombin. (d) Alexander Schmidt (1892, 1895): Formation of thrombin involved an essential constituent from tissue cells.

These findings were used by Morawitz (1940) and Fuld & Spiro (1904) in the formulation of the ‘classical theory’, which survived relatively unchallenged for about 40 years (see Fig 1).

#### *Classical theory and era*

Paul Morawitz (1879–1936), a 26-year-old Strasbourg physician, confirmed that tissue extracts did not coagulate fibrinogen (Morawitz, 1904; Ratnoff, 1980). Morawitz renamed Schmidt’s zymoplastic substance thrombokinase. Later, Nolf (1908) in France called this activity thromboplastic and Howell (1914) used the term ‘tissue factor’, both of which terms are still in use.

Thus at the beginning of the twentieth century the theory was that prothrombin was converted to thrombin under the

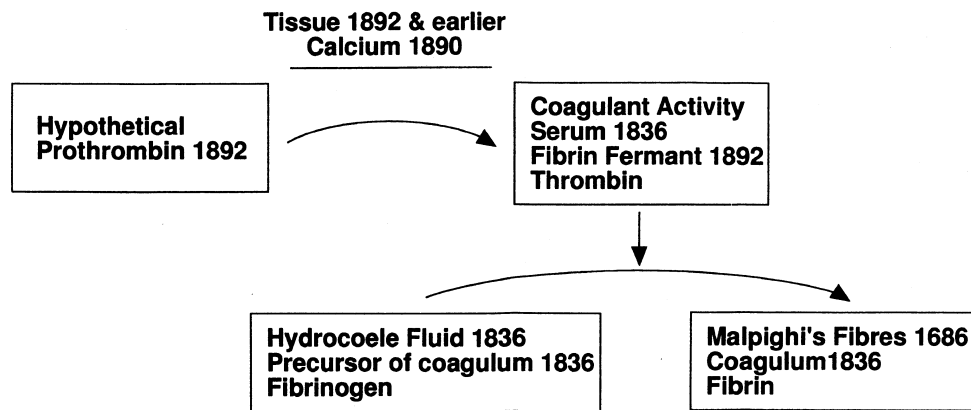


Fig 1. Classical theory. The authors were Schmidt (1892), Arthus & Pagès (1890), Buchanan (1836) and Malpighi (1686).

influence of thrombokinase (thromboplastin) and calcium, whereas thrombin converted fibrinogen to fibrin (see Fig 1). The mechanism for converting prothrombin using tissue was called the extrinsic pathway. It was known (see above) that blood clotted without tissue, albeit rather less quickly. This was the intrinsic pathway and was much neglected for almost half a century.

#### *The 40 years reign of the classical theory (1904–42)*

After the activity which culminated in the classical theory at the end of the nineteenth century there was a relative lull until the early 1940s. However, during that period there were some important events, the significance of which were not recognized until later, and an account of these events now follows.

Whipple (1912) reported the absence of prothrombin in a case of melaena neonatorum, and later (Whipple, 1913) he found similar features in a case of jaundice. In the earlier paper he described a 3-day-old infant who developed melena and other haemorrhagic symptoms. The blood remained fluid after prolonged contact with tissue juice. Using Schmidt's alcohol-precipitation technique (as described above), no thrombin could be extracted from the infant's blood, and a deficiency of prothrombin was postulated. In his later paper, Whipple (1913) described abnormal haemorrhage in a patient with terminal obstructive jaundice due to carcinoma of the head of the pancreas. He collected oxalated plasma, and to this he added tissue (spleen extract) and calcium. The clotting-time was slower than that obtained from his preferred normal (canine) plasma. In using this technique, Whipple was almost quarter of a century ahead of Quick, who developed the one-stage prothrombin-time more definitively, using brain extract (Quick, 1935).

*Heparin.* In 1916 the story of heparin began when Jay McLean (McLean, 1916), a medical student, was working at Johns Hopkins in the laboratory of the great coagulationist William H. Howell (1860–1945). Again, the finding was a chance one. He studied ether/alcohol extracts of brain, heart and liver. These extracts accelerated blood clotting, but after storage for some months they had lost their coagulant activity, and indeed were now *anticoagulant*. Howell & Holt

(1918) studied this phenomenon further, and coined the term heparin for the anticoagulant.

*Coumarins.* 'Sweet clover disease' in cattle was first noted in Canada (1921–23) when excessive or fatal haemorrhage occurred after castration or dehorning in cattle. Later Roderick (1931) traced the disease to improperly cured hay made from common types of sweet clover. The disease was reversible when cattle ceased being fed on spoiled hay. Roderick found slow clotting of the blood, but measurement of prothrombin had not been adequately developed at the time. Howell (1914) had shown that a crude preparation of prothrombin could be made by acetone-precipitation of bovine plasma. Such solutions from normal bovine plasma corrected the deficit in the blood of cattle suffering 'sweet clover disease', but not when the same solutions were made from blood of animals with the disease. In the early 1940s Link isolated and synthesized dicoumarol from 'spoiled sweet clover'. The synthesis of other coumarins such as warfarin followed; these important historical events have been described by Douglas (1962).

*Vitamin K.* A new burst of activity was induced by the discovery of vitamin K, an event which highlighted the need for an improved method of measuring prothrombin. Henrik Dam in 1935 fed an ether-extracted diet to chickens, which developed a haemorrhagic diathesis (Dam, 1935). None of the vitamins known at the time would correct this nutritional deficiency. The missing fat-soluble vitamin K was found in the photosynthetic portion of plants, e.g. alfalfa grass and horse chestnut leaves (Douglas, 1962). Following the discovery of prothrombin deficiency in obstructive jaundice (Whipple, 1913), it was established that bile salts were needed to absorb vitamin K. Appropriate preparations of vitamin K came into use in the prophylaxis and treatment of bleeding in haemorrhagic disease of the newborn, obstructive jaundice and excessive coumarin therapy.

*Measurement of prothrombin.* (i) *One-stage prothrombin-time.* This technique was described by Quick (1935) and depended on the classical theory; this suggested that, given enough tissue, calcium and fibrinogen, there is only one factor limiting the time of clotting: prothrombin. It is now recognized that this result is limited by deficiencies of factors

additional to prothrombin, but it has been of the greatest importance in advancement of knowledge, and it is used world-wide in the control of coumarin therapy.

Armand J. Quick (1894–1977) made immense contributions to our understanding (Ratnoff, 1980). Of the many distinguished coagulationists I visited later in my life, he was the only one to set aside a whole day taking the history from a patient with an obscure bleeding disorder and, with the help of one technician, to work at the bench doing the experiments he thought were relevant. I understand that Oscar Ratnoff, when possible, also treated his visitors to this privilege; Oscar's experiments and thinking dominated the field for much of the second half of this century.

(ii) *Two-stage prothrombin time*. At Iowa University, Warner, Brinkhous & Smith (1936) developed a method of measuring prothrombin which became known as the two-stage technique, in contrast to the one-stage method of Quick. Plasma (test or normal), tissue and, subsequently, calcium were mixed, and the technique measured formation of thrombin, by transferring an aliquot to fibrinogen, the coagulation time being a measure of thrombin. In the thrombin–fibrinogen reaction the shorter the clotting-time the higher the concentration of thrombin, and using this reciprocal relationship thrombin was measured in units.

*Consumption of prothrombin in glass tubes*. Ken Brinkhous (1939) reported experiments on the rate of utilization of prothrombin in whole blood delivered to a glass tube. He found that prothrombin consumption in haemophilic blood was defective; even after the start of clotting, prothrombin was utilized slowly, and the fibrin formation in haemophilic blood, once started, progressed very gradually. The time from the formation of the first fibrin to the last was long. In normal blood, in contrast, all the fibrinogen was coagulated rapidly, and the prothrombin was converted to thrombin soon after clotting was complete. Brinkhous used the two-stage technique to measure the residue of prothrombin in serum.

Quick (1947) reported similar studies; at specified times up to 1 h after blood had clotted, an aliquot of the serum was added to brain adsorbed plasma (see below) and calcium, and the clotting time recorded. In addition to confirming Brinkhous' finding in haemophilia, he also showed that the same result could be obtained in thrombocytopenia. An earlier study by the great French coagulationists, Bordet & Delange (1912), had reported this in thrombocytopenic plasma. In haemophilia and thrombocytopenia the one-stage prothrombin time was normal, but the prothrombin consumption test was abnormal.

#### Post-classical findings

*Factors V and VII*. (i) *Factor V*. The one-stage prothrombin-time as initiated by Quick (1935) made possible the immediate differentiation between the coagulation defect in haemophilia and that in obstructive jaundice. Within a few years, Quick (1943) had realized that there might be an additional clotting-factor needed to give a normal result in his test. When he stored oxalated (but not citrated) plasma, the clotting-time was prolonged and he called this phenomenon 'labile factor'. During the war in 1942, while in the Norwegian resistance, Paul Owren (1947) examined a

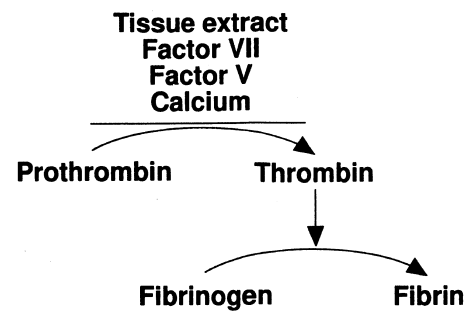


Fig 2. 1951. Prothrombin conversion to thrombin. The classical theory had been amended by the discovery of factor V and factor VII.

patient with a lifelong-coagulation defect in whose plasma there was a prolonged one-stage prothrombin-time, and found that this result could be corrected by adding adsorbed plasma where the prothrombin had been removed by an inorganic precipitate (Bordet & Delange, 1914; see below for a description of the procedure). The missing coagulation factor was called factor V (see Fig 2). Several workers, including Owren, found that factor V was not present in serum, it being, like prothrombin, used up during coagulation.

(ii) *Factor VII*. One of the earliest observations leading to the discovery of factor VII was by Owen & Bollman (1948), who showed that the addition of normal serum (containing neither prothrombin nor factor V) shortened the one-stage prothrombin-time of coumarin plasma. Within 3 years four other groups had described related phenomena to which the name factor VII was given (a term first used by Koller *et al.* 1952) (see Fig 2).

*Early plasma fractionation procedures*. In Oxford, in the work during 1951–53, simple plasma-fractionation procedures, however crude by today's standards, were most valuable.

*Adsorption on inorganic precipitates*. The nature of the hypothetical prothrombin was clarified by experiments in France (Bordet & Delange, 1914). They added an inorganic precipitate (calcium phosphate) to plasma, and the supernatant came to be called adsorbed plasma. This supernatant would not coagulate following the addition of tissue and calcium. The removal of prothrombin from the inorganic phosphate was proved by eluting a fraction containing prothrombin from the calcium phosphate, and by adding this back to the adsorbed plasma, which then coagulated normally. In our work we used aluminium hydroxide as the adsorbing agent.

(ii) *Ammonium sulphate precipitation*. Ammonium sulphate precipitation can be used to make some degree of separation of components in whole or adsorbed normal plasma (Quick, 1947). The globulin precipitate made by adding 33% ammonium sulphate contains fibrinogen and the substance missing in haemophilia (antihaemophilic globulin, AHG). The supernatant after this first precipitation can be treated by 50% saturation with ammonium sulphate to provide a factor V rich solution (Quick, 1947). These preparations can only be viewed as crude, but their use advanced knowledge.

*What was known in 1951*

Quick (1947) made two important advances in our knowledge of the blood's intrinsic pathway; we called this blood or plasma thromboplastin. Dilutions of tissue thromboplastin were made and used in a one-stage prothrombin-time test; Quick (1947) reported that more diluted tissue-preparation clotted haemophilic blood less well than normal blood, suggesting that there was a missing intrinsic activity in haemophilic blood. The experiments were confirmed by Biggs & Macfarlane (1951). As described above, Quick (1947) had also found defective consumption not only in haemophilia, as had Brinkhous (1939), but also in thrombocytopenia. The missing factor in haemophilia was called antihæmophilic globulin (AHG) (or antihæmophilic factor, AHF) because, together with globulins and fibrinogen, it was present in the precipitate in diluted acidified plasma (see Mellanby, 1909). From the preceding prothrombin-consumption studies it seemed reasonable to assume that the substances missing in haemophilia and platelets were components of blood thromboplastin, the intrinsic pathway for prothrombin conversion.

From work done just ahead of this in Glasgow Royal Infirmary, I knew that the tissue pathway in haemophilia was normal. The brain from a hæmophilic, taken at post-mortem, was used to make tissue extract; this coagulated hæmophilic plasma (or normal plasma) as efficiently as normal brain extract.

*Thrombin generation test.* The next step forward was the thrombin generation test (Macfarlane & Biggs, 1953). In this test whole blood was delivered to a glass tube, and samples were removed at minute intervals and tested for thrombin activity by addition to fibrinogen. When thrombin starts to form at 4–5 min it appears very suddenly, and the rise in concentration is precipitous in normal blood. This suggested that there is a delay-phase before a powerful intrinsic (i.e. blood) thromboplastin is developed. Macfarlane (1967), in an historical account, gives credit to Alastair Robb-Smith, head of the Department of Pathology at the Radcliffe Infirmary, Oxford, for suggesting this experiment. The delay-phase is a safety-mechanism to prevent unwanted intravascular coagulation.

*Patient with 'prothrombin' deficiency.* At the beginning of 1952 we were very fortunate to study a 26-year-old male patient who had an acquired coagulation-defect. In many respects the study of this patient made at least as important an historical contribution as the Christmas patient investigated some months later. He did not have a lifelong bleeding problem, having had tonsillectomy and tooth extraction with no excessive bleeding; he suffered hæmaturia, gum bleeding and spontaneous bruising of arms, legs and neck. The patient's one-stage prothrombin time was 18–22 s, compared to a normal time of 14 s. Using a modified two-stage prothrombin technique, plasma with brain added was recalcified and the released thrombin was examined by transferring aliquots into fibrinogen at intervals to provide a measure of thrombin. Plasma from the patient, a control, and a coumarin-treated patient were examined. The pattern of development of thrombin from these three plasmas was plotted. Thrombin from the patient's plasma appeared

immediately, and had disappeared within 1–2 min. In the normal plasma it appeared equally quickly, but the total thrombin-evolution was not completed for 4–5 min, whereas from the coumarin plasma it took 8–9 min. There was obviously a large amount of prothrombin in the coumarin plasma, but this evolved much more slowly. We measured prothrombin as the area within the curve, and also by using Mellanby's globulin fraction (dilution and acidification of plasma). This patient's plasma provided the next evidence of a powerful blood thromboplastin.

*Publication in Nature (Biggs, 1952): 'Plasma thromboplastin'*

Rosemary described her further examination of the diluted plasma of this patient, who had very little prothrombin. She saw how his plasma might provide evidence of a blood-thromboplastin. A few minutes after recalcification she removed an aliquot and used it in lieu of brain in a one-stage prothrombin test. The aliquot was as powerful as brain. At the same time a separate aliquot added to fibrinogen showed only trace amounts of thrombin.

Rosemary also used, as a crude preparation of anti-hæmophilic globulin (AHG) (see above), the ammonium sulphate precipitate made by 33% saturation of adsorbed normal plasma. A preparation of platelets could be made by differential centrifugation of citrated blood, i.e. by obtaining first a platelet-rich plasma, and then, after further centrifugation, separating and washing the platelets in saline. Owren (1947) had shown that factor V was mainly precipitated by the second ammonium precipitate at 50% saturation. Rosemary adsorbed and eluted from normal serum a preparation containing the activity then known as factor VII. When the AHG preparation, the serum eluate, platelets and calcium were added together, there was a powerful thromboplastin, clotting normal plasma in 13 s. This 'thromboplastic' strength was bettered later in our work.

Lupus-type circulating anticoagulants had not been discovered by 1952 and it seems likely, and with the wisdom of hindsight, that this patient was an example where prothrombin was deficient. Adsorbed normal plasma (a source of factor V) and normal serum (a source of factor VII) failed to shorten the patient's one-stage test. Many years later I appreciated that this type of anticoagulant was easily diluted out, and it was this property that allowed powerful blood-thromboplastin to be discovered (see above). Some years afterwards the patient's blood was re-examined by the late Roger Hardisty, and he reported finding a circulating anticoagulant. It is likely he was correct.

*Thromboplastin generation test (Fig 3, Table I)*

Between March and June 1952 the observations published in *Nature* were extended. The research at that stage had indicated that an incubation mixture containing: (i) source of antihæmophilic factor (adsorbed normal plasma) diluted 1 in 5 with saline (alumina was the adsorbing agent); (ii) source of factor VII (normal serum), diluted 1 in 10 with saline; (iii) platelets; (iv) calcium, would generate a powerful blood thromboplastin. The serum had been incubated for 1 h after coagulation at 37°C to ensure consumption of prothrombin, factor V and antihæmophilic factor. Aliquots

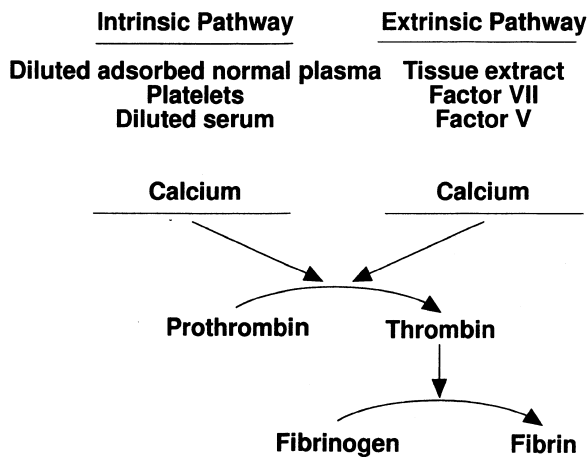


Fig 3. Early 1952. Prothrombin conversion to thrombin. Adsorbed normal plasma was used as a source of antihæmophilic factor. Serum was used as a source of factor VII; platelets and then calcium were added to the reaction mixture.

were removed at minute intervals. The potency of the plasma (or blood) thromboplastin was tested in a one-stage test, in lieu of brain, on high-spun platelet-free plasma (Table I-i).

Substitution of the patient's plasma or serum for the corresponding normal reagent gave abnormal results. The first abnormalities were demonstrated in hæmophilia where the adsorbed plasma was abnormal (Table I-ii) and coumarin therapy when the serum was abnormal (Table I-iii).

This was the first demonstration that coumarin therapy interfered with the intrinsic prothrombin-converting pathway:

previously, only interference with the extrinsic system had been recognized. This was an early hint of the other activities later defined as factors IX and X.

With the knowledge existing at the time, it was assumed that the adsorbed-plasma activity was due to antihæmophilic globulin (later factor VIII) and that the serum activity was due to factor VII. This was an example of the advantages of false assumption. These findings were submitted (June 1952) to the *Journal of Physiology*. By the time of the publication, early in 1953 (Biggs *et al*, 1953b), it was clear that some other factor or factors were present in the serum and the summary of the paper was amended at the proof stage to allow for this.

*The discovery of factor IX deficiency (called Christmas disease)*

*Creating the habit of mixing blood from two hæmophiliacs* (Merskey, 1950, 1951). Clarence Merskey, a travelling scholar from South Africa, was working in Dr Macfarlane's Oxford department in 1949–50 and established that hæmophilia was not necessarily always associated with severe recurrent hæmarthropathy and major spontaneous bleeding. The patient might have a hæmorrhagic problem only if there was a major hæmostatic challenge, such as circumcision, tonsillectomy or tooth extraction, and the disease could have several grades of severity. He demonstrated that the plasma of a mild hæmophiliac would not correct the recalcification time of plasma from a severe hæmophiliac as well as would normal plasma. Workers investigating patients with an undiagnosed 'bleeding' disorder acquired the habit of using this test procedure, and it became apparent that occasionally two hæmophilic plasmas were mutually corrective. In the middle of the

Table I. Thromboplastin generation test.

	Adsorbed plasma	Serum	Normal platelets	Incubation time (min)					
				1	2	3	4	5	6
June 1952				Clotting time (s)					
i	Normal	Normal	Normal	30	15	11	10	10	10
				(Normal)					
ii (Hæmophilia)	Patient	Normal	Normal	78	69	57	34	34	33
				(Abnormal)					
	Normal	Patient		45	20	13	13	11	11
				(Normal)					
iii (Coumarin)	Patient	Normal	Normal	Normal					
	Normal	Patient	Normal	Abnormal					
July 1952 (and later)									
iv (Christmas disease)	Patient	Normal	Normal	Normal					
	Normal	Patient	Normal	Abnormal					
v (Circulating anticoagulant or heparin)	Patient	Patient	Normal	Abnormal					
	Normal	Normal	Normal	Abnormal					
vi (Another disease)	Patient	Normal	Normal	Normal					
	Normal	Patient	Normal	Normal					
	Patient	Patient	Normal	Abnormal					

summer in 1952 we were engaged in developing the thromboplastin generation test. I was asked to examine the blood of two patients whose plasma corrected the recalcification-time of severe haemophilia as effectively as did normal plasma. I was sent to London to bring back blood collected by John Dacie (later Sir John Dacie FRS) and myself in the patient's home; the boy's surname was Christmas. The late Bob Pitney in the laboratory at the Postgraduate Medical School had observed 'cross correction' by the plasma from this boy when added to the plasma of other haemophiliacs. Around the same time an Oxford patient previously investigated by John Poole (Poole, 1953) was also re-tested by me, using the thromboplastin generation test. This showed that, in the new 'haemophilia' called by us 'Christmas disease', the serum was defective in the test system (Table I-iv); the previous, and much more common, disease with defective adsorbed plasma retained the original term, haemophilia. Other patients were provided by those who had recently worked in Oxford: Clarence Merskey, by then back in Cape Town, and John O'Brien, by then in Portsmouth. Two of the patients belonged to a family with sex-linked recessive-type inheritance, making the two disorders even more alike. The agent defective in this new group of patients was named Christmas factor (Biggs *et al*, 1952) and subsequently factor IX.

Some objected to the use of the name 'Christmas', but it was stoutly defended: it was better to use this term than to invent an elusory functional name. In terms of subsequent specific therapy, it was essential to distinguish the two types of haemophilias. Although agreeing in 1952 to this decision, I remained slightly uncomfortable about it and it was unfortunate that the paper appeared in the 1952 Christmas issue of the *British Medical Journal*.

The availability of the thromboplastin generation test enabled investigators world-wide to see in which category each of their patients belonged.

*Earlier or contemporaneous discovery of two haemophilias* (Brinkhous, 1965; Forbes, 1997). Some have given first credit to Feissly (1924) for observing cross correction between two haemophiliacs. In his papers (Feissly, 1923, 1924) he gave excellent and important results showing correction of prolonged whole blood clotting time in haemophilia by transfusion of citrated normal blood or plasma. In addition, he transfused haemophilic serum, i.e. unclotted plasma, 'rich in prothrombin and thrombin' to a second haemophiliac; The published results did not provide convincing evidence of cross correction.

In 1944 Castrex, Pavlovsky and Simonetti, three Argentinian investigators (see Pavlovsky, 1947), reported the results of mixing the blood of two haemophiliacs *in vitro* and by cross transfusion. In 1952 two other groups reported separate cross corrections; Aggeler *et al* (1952) and Schulman & Smith (1952) submitted their papers within a few days of each other. In neither of these reports were the kindreds sufficiently large for confidence about the pattern of inheritance. The laboratory experiments described by Johnson *et al* (1954) in Walter Seegers' laboratory yielded another important early observation.

#### *The nature of the coagulation defect during coumarin therapy*

The patient with the very low prothrombin level also contributed to the understanding of the nature of the coagulation defect in coumarin therapy.

Despite the discovery of factors V and VII, a prolonged one-stage test in 1952 was still loosely described as 'hypo-prothrombinaemia'. We thought at first that prothrombin was not deficient following interference with vitamin K by coumarin therapy. We were somewhat hasty, having based this conclusion on the early days of therapy, only realizing later that the prothrombin level did fall to some degree over the following 2 weeks of continued therapy. On testing daily plasma specimens after starting coumarin therapy, and using prothrombin assays as described in our papers (Biggs & Douglas, 1953a, b), we found that prothrombin took about 1–2 weeks to fall to about half its value, and that even then there was sufficient prothrombin left, rendering it unlikely to be contributing in a major way to the prolonged one-stage test result during coumarin therapy.

We had also demonstrated that coumarin serum was deficient in the thromboplastin generation test (see above). Only later was it established that this serum not only lacked factor VII but also factors IX and X. The following is a quotation from Biggs & Macfarlane (1953): 'Then the substance isolated from plasma is pure in a very limited sense. A factor may be freed from constituents recognized by a particular worker, but it cannot be known to be free from unrecognized substances. Thus prothrombin prepared by modern methods is free from antithrombin, the antihemophilic globulin, factor V and fibrinogen, but this does not imply the isolation of a functionally pure substance'. In the early stages of our work we made just such a mainly false assumption about the activity needed in blood thromboplastin found in serum and adsorbed on alumina. We attributed normal serum activity in thromboplastin generation to factor VII, realizing only after the events of the summer of 1952 that this could be due to Christmas factor alone or with factor VII. We did not know at that time that Christmas factor was vitamin K dependent. I was not convinced of the fall in concentration of factor IX during coumarin therapy until later (Douglas & Mair, 1958). It was in 1956/57 that others established that factor X (another factor adsorbed on alumina) was also present in serum and participated in its thromboplastic activity.

*The rise and fall of the thromboplastin generation test.* Because of urgent need in 1953 for clinicians around the world to define the nature of an individual patient's defect, the thromboplastin generation test became very widely used. Rapidly thereafter it was important in treatment of the haemophilias as materials became available. Assay methods for factors VIII and IX were designed on the basis of this test. I recognize that it is now used only very occasionally and that one-stage methods using factor VIII or factor IX deficient plasma with kaolin activation and a lipid source have taken its place. It was also used to examine for platelet function and the detection and study of circulating anticoagulants (Table I-v). Faced with a new patient with an undefined haemorrhagic state, it was for some years an important laboratory tool.

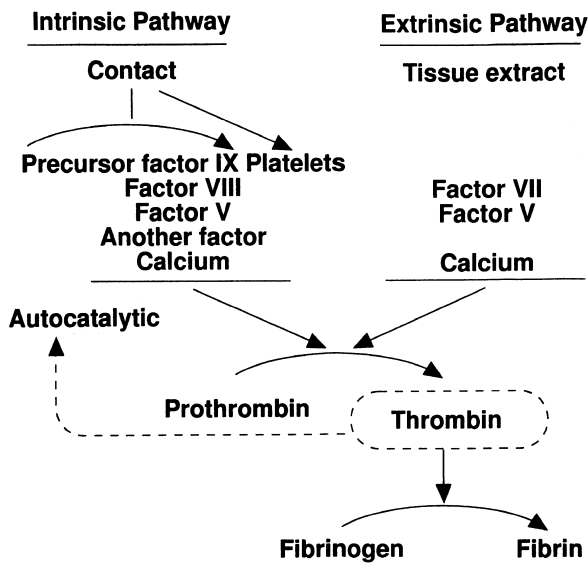


Fig 4. 1953. Prothrombin conversion to thrombin. Factor IX (as Christmas factor) had now been discovered and factor V was needed as well as antihæmophilic factor. Another factor (later to become factor XI) was recognized but not studied. Early steps to study contact are recorded.

*Later experiments*

In our second *Journal of Physiology* paper (Biggs *et al*, 1953c) we took adsorbed plasma and, by using ammonium sulphate precipitation, divided this into factor V and factor VIII and showed that both were required to make thromboplastin. The role of adsorbed normal plasma was not due to factor VIII alone.

We realized that all the then known constituents of blood thromboplastin – AHG (factor VIII) platelets, factor V, Christmas factor (factor IX), and possibly factor VII – were unlikely to react together simultaneously, but it was more likely that they reacted in some form of sequence. Since we did not have pure reagents, and some of the constituents (particularly factor X) were unknown, it was almost inevitable that, as time passed, any tentative conclusions would be overturned. We wrote of various hypothetical intermediate products, and in these various experiments we studied the contact effect. Exposure to glass accelerated thrombin formation in plasma and this needed factor IX or platelets to be present; at least we suggested these two components were needed earlier rather than later.

In our third *Journal of Physiology* paper (Biggs *et al*, 1953a) preincubation of brain with factor VII (serum) and factor V made the prothrombin converting property of brain more powerful. Heparin interfered with blood thromboplastin formation; at the time heparin was believed to act mainly as an antithrombin, although others had suggested that it might act earlier.

Figs 3 and 4 show how coagulation theory was changed by the events of 1951–53, illustrating the extrinsic and intrinsic pathways as separate entities. By 1998 (Fig 5) it

had become clear that the two pathways interact, well ahead of the final conversion of prothrombin.

*Another blood thromboplastin component.* Towards the end of my time in Oxford another patient with a life-long but mild hæmorrhagic disorder was studied. This was neither Christmas disease nor hæmophilia, the abnormality in the thromboplastin generation test being seen when the patient's adsorbed plasma and his serum were reacted together in the system (Table I-iv). With hindsight, this was factor XI deficiency. However, all we knew at the time was that there was at least one other factor (see Fig 4) needed in the blood's own thromboplastic system. Later it was recognized that factor XI was critical in the contact reaction.

*Gwyn's amplifier.* Throughout the time that this work was progressing, Gwyn often talked about blood coagulation being like an 'amplifier', each stage triggering off the next. He published this concept in *Nature* (Macfarlane, 1964), a decade later, in a paper entitled 'An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier'. Together with Davie & Ratnoff (1964) these hypotheses were critically important for future blood-coagulation research. Although their initial schemata have had to be modified, they formed the basis of much of what followed. Though it was not specifically studied, we knew that thrombin accelerated earlier stages. Obtusely, I thought this was the reason for Gwyn's amplifier, and turned a deaf ear.

*Absence of knowledge of factor X.* A large 'hole' in our understanding of the mechanism in 1951–53 was the absence of knowledge of the undiscovered factor X. This followed from the work of Bergsagel & Hougie (1956), Telfer *et al* (1956) and Hougie *et al* (1957). Gwyn had some premonition of this, and, in addition to talking about amplifiers, he pressed in 1952 for the further pursuit of the role of Russell's Viper venom, in order to make fundamental advance. A decade later the venom was shown to react with factor X and nothing else.

*Thrombin–fibrinogen reaction.* Our coagulation experiments depended on one reaction: the time taken for thrombin to coagulate fibrinogen. The more one studied much earlier reactions, the less confident one could be about the result. I realized by the end of my time in Oxford that the problems could not be taken much further without the contribution of basic scientists, particularly at that time in biochemistry. In the U.S.A. this was already much more widely understood, and was financially supported, resulting in distinguished contributors in basic science such as Walter H. Seegers and Earl Davie. In the British system it proved much more difficult to create established posts for scientists in a clinical department because the limited funding had to be given in the first place to those who cared for patients and were responsible for undergraduate teaching.

It is easy to exaggerate the importance of scientific advances with which one is familiar on the personal and technical level. Gwyn and Rosemary often spoke of the need to treat hæmophilia more effectively. The contribution, however, to the discovery of factor IX deficiency, and the advance in understanding of the intrinsic pathway, was important. The thromboplastin generation test made

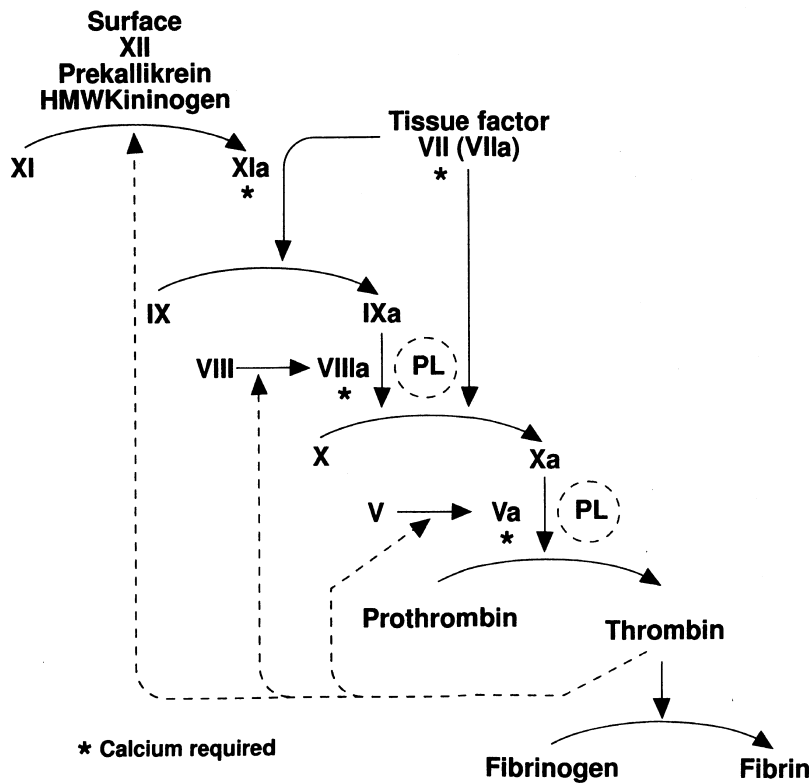


Fig 5. 1998. Prothrombin conversion to thrombin. By this time factors XII, XI and X had been added and much more detail of the interactions elucidated. An important development was the appreciation that the intrinsic and extrinsic pathways had a common pathway well ahead of the conversion of prothrombin to thrombin. The discontinuous lines show the autocatalytic action of thrombin. Factor VII with tissue in 1998 is believed to act early as well as late. PL = phospholipid (such as that in platelets).

possible improved diagnostic accuracy, and led to the development of assays of factors VIII and IX. There was a long tough road ahead in haemophilia management, but the door had been opened. The story up to 1967 has been told by Rosemary (Biggs, 1967).

Haemophilia is an important disease, but thrombosis too is serious and far more common. There have been enormous advances in the understanding and management of thrombo-embolic disease during the half-century since our work. The importance of platelets in the intrinsic pathway as a source of phospholipid was demonstrated in our work and there followed much more important work on platelet pathophysiology.

What general issues did I learn from my time in Oxford? (i) Theories may be accepted as fact especially if presented by eminent people. (ii) Theory tends to outstrip facts. (iii) When well-founded facts outstrip theory, then the theory has to be extended. (iv) A few facts are much more valuable than many hypotheses. (v) When an experiment gives the 'wrong' results it may well lead to new and more important pathways for investigation. It is usually the unexpected development that turns out to be more important. Nourish the exception. (vi) Because biology is so complex, if enough work and experiments are done, sooner or later the investigator is likely to strike gold. (vii) The best research comes from a laboratory where the atmosphere is relaxed and there is no pressure from the top or the side to publish. (viii) Try to identify and encourage those of the young who are seen to enjoy the intellectual pursuit of new knowledge. (ix) Apparently brilliant ideas on their own are of little use,

unless the originator is motivated to live laborious days, either doing the relevant experiments with his own hands or by organizing and supervising their execution. (x) Once a new scientific fact has been established with confidence, the investigator should be open about it and not secretive, and should publish it as soon as possible, whatever other pressures exist. (xi) Know the previous literature, and not only that of recent years.

#### Conclusion and apology

I have set down my reminiscences of the time; the judgement of their importance belongs to a later generation. It is not a comprehensive history and I apologize to those whose contributions have been omitted. I have tried to provide the reader with an historical step-ladder so that he can appreciate the sequence of events which led to understanding blood coagulation in 1953.

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