

Historical Review

TOWARDS MOLECULAR MEDICINE; REMINISCENCES OF THE HAEMOGLOBIN FIELD, 1960–2000

When historians of medicine in the twentieth century start to piece together the complex web of events that led from a change of emphasis of medical research from studies of patients and their organs to disease at the levels of cells and molecules they will undoubtedly have their attention drawn to the haemoglobin field, particularly the years that followed Linus Pauling's seminal paper in 1949 which described sickle-cell anaemia as a 'molecular disease'. These personal reminiscences of some of the highlights of those exciting times, and of those who made them happen, make no attempt to provide a comprehensive account of how the field evolved. Readers who are interested to learn more of the history of haemoglobin and its diseases are referred to the short bibliography which follows this essay.

WHY THALASSAEMIA AND HAEMOGLOBIN? A CONFESSION

Why, any aspiring young research haematologist might ask, should an English medical graduate become obsessed with a family of diseases which are restricted mainly to tropical climes? Although I have been asked this question many times, by far the most memorable was in 1962 during the oral examination of my Liverpool MD thesis on thalassaemia, conducted by the external examiner, Newcastle neurologist Henry Miller. 'Well', he said, 'it looks alright but you must stop working on a disease that none of us has heard of; go into psychiatry before it is too late'.

But it was already too late. My completely unplanned entry into the haemoglobin field 4 years earlier was due almost entirely to the administrative eccentricities of the British Army. Two years after qualifying in medicine in Liverpool, and armed with a Membership of the Royal College of Physicians (MRCP), obtained only because it took the Army a full year after my house jobs to find the correct address to dispatch my call-up papers, I had to serve for 2 years in the Royal Army Medical Corps (RAMC) as part of compulsory National Service. Terrified of aeroplanes, bullets and snakes, I volunteered to serve in the United Kingdom; 2 weeks later I was on a troop ship bound for Singapore, where, as I had no paediatric training, I was put in charge of the children's ward at the British Military Hospital. One of my first patients was a Nepalese Gurkha child who had been kept alive from the first few months of life with regular blood transfusion and who had defied the diagnostic might of the RAMC.

Correspondence: D. J. Weatherall, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK. E-mail: liz.rose@imm.ox.ac.uk

I soon took to spending my spare time at the General Hospital in Singapore, where I met a lively biochemist from Malta, Frank Vella, who, after travelling the world, subsequently had a distinguished career in biochemistry in Canada. Frank, who had initiated a filter-paper electrophoresis survey for abnormal haemoglobins in Malaya, told me about a publication from Thailand which described some children with thalassaemia, suggesting that this disease is not, as previously had been thought, restricted to the Mediterranean region. Luckily, another paper had also just appeared, from the American immunologist, Henry Kunkel, which described how, using electrophoresis in slabs of starch, he had found a minor component of human haemoglobin (Hb), Hb A₂, the proportion of which was elevated in some carriers of thalassaemia. After several weeks spent knee deep in potato starch, we found that the Gurkha child's parents had increased Hb A₂ levels and, hence, that she was likely to be homozygous for thalassaemia. Frank and I rushed this breakthrough to the British Medical Journal, but my excitement on seeing my first paper in print was short lived; I was hauled up before the Director General of Medical Services for the Far East Land Forces and told that I could be court marshalled for not getting permission from the War House (Office) to publish information about military personnel. 'And, in any case', he added, 'it is bad form to tell the world that one of our pukka regiments has bad genes; don't do it again'.

After a year in Singapore, I was sent to look after the medical wards at the British Military Hospital in Taiping, North Malaya. By now, completely bitten by the haemoglobin bug, I devised a simple electrophoresis system using car batteries to look for haemoglobin variants. Vella had left Singapore and so whenever I thought I had found one I posted it to Hermann Lehmann in London. Just before the end of my National Service I arranged to go to Johns Hopkins Hospital in Baltimore to train in genetics and haematology. While passing through London en route for the USA I visited Hermann to thank him for all his help. Encouragingly, he told me that I was wasting my time working on haemoglobin because there was 'nothing left to do'. 'Start exploring red cell enzymes', he suggested. In the event, on arriving in Baltimore in 1960 it turned out that human genetics, and the haemoglobin field in particular, were bubbling with excitement and potential.

The only lessons for those contemplating careers in medical research from this chapter of academic and military gaffs are that, regardless of the working conditions, when there are sick people there are always interesting research questions to be asked, and only take career advice along the way if you are determined to ignore it.

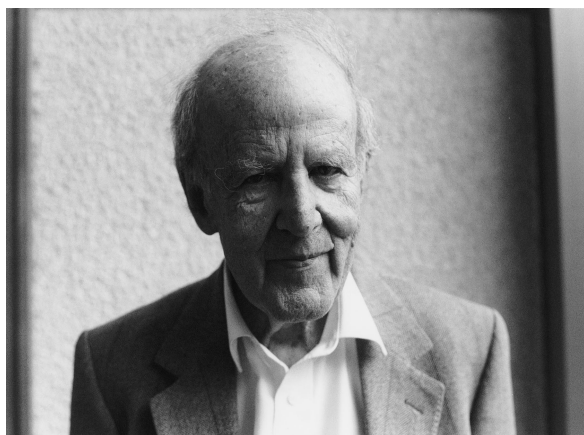


Fig 1. Max Perutz. Photograph by B. Meyra, Pressebild, Berlin.

THE DIVERSE ROUTES OF THE HAEMOGLOBIN FIELD IN THE 1960s

The excitement of the haemoglobin field in 1960 reflected the chance amalgamation of several disciplines in the 1950s, particularly X-ray crystallography, protein chemistry, human genetics and haematology.

X-ray crystallography and protein chemistry

From the early 1930s the structure of proteins became one of the central problems of biochemistry. At that time, the only way of tackling this problem was by X-ray crystallography. In 1937 Felix Haurowitz suggested to Max Perutz (Fig 1) that an X-ray study of haemoglobin might be a good subject for his doctoral thesis. He was given some large crystals of horse methaemoglobin which gave excellent X-ray diffraction patterns. However, there was a major snag; an X-ray diffraction pattern provided only half the information



Fig 2. Attendees at the 1st Conference on Problems of Cooley's Anemia held in New York in December 1963. The lady in the centre and her companion on her left are Officers of the Cooley's Anemia Blood and Research Foundation for Children. The attendees are (left to right) (1) unrecognizable, although possibly the present author; (2) Donald Rucknagel; (3) Arno Motulsky; (4) Vernon Ingram; (5) Paul Marks; (6) Ed Burka; and (7) Lucio Luzzatto.



Fig 3. Five generations of Boston haematology. Seated is William Castle. Standing (left to right) are Stuart Orkin, David Nathan and Alan Michelson. The picture on the left is of Dean David Edsall of Harvard Medical School who established the Thorndyke Laboratory at the Boston City Hospital. He was succeeded by Dean Peabody, who recruited both George Minot, who won the Nobel Prize for his work on pernicious anaemia, and William Castle, who should have also received it.

required to solve the structure of a protein, that is the amplitudes of diffracted rays, while the other half, their phases, could not be determined.

After numerous setbacks extending over many years, Perutz and his colleagues finally cracked the phase problem in 1953, when they discovered that it could be solved in two dimensions by comparison of the diffraction patterns of a crystal of native haemoglobin with that of haemoglobin reacted with mercuribenzoate, which combines with its two reactive sulphhydryl groups. In short, to solve the structure in three dimensions required the comparison of the diffraction patterns of at least three crystals, one native and two with heavy atoms combined with different sites on the haemoglobin molecule. In 1959 this approach yielded the first three-dimensional model of haemoglobin, at 5.5 Å resolution.

Protein chemistry evolved side-by-side with X-ray crystallography during the 1950s. In 1951 Fred Sanger solved the structure of insulin, a remarkable *tour de force* which showed that proteins have unique chemical structures and amino acid sequences. Sanger had perfected methods for fractionation and characterization of small peptides by paper chromatography or electrophoresis. In 1956 Vernon Ingram (Fig 2), who, like Max Perutz, was a refugee from



Fig 4. Hermann Lehmann. This group photograph was taken during a visit with the author to China in July 1979. Hermann (centre) is being shown some haemoglobin variants by Professor Shie Liang, Professor of Paediatrics at Kwangsi Medical College, Nanning.

Germany, was set the task of studying the structure of haemoglobin from patients with sickle-cell anaemia, a story that we will return to later. As part of this work Ingram separated the peptides produced after globin had been hydrolysed with the enzyme trypsin, which cuts only at lysine and arginine residues. Although these amino acids accounted for 60 residues per mol of haemoglobin, only 30 tryptic peptides were obtained, indicating that haemoglobin consists of two identical half molecules. Re-examination of the amino-terminal sequences of haemoglobin by groups in the United States and Germany showed 2 mols of valine-leucine and 2 mols of valine-histidine-leucine per mol of globin. These findings, which were in perfect agreement with the X-ray crystallographic results, suggested that haemoglobin is a tetramer composed of two pairs of unlike peptide chains, which were called α and β . It was soon found that the non- α chains of fetal haemoglobin, which had been known for over 100 years to be different to adult haemoglobin, have different structures and these were designated γ chains.

Sickle-cell anaemia; a molecular disease

A seminal advance, and one which was to mark the beginning of molecular medicine, was the chance result of an overnight conversation on a train journey between Denver and Chicago. Linus Pauling, the protein chemist, and William Castle (Fig 3), one of the founding fathers of experimental haematology, were returning from a meeting in Denver and Castle mentioned to Pauling that he and his colleagues had noticed that when red cells from patients with sickle-cell anaemia are deoxygenated and sickle they show birefringence in polarized light. Pauling guessed that this might reflect a structural difference between normal and sickle-cell haemoglobin which could be detected by a change in charge. He gave this problem to one of his post-doctoral students, a young medical graduate called Harvey Itano. At that time they knew that a Swede, Arne Tiselius, had invented a machine for separating proteins according to

their charge by electrophoresis. As there was no machine of this kind in Pauling's laboratory, Itano and his colleagues set to and built one. Eventually they found that the haemoglobin of patients with sickle-cell anaemia behaves differently to that of normal people in an electric field, indicating that it must have a different amino acid composition. Even better, the haemoglobin of sickle-cell carriers was a mixture of both types of haemoglobin. This work was published in *Science* in 1949, under the title 'Sickle-cell anaemia: a molecular disease'.

The scene now shifts across the Atlantic to Cambridge, where Perutz had become interested in sickle-cell anaemia. He has recounted how, after publishing a paper suggesting that the distortion of sickle cells is due to the crystallization of deoxyhaemoglobin, he showed his findings to a group at the Pasteur Institute in Paris. They had also studied X-ray scattering of deoxygenated sickle cells to see if they could obtain a crystalline pattern but found that it was exactly the same as that of oxygenated sickle cells and normal cells. They implied that Perutz and his colleagues must have faked their results! Twenty years later Beatrice Magdoff obtained beautiful crystalline X-ray patterns from sickle cells and showed that the French group had failed because they had used leaky capillaries which let in oxygen!

Perutz and Crick suggested to Ingram that he should apply Sanger's techniques of peptide analysis to see if he could find any difference between normal and sickle cell haemoglobin. After digesting haemoglobin with trypsin, Ingram separated the peptides by electrophoresis and chromatography in two dimensions to produce what he later called 'fingerprints'. He recalls that his first efforts looked like a watercolour that had been left out in the rain. But gradually things improved and he was able to show that the fingerprints of Hbs A and S were identical except for the position of one peptide. Using a method that had been developed a few years earlier by Pehr Edman, which allowed a peptide to be degraded one amino acid at a time in a stepwise fashion, Ingram found that this difference was due to the substitution of valine for glutamic acid at position 6 in the β chain of Hb S.

As well as demonstrating how a crippling disease can result from only a single amino acid difference in the haemoglobin molecule, this beautiful work had broader implications for molecular genetics. Although nothing was known about the nature of the genetic code at the time, the findings were compatible with the notion that the primary product of the β -globin gene is a peptide chain, a further development of the one-gene-one-enzyme concept, suggested earlier by Beadle and Tatum from their studies of *Neurospora*, and a prelude to the later studies of Yanofsky on *Escherichia coli*, which were to confirm this principle.

The rapid spread of haemoglobin research

With the advent of simple filter paper electrophoresis, haemoglobin analysis became the province of clinical research laboratories during the 1950s and 'new' abnormal haemoglobins appeared almost by the week. Although many scientists were involved it was Hermann Lehmann (Fig 4) who became the father figure. Like Handel,

Hermann was born in Halle and, also like the composer, made his home in Great Britain. He came to England as a refugee and at the beginning of the Second World War had a short period of internment as a 'friendly alien' at Huyton, close to Liverpool, an experience shared with many others, including Max Perutz. He travelled widely during his later war service in the RAMC and developed a wide international network which enabled him to discover 81 haemoglobin variants during his career. In 1979 Hermann and I were invited by the Chinese Academy of Sciences to tour China to advise on developments in the haemoglobin field (Fig 4). He was a remarkable travelling companion, constantly disappearing (even into Tibet) to collect blood samples and make new contacts. The trip was only marred for me by having to share a bedroom with him; he appeared to have an inverted sleep rhythm and spent all night wide awake, smoking evil-smelling Swiss cigars and noisily applying emery paper to his glass slides in the vain hope that they might fit into the narrow confines of Chinese projectors.

These early studies on human haemoglobin variants provided some valuable information about the genetic control of haemoglobin. While the first to be described appeared to be alleles of Hb S, in 1958 Smith and Torbert, research fellows working at Johns Hopkins Hospital in Lockard Conley's Department, discovered a family in which two haemoglobin variants, Hbs Hopkins 2 and S, segregated independently. Shortly afterwards, in a series of ingenious dissociation-reassociation experiments, Harvey Itano and Elizabeth Robinson showed that Hb Hopkins 2 is an α chain variant. Hence, it was now clear that there must be at least two unlinked loci involved in regulating haemoglobin production, α and β . The discovery of the γ and δ chains of Hbs F and A₂, respectively, meant that there must be at least four loci involved. Subsequent family studies and analyses of unusual variants resulting from the production of $\delta\beta$ or $\gamma\beta$ fusion chains led to the ordering of the non- α globin genes.

The thalassaemias as disorders of haemoglobin production

By the early 1950s it was clear that the thalassaemias are heterogeneous and are inherited in a Mendelian recessive fashion. The notion that they, like sickle-cell anaemia, are inherited disorders of haemoglobin arose from a series of totally unconnected observations. It had been known for some years that children with severe forms of thalassaemia might have persistent production of HbF and it was found later that some carriers might have elevated levels of Hb A₂. Could these findings mean that thalassaemia results from a defect in Hb A production? The seminal observation in favour of this notion came from the study of patients who had inherited the sickle-cell gene from one parent and thalassaemia from the other.

Sickle-cell thalassaemia was first described by Ezio Silvestroni and his wife Ida Bianco in 1946, although at the time they could not have known the full significance of their finding. Indeed, because of lack of scientific communication during the Second World War, and difficulties of language, many of the invaluable genetic studies of this

remarkable Italian couple remained unknown to English-speaking scientists for many years. After the advent of haemoglobin electrophoresis, Phillip Sturgeon and his colleagues in the USA found that the pattern of haemoglobin production in patients with sickle-cell thalassaemia is quite different to that of heterozygotes for the sickle-cell gene; the effect of the thalassaemia gene is to reduce the amount of Hb A to below that of Hb S, i.e. exactly the opposite to the ratio observed in sickle-cell carriers. As it was known that the sickle-cell mutation occurs in the β globin gene, it could be inferred that the action of the thalassaemia gene was to reduce the amount of β globin production from the normal allele. Indeed, from the few family studies available in 1960 there was a hint that this form of thalassaemia might be an allele of the β globin gene. Another major observation that was made in the mid-50s was the association of unusual tetramer haemoglobins, β_4 (Hb H) and γ_4 (Hb Bart's), with a thalassaemia phenotype. Could these variants reflect the results of defective α chain production?

In 1959 Vernon Ingram and Tony Stretton crystallized many of the ideas that had evolved over the late 1950s in a seminal paper which proposed a model for the genetic basis of thalassaemia. In essence, they suggested that there are two major classes, α and β , just as there are two major types of structural haemoglobin variants. They extended the ideas of Linus Pauling and Harvey Itano, who had suggested that defective globin synthesis in thalassaemia might be due to 'silent' mutations of the β globin genes, and postulated that the defects might lie outside the structural gene in the area of DNA in the connecting unit which proceeds it.

Ingram and Stretton's paper did not please Itano and Pauling, who wrote a letter to *Nature* in which they claimed that the genetic and chemical studies on haemoglobin reported since 1957 had not substantially altered their inferences about the nature of thalassaemia. Twisting the knife, they added that Ingram's papers were remarkable for the extent to which the custom of giving pertinent reference to the ideas and findings of previous workers had been ignored! This was completely unfair. In fact, work on the interactions of thalassaemia and haemoglobin variants in the late 1950s had moved the field to a considerably higher level of understanding than is apparent in the earlier papers of Pauling and Itano. In any case, in their paper Ingram and Stretton generously acknowledged the ideas of other workers, including Lehmann, Gerald, Neel and Ceppellini, that had allowed them to develop their conceptual framework of the general nature of thalassaemia. This interpretation of events, and the input of scientists from many different disciplines into these concepts, is supported by the published discussions of several conferences on haemoglobin held in the late 1950s.

The end of the beginning

By 1960 therefore, although there were only a limited number of answers, the central questions about the molecular pathophysiology of haemoglobin had been clearly defined, an enormous advantage to those of us who entered the field at about that time. Although some of the scientists

who had helped to develop it stayed on, many moved on to new pastures, motivated to some extent by what they saw as more exciting questions in the rapidly developing field of molecular biology, but also by the belief that unless there were major developments in technology it would not be possible to make further progress towards an understanding of the molecular genetics and pathology of haemoglobin, particularly the thalassaemias. A small core of basic and clinical scientists were not deterred by these problems, however, and over the next 20 years were able slowly to set the scene for the arrival of a second wave of basic science, which descended on the field in the early 1980s when haemoglobin became a major target for the new technology of molecular biology.

THE LONG PREPARATION FOR THE MOLECULAR ERA: 1960–1980

Research into the genetic control and structure/function relationships of haemoglobin ran along aside the investigation of the thalassaemias during the 1960s and 1970s and there was frequent cross-fertilization between the two endeavours.

Structure/function relationships and the molecular pathology of haemoglobin

During the 1960s Perutz and his colleagues made steady progress towards an atomic model of haemoglobin, which they finally published in 1970, 33 years after the first X-ray diffraction pictures of haemoglobin were obtained. The first models were of horse methaemoglobin; horse and human deoxyhaemoglobin soon followed. Over the same period structural analysis of haemoglobin variants had become more or less routine and some indication of the broad clinical spectrum of disorders with which they are associated became apparent. The hereditary methaemoglobin-aemias were discovered by Gerald, haemolytic anaemias due to unstable variants were identified by groups led by Dacie and Lehmann, and Charache, Clegg and I described the first case of familial polycythaemia due to a haemoglobin variant with a high oxygen affinity; many others followed. This plethora of structural variants, taken together with increasing knowledge of the molecular structure of haemoglobin, allowed Perutz and Lehmann to publish their classic paper, *The Molecular Pathology of Human Haemoglobin*, in 1968. This landmark publication, which came complete with stereoscopic pictures of different parts of the haemoglobin molecule, provided an interpretation of the different clinical disorders associated with haemoglobin variants at the molecular level. Although some of this work has been refined and extended since then, very few new concepts have been established which are not to be found in this paper.

The success of the Perutz/Lehmann collaboration reflected the different but totally compatible abilities of these two scientists. Perutz's remarkable insights and persistence ultimately led to a clear understanding of the relationship between the structure and function of haemoglobin, a truly remarkable achievement. Lehmann, equally

dogged in his determination to scour the world for the last remaining human haemoglobin variant, was not simply a stamp collector, but had a clear vision of both the clinical and broader biological implications of what he was doing. He also had the unusual ability as a clinical scientist of being able to communicate with Perutz, a valuable lesson of the importance of nurturing the careers of clinicians who are able to cross the barrier into the world of the basic sciences. And, as Perutz has recalled, Lehmann's attention to detail was formidable. While this paper was being written, Perutz went on vacation in Switzerland and was rung up daily by Lehmann with concerns about minute errors in its contents. My much-thumbed reprint still has stapled to it a typed list of minor errata which, I suspect, would have been ignored by the majority of authors.

Progress in understanding the genetic and molecular basis for the thalassaemias

In the 1960s the thalassaemias became of increasing interest because of their extraordinary frequency and clinical heterogeneity, combined with the possibility that they might reflect the action of mutations involved in the regulation of protein synthesis. First, we focused our attention on trying better to define their genetic heterogeneity using more sensitive electrophoretic techniques, particularly starch gel electrophoresis. It soon became clear that the β thalassaemias are extremely heterogeneous, some associated with no β globin, others with a varying reduction in the amount produced. Another family of β -like thalassaemias, in which there is no β or δ chain synthesized, the $\delta\beta$ thalassaemias, soon appeared on the scene, and the α thalassaemias were equally heterogeneous; although milder forms were extremely common in African populations, none of the severe manifestations of the disease which were observed in Asia, hydrops fetalis and the moderately severe form of haemolytic anaemia associated with Hb H disease, were found in Africans.

In a small monograph written in 1965, I attempted to summarize up to the point we had reached. Although a great deal of progress had been made at the phenotype level, the genetics of the thalassaemias, particularly the α thalassaemias, remained very confusing and, although there were no lack of hypotheses, we had only the haziest ideas about their pathophysiology or molecular pathology. On the other hand, it was already clear that they had the potential to teach us a great deal about abnormal gene regulation as the basis for disease.

α thalassaemia remained something of a geneticist's nightmare for many years. Lie-Injo described stillborn, hydropic babies with large amounts of Hb Bart's, and HbH disease had been well characterized as a moderate form of haemolytic anaemia associated with varying amounts of HbH in the red cells. Family studies which attempted to describe the genetic transmission of these conditions gave confusing results because the haematological changes in carriers were so mild. However, the elegant population studies of Prawate Wasi and his colleagues in Thailand started to make some sense of the scene and, when Susan Hollan's group in Hungary discovered a family with two

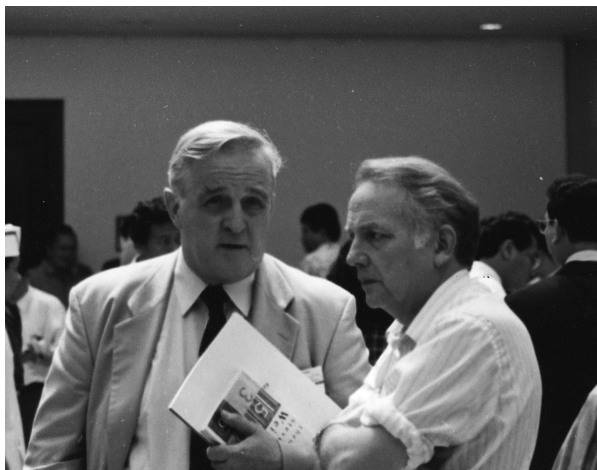


Fig 5. The author (left) with John Clegg at a thalassaemia meeting in Indonesia, 1993.

α -chain structural variants, indicating that the α globin genes must be duplicated, things started to become clearer. If this family had turned up 10 years earlier, many hours of futile speculation about the genetics of α thalassaemia would have been avoided; those who work in microbial genetics, in which their subjects breed to order in a few hours, should have some sympathy for human geneticists.

Globin synthesis in thalassaemia

Paul Marks (Fig 2), Dick Rifkind, Ed Burka and Arthur Bank demonstrated defective globin synthesis in thalassaemic red cells in the early 1960s, but it proved difficult to take this work further because there was no way of measuring the relative rates of globin chain synthesis in a quantitative fashion. This problem became particularly pressing when, in 1963, Phaendon Fessas described irregular inclusion bodies in the red-cell precursors of patients with β thalassaemia, showing, incidentally, just how much can be learnt by an acute observer with the simplest of equipment. Were these excess α chains that had precipitated, or was this some form of highly unstable haemoglobin which was the basic cause of defective β chain production?

Determined to try to crack this problem, in 1963 I started to study *in vitro* haemoglobin synthesis by analysing the incorporation of radioactive amino acids into globin after incubation of reticulocytes or bone marrow. It was relatively easy to obtain linear synthesis for several hours but the problem was how to separate the globin chains. Experiments in which they were separated by hybridization with canine haemoglobin, or by the even more tedious approach of countercurrent distribution, always showed unequal labelling of α and β chains in β -thalassaemia reticulocytes, but the results were not quantitative.

At about this time John Clegg (Fig 5) came to work with Mike Naughton in the Biophysics Department at Johns Hopkins University. John had come to Hopkins as a post-doctoral refugee from the laboratory of the distinguished protein chemist Hans Neurath; the rather rigid regimen of

Neurath's laboratory did not suit his more relaxed approach to science and they had decided to part company. His first project was an abortive attempt to study the synthesis of insulin and, as I had started to spend time in the Biophysics Department learning some protein chemistry from Naughton, we soon started to meet for tea to sympathise with each other over our mutual lack of results. During one of these sessions John suggested that it might be possible to obtain better separation of globin chains using an approach he had developed for separating the chains of fibrinogen while a research student in Sanger's laboratory in Cambridge. So, again by chance, began a friendship and scientific partnership which has lasted to this day.

Much to our delight we were soon able to obtain excellent separations of the globin chains, with full recovery of radioactivity, in experiments in which we incubated reticulocytes from normal persons or those with different forms of thalassaemia. It was soon clear that the major finding in all thalassaemic red-cell precursors was imbalanced globin-chain synthesis; many groups subsequently adapted this method to define different forms of thalassaemia. The idea that the characteristic damage to red-cell precursors and mature red cells in thalassaemia might be due to the deleterious effects of the globin chains which are produced in excess was developed by David Nathan (Fig 2) and his colleagues in Boston and later by others, notably Eliezer Rachmilewitz in Israel and Stanley Schrier at Stanford, work which eventually led to a much better understanding of the pathophysiology of the thalassaemias.

But why were the globin chains not effectively produced in thalassaemic patients? In the early 1960s I had attended a seminar by Howard Dintzis in which he described how, by labelling rabbit reticulocytes for a short period, it was possible to demonstrate how globin chain synthesis begins at the amino-terminal end and continues sequentially, to end at the C terminus. In essence, he was able to plot the growth of a globin chain both in the soluble cell fraction and on the ribosomes. Why, I wondered in my innocence, couldn't similar experiments be done in thalassaemic red-cell precursors to measure the time it takes to make a globin chain and to see whether synthesis is linear, or perhaps even non-linear as would be expected if Pauling and Itano's structure-rate hypothesis was correct? However, Dintzis had carried out his experiments on phenylhydrazine-treated rabbits with reticulocyte counts of nearly 100%, which were therefore very active in protein synthesis, very different to the paltry dog-eared reticulocytes of patients with thalassaemia. However, after 2 or 3 years of frustration we were able, with the help of Prawate Wasi in Bangkok, to obtain reasonable short-term labelling of globin chains from both normal and thalassaemic reticulocytes and to determine both the timing and pattern of their synthesis. In the event, there was no difference between normal and thalassaemic β -globin production. It seemed, therefore, that the mechanisms of translation and chain termination were not affected in β thalassaemia; later it was possible to show that the rates of initiation were normal. Thus, it appeared that, at least in the cases of β thalassaemia that we had studied, the primary defect must reflect a reduced amount of normal



Fig 6. Y. W. Kan.

messenger RNA for the globin chain that is ineffectively synthesized.

Although this was more or less the end of the road for the biosynthetic approach to the molecular pathology of thalassaemia, studies of the structure of two haemoglobin variants which had been discovered over this period also provided a hint to what was in store in the rapidly approaching molecular era. Corrado Baglioni described a variant called Hb Lepore, which he found was composed of normal α chains combined with non- α chains which consisted of part δ and part β chains. He suggested that this was the product of a $\delta\beta$ fusion gene which resulted in a reduced output of its product and, hence, the clinical features of thalassaemia. After carrying out this and a great deal more beautiful science in the USA, Corrado attempted to return to his native Italy; there he failed the public examination required before achieving the rank of Professor because he was unable to define what is meant by pH!

In 1973 we were referred a family from Jamaica by Paul Milner in which several patients with Hb H disease had trace amounts of an abnormal haemoglobin. We guessed that this was an α chain variant which is produced in very low amounts and, hence, results in the phenotype of α thalassaemia. The variant, which we christened Hb Constant Spring, the name of the suburb in Kingston in which the affected family lived, turned out to be unusual in more than its name, in that it had an α chain which was extended by 31 amino acid residues. We suggested therefore that this might result from a base substitution in the α -chain termination codon, UAA to CAA, as residue 142 in the

elongated chain was glutamine which is encoded by CAA. It followed that there must be messenger RNA which is not normally utilized which, in this case, is read through until another in-phase stop termination codon is reached. From such knowledge of the genetic code for proteins as was available then, it was possible to hazard a guess at what the structure of this normally untranslated messenger RNA might be. This interpretation, which was greeted with great scepticism (to put it mildly) by our molecular-biologist colleagues at the time, was fully vindicated when human messenger RNA was sequenced a few years later. Thus, this form of thalassaemia seemed to reflect the effects of the translation of normally unread messenger RNA, leading to a marked reduction of synthesis of the variant haemoglobin.

Moving to RNA and DNA

In the early 1970s several groups attempted to explore globin messenger RNA in thalassaemia by isolating it from thalassaemic reticulocytes and assaying its activity in cell-free systems. These pioneering experiments were carried out by Nienhuis and Anderson at the National Institutes of Health (NIH) and Benz and Forget in Boston. They, and later others, found that there was a reduction in β globin mRNA in this system. However, because they were measuring activity and not quantity it was not possible to distinguish whether the mRNA was present in decreased amounts or whether it was functionally abnormal. However, following the discovery by Howard Temin and David Baltimore that an enzyme called reverse transcriptase, obtained from certain tumour viruses, can synthesize complementary DNA (cDNA) from messenger RNA templates, it became possible to synthesize radioactively labelled α and β cDNA which could then be used to analyse the levels of mRNA in reticulocytes by cDNA/RNA hybridization. Initially, Kacian and Housman and their colleagues, and later others, were able to show that there was a reduced level of β globin messenger RNA in some β thalassaemic red cells. Further studies demonstrated cases in which there was no detectable β globin messenger RNA, or in which there was apparently normal amounts of full-length but non-functional messenger RNA. In 1979 Chang and Kan (Fig 6) determined the nucleotide sequence of mRNA from one of the latter cases and found that an AAG codon for lysine had mutated to the chain termination codon UAG.

During the early 1970s it seemed increasingly likely that some forms of thalassaemia might be caused by gene deletions, and thoughts turned to using cDNA/DNA hybridization to probe for the presence or absence of globin genes. After we had found that stillborn babies with severe α thalassaemia synthesize no α globin chains at all, or any α -globin fragments, we travelled to Glasgow to meet John Paul, who had had some experience of molecular hybridization, to ask whether it might be feasible to determine whether the α globin genes were deleted in infants of this type. John gave the problem to two molecular biologists in his group, Sergio Ottolenghi and Bob Williamson. With the help of our Thai colleagues we obtained tissue from a stillborn infant with the Hb Bart's hydrops fetalis syndrome and sent it together with normal fetal tissue to Glasgow,

after first determining that there was no α chain synthesis in this particular case. It was found that the hydropic fetal liver cells contained normal β globin genes but that there was a more or less complete absence of α -globin genes. Y. W. Kan had set up a similar collaboration in San Francisco and our two papers, describing a deletion as the basis for human disease for the first time, appeared back to back in *Nature* in 1974.

By the late 1970s, techniques for the direct analysis of the globin genes had become available. It had been an exhilarating although often frustrating time to be working in this rapidly moving field but, considering the complexity of the central problem, it is surprising how much progress was being made by approaches which were so far removed from the defective genes. Already we knew that the thalassaemias were extremely heterogeneous and could result from gene deletions, nonsense or chain-termination mutations, fusion genes, or highly unstable gene products. It was also gratifying to see that the work on haemoglobin synthesis had moved rapidly into the clinic for the successful prenatal diagnosis of the haemoglobin disorders, and that our better understanding of the pathophysiology of the thalassaemias was leading to more rational forms of transfusion and chelation therapy.

THE MOLECULAR ERA

With the advent of Southern blotting and gene cloning in the late 1970s, it was not surprising that an army of molecular biologists descended quickly on the haemoglobin field. The knowledge that had been gained about the organization and function of the globin genes in the preceding years meant that the system was virtually handed to them on a plate! Soon, the haemoglobin genes were sequenced and their arrangement determined. Gratifyingly, it was found that their arrangement was precisely that anticipated by the earlier genetic studies.

However, there were some surprises which, again, put haemoglobin in the forefront of the understanding of human gene structure and function. In the late 1970s, the groups of Leder, and Jeffreys and Flavell independently observed that the coding regions of the globin genes are broken up by non-coding regions called intervening sequences, or introns, an observation which was later shown to be true for most mammalian genes. and in 1979 Jeffreys found two polymorphisms for a restriction enzyme in the γ globin genes and estimated that perhaps 1% of nucleotides might be polymorphic. Subsequently it was found that these restriction fragment length polymorphisms (RFLPs) are spread throughout the globin gene clusters, but not in a random fashion. In 1978 Kan and Dozy, using the restriction enzyme *Hpa* I, found that the β globin gene of normal individuals is located on a 7.6-kb fragment, while that of patients with sickle cell disease it is on 13 kb fragment. It appeared that the sickle mutation was on a chromosome that also carried a RFLP outside the coding area for the β chain; further studies showed that this is a very strong association in some populations. This was the beginning of the use of DNA linkage analysis for prenatal

diagnosis and, at the same time, increased the impetus towards finding genes of unknown function by linkage analysis, first called reverse genetics and later rechristened positional cloning.

There were other surprises in the early 1980s. For example, we stumbled on the association between α thalassaemia and mental retardation in some families, an observation which opened up a new field of investigation into causes of developmental abnormality and which, ultimately, defined the importance of subtelomeric deletions in generating retarded development and disclosed a new gene on the X chromosome which, when mutated, is associated with widespread developmental abnormalities and defective α chain synthesis.

During the 20 years of the molecular era, and led by the groups of Orkin (Fig 3), Antonarakis, Kan, Forget, Bank, Higgs and others, hundreds of genes from patients with different forms of thalassaemia were cloned and sequenced and a remarkable picture of the global diversity of the disease emerged. Further work confirmed what we had long suspected, that is, the existence of multiple genetic modifiers of the different forms of thalassaemia. Blood samples from unsolved diagnostic mysteries, some that had lain in deep-freezes for over 20 years, were disinterred and DNA extracted; one such sample, from an old Irishman who was the first reported case of dominant β thalassaemia from our group in Liverpool back in the early 1970s, helped Swee Lay Thein to elucidate the molecular basis for this particular intriguing family of thalassaemics. and the ability accurately to define the different thalassaemia genotypes finally enabled us to confirm JBS Haldane's suggestion that at least some of these diseases are particularly common because of heterozygote or mild-homozygote advantage against malaria. Both RFLP linkage and direct gene analysis rapidly took the place of globin synthesis in prenatal diagnosis of the severe forms of thalassaemia, and there was a remarkable reduction in their frequency, particularly in the Mediterranean Island populations, a success story which owes much to Kan, Dozy, Kazazian, Cao, Alter, Old and others.

It had been a glorious time to be involved in research in haemoglobin and its disorders. Progress had depended upon the work of a relatively small number of mainly clinically based groups in several different countries, all of which met regularly and among which there was a remarkable degree of friendly and open collaboration. Although it is invidious to mention one of them, the breadth and volume of the products of David Nathan's stable in Boston reads like a Who's Who of haemoglobin; Kan, Orkin, Forget, Benz, Propper, Alter, Olivieri, Platt and many others. While receiving honours for their work, their experiences were equally broad ranging; the presentation of the National Medal for Science to David Nathan severely taxed the medical vocabulary of the then President of the United States, Y.W. Kan discovered that he was only the second Chinese scientist to sign the admissions book of the Royal Society, the first having been an obscure Chinese Emperor sometime in the dark ages, and the Joe A. Calloway Award for Civic Courage, bestowed by the Nader Foundation in the

USA to Nancy Olivieri for her lone stand against the pharmaceutical industry and the University of Toronto, was one of the important catalysts in changing the policies of many of the world's leading medical journals in the publication of drug trials.

ENVOI

To have spent the last 40 years as a player, albeit sometimes peripheral, in the human haemoglobin field has been an enormous privilege and has provided a ringside seat at the extraordinary developments in molecular and cell biology and the attempts to move some of these advances into clinical medicine.

The one regret is that the remarkable advances in human genetics, and in the evolution of molecular medicine in general, have, with the exception of the development of more precise methods of diagnosis and prenatal detection, done little to help patients with these distressing diseases. The principles of the management of thalassaemia by transfusion and iron chelation were laid down in the 1960s, and, in truth, with the exception of bone-marrow transplantation, there have been no major conceptual advances in therapy. There is one exception, however. From the 1960s onwards it became clear that it might be possible to control the β thalassaemias and sickle-cell anaemia by augmenting fetal haemoglobin production. Studies on the complex mechanism of the regulation of the switch from fetal to adult haemoglobin by the groups of Grosfeld, Stamatoyannopoulos, Groudine, Wood and others, and their clinical applications by those of Nathan, Charache and others, have achieved at least some limited successes, more, however, in the management of sickle-cell anaemia than β thalassaemia.

On the other hand, the quality of life and prognosis for children with β thalassaemia, at least in the richer countries, has been transformed by some excellent clinical research. Prime examples in the thalassaemia field include the development of effective transfusion regimens by Wolman, Modell, Piomelli and Cazzola, evolving the most effective approaches to chelation therapy by Propper, Hussein and Pippard, and the establishment of more rigorous and scientifically based approaches to the assessment of body iron load by Barry, Letsky, Olivieri and Brittenham. Similar progress had been made in the symptomatic management of sickle-cell anaemia.

The lack of a more definitive approach to the cure of the haemoglobin disorders, despite so much knowledge about their molecular pathology, should not surprise us. The story of the advancement of medical practice over the last century tells us that there is always a long lag period before developments in the basic science reach the clinic. Somatic-cell gene therapy has proved to be an extremely difficult nut to crack, and for diseases like thalassaemia in which high-level, accurately regulated gene expression is required, it seems likely that it will be a long time before these formidable problems are solved. and when they are, the technology required may be far too expensive to transmit to

the developing countries where these diseases are so common.

Western medicine, and clinical genetics in particular, has benefited enormously from lessons learnt from the haemoglobin field. We now need to repay the developing countries where these extraordinary experiments of nature pose an increasing health problem, by transferring the technology that has resulted from their study for their better control and management. Given the will on the part of the international research community, governments and international health agencies, this could be relatively easy to achieve over the next few years. If this is not done soon, and hundreds of thousands of children with sickle-cell anaemia and thalassaemia continue to die in poorer countries, 40 years of elegant science may start to have a hollow ring about it.

Weatherall Institute of Molecular Medicine, University of Oxford,
Oxford, UK

D. J. WEATHERALL

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