HAEMOGLOBIN

HAEMOGLOBIN

HAEMOGLOBIN

A SYMPOSIUM based on a Conference
held at CAMBRIDGE in June 1948
in memory of SIR JOSEPH BARCROFT

Editors
F. J. W. ROUGHTON
J. C. KENDREW

LONDON
BUTTERWORTHS SCIENTIFIC PUBLICATIONS
NEW YORK
INTERSCIENCE PUBLISHERS INC.
1949

BUTTERWORTHS SCIENTIFIC PUBLICATIONS LTD
BELL YARD TEMPLE BAR LONDON, W.C.2

BUTTERWORTH AND CO. (AFRICA) LTD : DURBAN

BUTTERWORTH AND CO. (AUSTRALIA) LTD
SYDNEY : MELBOURNE : WELLINGTON : AUCKLAND

BUTTERWORTH AND CO. (CANADA) LTD : TORONTO

American Edition published by

INTERSCIENCE PUBLISHERS INC.
215 FOURTH AVENUE, NEW YORK 3

First Edition May 1949

Printed in Great Britain by Love & Malcomson Ltd., Redhill, Surrey

CONTENTS

PREFACE

WHEN Joseph Barcroft returned to Cambridge after World War I, he resumed the series of researches on haemoglobin on which he had been so actively engaged in the Cambridge Physiological Laboratory before that war. His enthusiasm, energy and inspiration acted like a magnet on workers in many other laboratories, so that by the end of World War II active projects on haemoglobin were being undertaken in no less than six different Cambridge laboratories. Two annual meetings of the various workers were subsequently held under Barcroft's chairmanship, and at the latter of these meetings in November 1946 he himself fixed the next meeting for the last Friday of November 1947. His sudden death on 21 March 1947, however, prevented him from keeping this engagement to which he had looked forward so much. Soon after his death some of his colleagues in Cambridge met together and decided that the next of the haemoglobin meetings should be on a much larger scale, and should be devoted to his special memory and honour. Invitations were sent out to leading workers on haemoglobin in many parts of the world, and a three-day conference, of which this book is the outcome, was held in Cambridge from 15 June to 17 June 1948, inclusive.

The proceedings opened with a morning devoted to biographical tributes by eight physiologists who had known Barcroft intimately at various stages of his life. At the end of the session a coloured cinema film with sound accompaniment was shown of Barcroft performing one of his typical haemoglobin experiments. The remaining sessions, five in number, were given to specialist papers on recent advances in different aspects of the subjects. These papers are grouped together in this book in almost the same way as they were arranged at the conference. On the afternoon of Tuesday, 15 June, Lady Barcroft and Professor Henry Barcroft entertained the members of the conference to tea in the Physiology Laboratory, and on the Thursday afternoon demonstrations, followed by tea, were given in the Molteno Institute. Social parties also took place on each of the two first evenings of the conference. The total number of visitors from outside Cambridge was about fifty, and the attendance at the meetings was sometimes as much as one hundred or more. Some of the visitors were accommodated at King's College, by the kindness of the Provost and Fellows.

At the end of the conference, which all agreed had been extraordinarily active and successful, it was unanimously resolved that the local Committee (Mr. G. S. Adair, Dr. R. Hill, Professor D. Keilin,

Mr. J. C. Kendrew, Dr. M. F. Perutz and Professor F. J. W. Roughton) should take steps to publish the whole proceedings in the form of a memorial volume within a year's time if possible. Professor F. J. W. Roughton and Mr. J. C. Kendrew were appointed editors, and Messrs. Butterworths Scientific Publications Ltd publishers. We believe that the astonishing range of subjects comprised in this volume would have given the utmost delight to the late Sir Joseph Barcroft, who was indeed the fountain-head of so much of all this varied work. If so, there can be no more fitting memorial to him.

F. J. W. R.
J. C. K.

Cambridge
March 1949

BIOGRAPHICAL NOTE

JOSEPH BARCROFT was born on 26 July 1872, at the Glen, Newry, Co. Down, N. Ireland. He came of a Northern Ireland Quaker family and was the second of the five children of Henry Barcroft, D.L. and Anna Barcroft. He was educated at the Friends School at Bootham, York, and the Leys School, Cambridge. In his last school year he passed the London B.Sc. Examination. He went up to King's College, Cambridge in 1893 and took a First Class in the Natural Sciences Tripos Part I in 1896, and again a First Class in Part II in Physiology in 1897.

In 1899 he won the Walsingham Gold Medal for research in Physiology and a Prize Fellowship at King's College. Shortly afterwards he was appointed to a Lectureship in Natural Sciences at that College, and in 1904 to a University Demonstratorship in Physiology. In 1910 he was elected F.R.S. and between this year and 1914 took part in, or led several high altitude expeditions. During World War I he was head of the Physiological Branch of the Chemical Warfare Service at Porton, and was awarded the C.B.E. He returned to Cambridge after World War I, first as Reader in Physiology, and then, six years later on the death of Professor Langley, he succeeded to the Chair of Physiology at Cambridge, which he held till he retired in 1937 under the age rule. During this period he was knighted and received many academic honours. Earlier in the inter-war period he had held the chairmanship of the Medical Research Council committee on haemoglobin, the Fullerian Professorship of Physiology at the Royal Institution, and had led an Anglo-American High Altitude Expedition to the Andes (1922). He also visited America on several occasions to deliver lectures sponsored by distinguished foundations or to receive other academic honours.

He continued in active research at Cambridge after retirement from his Chair until the outbreak of World War II, when he was promptly called back to his old service at Porton, with which indeed he had maintained active contact between the wars. Fortunately gas warfare did not materialize in World War II, so he returned in 1941 to the Cambridge Physiological Laboratory as head of the Agricultural Research Council Unit in Animal Physiology. During this period he was also deeply concerned with food and nutrition problems, being President of the Nutrition Society for several years. In 1943 he was awarded the Copley medal, which is the highest research honour in the gift of the Royal Society. After World War II he resumed active research on foetal physiology and on haemoglobin in which he had

been a leader for fifty years. He died suddenly of a heart attack, in the midst of active work on 21 March 1947.

Barcroft published three to four hundred original papers and several text books, including two classics, *The Respiratory Function of the Blood* and *Features in the Architecture of Physiological Function.* It is unnecessary here to refer to his personal qualities, as these are reflected so clearly in the tributes given in the first section of this book.

In 1903 he married Mary Agnetta (Minnie) Ball, a daughter of the famous astronomer Sir Robert Ball. She survives him with two sons, Henry (now Professor of Physiology at St. Thomas's Hospital, London) and Lieut-Col. Robert Ball Barcroft.

F. J. W. R.

I

TRIBUTES TO SIR JOSEPH BARCROFT

Professor E. D. Adrian

PROFESSOR ROUGHTON's conference on haemoglobin opens to-day with a special meeting in commemoration of Sir Joseph Barcroft and it takes place in the lecture theatre which he had built and in the laboratory where he worked for thirty years. It is a very great honour for me to welcome you in this laboratory. We are proud to have so many distinguished visitors in the field which he opened up. I said this was a special meeting, but of course in a sense your whole conference is a tribute to Barcroft—just the sort of tribute he would have liked, and he would have been eager for you to get down to the job of scientific discussion. In spite of that I do not think he would have minded our spending this morning talking about more personal things. He would not have been Joe Barcroft if he had not valued the friendships he made and the affection that everyone felt for him ; to-day's meeting gives us the opportunity of thinking of the man as well as of his scientific achievements. As to those, it is obviously right that the conference should be about haemoglobin ; that was his major interest and he never left it. But one of the most remarkable and characteristic things about him was the way in which he could open up one field after another. He could be commemorated by a meeting on foetal physiology, on the spleen, on adaptation to high altitudes or on many other subjects. When war broke out he was called down to Porton as an expert on defence against gas attack, and then to our great good fortune he came back to Cambridge to direct the Unit of Animal Physiology which had just been set up by the Agricultural Research Council. In those last four years, back in his own laboratory, he showed just the same power of inspiring his team and starting fresh lines of work. Just before his death he was thinking out a new attack on some of the problems of animal metabolism, and if he had lived you would have seen him busy with isotopes and mass spectrographs, developing quite new lines and going in search of the latest techniques to employ them. The fact was, of course, that he never really grew old and he never lost the knack of reducing a problem to its simplest elements and then finding an answer by the most direct method. One of his most fruitful methods was to look for help in all directions, to bring in new recruits and to act as a catalyst in translating their ideas into practical outcome. Many worked with him and experienced his

remarkable power of forging ahead all the time. I will not take up time in emphasizing what you know already, but I would like to say one thing. When he retired from the Chair of Physiology in 1937 he was sixty-five but still at the height of his powers as an investigator. It was quite unthinkable that he should not go on in the laboratory as before, but as you know it is not always an easy matter for an Emeritus and an acting Professor to work together in complete harmony, and I should like to put it on record that his presence here, besides being an immense asset to our research strength, was never anything but a great comfort and encouragement to his successor. Of course, I never thought it would have been otherwise. Barcroft was a very wise and kindly man as well as a great physiologist.

I have only one other thing to add and that is that when he retired from the Chair here, we thought we should like some record of him which would give a little more information than the ordinary portrait or photograph, so we asked him to let us make a film showing him doing an experiment on haemoglobin. It was made by Professor Winton and by John Freeman, our expert photographer, and although we had no sound equipment, we did the best we could by making some gramophone records of his voice to add as a commentary. If there is time at the end of the meeting, I think we might show that film.

Sir Henry Dale

I HAVE been asked to speak of our friend Barcroft's work up to about 1909–10. My friendship with him had begun nearly twenty years earlier, when we were boys together at the Leys School. At that first encounter I looked up to him with a certain awe, for he was a prefect, my senior by nearly three years, and I was a callow newcomer. By the time we came together again in the University, however, he was only a year ahead of me in academic ranking, since he had taken, under medical advice, a prolonged rest between school and university. The need for this was probably due to overwork in his last year at school, in which he had achieved the unusual feat, for a schoolboy, of graduating B.Sc. by examination at the University of London, then only an examining and degree-giving body. Whatever had been amiss, the medical advice seems to have been good, for Barcroft, when he had once begun the life of research, kept to it with unflagging energy and continued success right up to the day of his sudden death at an age

well beyond that at which the majority have begun to think of an easier time. So it came about that I found myself overlapping with him in what Cambridge then offered as a course of advanced physiological chemistry. Sheridan Lea had fallen out through illness, and Hopkins was not to arrive till two years later ; meanwhile the class was in the hands of a deputy, whom I found very uninspiring. I recall this, because I remember speaking to Barcroft one day in a mood of discouragement with the subject as thus presented, and I remember being surprised at the assurance with which he expressed his conviction that, when we came to the real thing, it would nevertheless be on the chemical side that the main advance of physiology would be found. I think that already, then, while still completing his preparation for the final examination, he must have begun to choose the general direction of what was to be his life's work. Certainly he was ready and eager to begin, when once the examination was out of the way.

The actual problem on which Barcroft began his researches was suggested to him by Langley, though probably not the method of approaching it. For Langley was not usually interested in chemical methods, but had long been concerned with the salivary glands and with the meaning of the contrasted effects of the chorda and sympathetic nerve-supplies on the secretory activity of the submaxillary gland. Heidenhain had attributed these to the relative predominance in those nerves of secretory and trophic fibres, while Langley believed that everything could be explained by one kind of secretomotor action and the widely different vasomotor effects of the two nerves. Others had suggested that the sympathetic action produced only a squeezing of the acini and ducts by contraction of plain muscle fibres, while others again had interpreted the sympathetic action as simply inhibitory. Langley asked for further light on this controversy, and Barcroft, we may suppose, thought that he could produce some by studying the gaseous metabolism of the gland and its changes with the different kinds of activity. His only predecessors in the attempt had been Chauveau and Kaufmann, eleven years earlier. They wished to test whether the increase of oxidative metabolism which they had observed with muscular activity, as also had Sczelkow and Schmidt twenty years or more earlier still, in Ludwig's laboratory, would be found also with secretory activity of the salivary gland. Chauveau and Kaufmann had used the horse and the natural stimulus of chewing and their results had not been striking or, indeed, uniform. Barcroft decided to use the sub-maxillary gland in the anaesthetized dog and to stimulate the nerves artificially.

Barcroft had thus chosen his first research objective, but, before he came within striking distance of it, he had to surmount the formidable

difficulties presented by the methods then available for his purpose. For it must be remembered that in 1897, when he began, the accepted method of obtaining the gases from a sample of blood, for analysis, was to pump them off into a vacuum produced by a Töpler mercury pump, the evolution of the gases taking place in a vessel having the form of a series of bulbs, to restrain frothing, while its progress was encouraged and its completeness ensured by bathing the vessel meanwhile in hot water. For his purpose, Barcroft needed further to store consecutive blood samples, collected during the course of an experiment without contact with air, in a series of such vessels, evacuated in advance ; and eventually he had to put each of these in succession, by turning one of a series of taps, into connexion with the pump through an unjointed run of glass tubing. So he arrived at a rather formidable, multiple apparatus, which had to be fused together by the blow-pipe before each experiment and cut into sections afterwards for cleaning. Modern standards, I suspect, might regard this first apparatus of Barcroft, when fully set up, as justifying a good sized room and a well drilled team for its proper manipulation. Barcroft had only a sort of cubicle, for which memory suggests a floor-space not more than eight to ten feet square, cut off from the corner of a room, which was also a highway through the laboratory, by means of a wooden framework draped with green baize. Within this tabernacle he had to install, dismantle and clean his apparatus, and eventually to prepare his animal and perform the whole experiment by himself, with such casual help as he could obtain on occasion from an attendant, or, quite frequently, from passing acquaintances whom he could entice into his service. This procedure was facilitated by the fact that the working space was shut off only by curtains, through which J. B. could put out his head and call anybody in sight to assist him, without leaving hold of whatever his hands were manipulating. I remember being caught thus myself when dressed for a lawn-tennis party, and having my only pair of clean white trousers bespattered with blood. Such conditions may well have delayed progress and it is hardly surprising that, though Barcroft was all the time steadily at work, and characteristically ready to demonstrate the progress he was making with his method, and the difficulties he was encountering and overcoming, to every meeting at Cambridge of the Physiological Society, or to the International Congress there in 1898, it was not until he had been at it for about three years that he published the first detailed account of his method. And yet another year was necessary before he had obtained satisfactory determinations of the oxygen and carbon dioxide contents of the arterial blood, and of the venous blood leaving the salivary gland, at rest and in activity caused by chorda stimulation, and had been able

from these and other relevant data to calculate the oxygen absorbed and carbon dioxide yielded to the blood per minute, under the varying conditions. For, on the way, he had obtained additional data, which enabled him to clear up various anomalies, such as the surprisingly small differences between arterial and venous blood gases in the active gland, which Chauveau and Kaufmann had left unexplained. There was the fact, for example, that the actual volume of oxygen obtainable, per unit volume, from the bright blood flowing rapidly from the vein of the active gland, might be but little less, or even slightly greater, than that from the arterial blood entering it. Barcroft found that so much fluid had been lost from the blood during its passage, to form saliva and lymph, as to effect a material concentration of the corpuscles, and that, when due allowance was made for this and for the greatly increased rate of flow, the amount of oxygen taken from the blood by the active gland was actually three or four times as much as that taken by the resting gland in the same time. Determinations made only on blood samples, again, had appeared to show that the increase in the output of carbon dioxide with activity was less than that in the calculated uptake of oxygen ; but Barcroft showed that, when the carbon dioxide extractable from the saliva was added to the total, the output of carbon dioxide with activity was increased by an even greater multiple than the absorption of oxygen, so that the respiratory quotient of the gland rose with activity.

I do not think that it can be claimed that he had solved the problem which he tackled at Langley's behest ; in particular, his blood-gas determinations seem to have left the disputed nature of the secretory action of sympathetic impulses as much a puzzle as ever. Undoubtedly sympathetic stimulation caused secretion ; in the cat this was moderate in amount and accompanied even by a mild vasodilatation. There seemed no reason to doubt that energy was needed for its production ; yet there was no definite increase in the uptake of oxygen by the gland, while its output of carbon dioxide was even diminished —perhaps by the output of carbonate into the saliva. And there Barcroft seems to have left that aspect of the matter.

I do not think, however, that I shall be suspected of exaggeration if I suggest that this first research which Barcroft did, begun with only his own student knowledge at his back and using what help he could get, during the four years of its progress, from occasional consultations with men who had more experience of the methods he was using, is entitled to rank as one of the classics of physiology. Remember that, when it was done, there was no real beginning of detailed knowledge concerning the way in which oxidation provides energy for functional activity ; the work of Fletcher and Hopkins on surviving muscle was

not published until 1907, six years after Barcroft's third and final paper in the *Journal of Physiology* on the gaseous metabolism of the salivary gland. He had, in fact, made the first approach to a really critical and quantitative study of the gaseous exchange with the blood of any organ, and its variation with controllable activity ; he had had to use practically single-handed, and to adapt to his own purposes, essentially cumbrous methods ; and yet he had carried it through to success, with characteristically cheerful optimism. To recall further the state of knowledge with which he started, we may note that Volume 25 of the *Journal of Physiology*, in which Barcroft published the first two of his full papers, contained also the description by Haldane and Lorrain Smith of the determination of haemoglobin in a blood sample by direct, colorimetric comparison. Barcroft was able to use this method to measure the loss of fluid by the blood in passing through the active salivary gland. It also contained Haldane's second and fuller description, with a defence of its accuracy, of his ferricyanide method for determining the oxygen held by haemoglobin in blood.

Barcroft was clearly accepted at once by his seniors as a new recruit of the first rank to experimental physiology. A year after he finished his work on the salivary gland by use of the existing cumbrous methods for pumping off and analyzing the blood gases, we find him in co-operation with Haldane describing the method of measuring the oxygen and carbon dioxide in a small sample of blood by liberating them chemically, the oxygen with ferricyanide and the carbon dioxide with tartaric acid, in a small closed bottle connected with a water manometer. This, of course, immediately provided Barcroft with an immensely more convenient method for studying the gaseous metabolism of other glands and organs, and in the following years we find him engaged on such studies of the kidney with Brodie and of the pancreas with Starling. In the pancreas they encounter again one of the anomalies, in the absence of any conspicuous rise and sometimes even a fall in the output of carbon dioxide to the blood, when the gland is made to secrete rapidly by secretin, and they find the explanation for this in the large amount of sodium carbonate in the secreted juice. And then, as the last of the studies in that particular series, came a paper with Dixon on the gaseous metabolism of the mammalian heart, in 1907, in the same number of the *Journal of Physiology* as the paper by Fletcher and Hopkins on surviving voluntary muscle, to which Barcroft and Dixon were able to make passing reference in dealing with their own results. The application of the method was not by any means exhausted, and we find Barcroft returning, as late as 1912, to a study of the gaseous metabolism of the liver with L. E. Shore. But he was, for the time, diverted from it by a further simplification of the method of

analysing small samples of blood for their contents of gases, by his introduction of the now familiar differential manometric method. This, I suppose, with the modifications and elaborations of detail which others have added to give it a more general application, may be regarded as one of Barcroft's greatest gifts to experimental physiology and biochemistry. In one form or another, with his own, or more frequently Warburg's, name attached to it, it has become a part of the standard equipment of almost any laboratory of experimental biology. In Barcroft's own hands, and in those of a series of collaborators, it found its first use, however, in the next and, perhaps the most important of the phases of his research activities, in which he dealt with the oxygen dissociation curve of blood, and brought this for the first time into clear and intelligible relationship with the dissociation curve of free haemoglobin, by demonstrating the effects on the latter of the presence of different salts and of carbon dioxide. Hitherto he had been dealing with blood simply as a vehicle, and his attention had been concentrated on the tissue activities and the consequent abstraction of oxygen from and addition of carbon dioxide to the blood. Now he turned to the factors controlling the behaviour of the vehicle itself and favouring the uptake or outlet of oxygen by the blood. This study of the dissociation curves of blood and of haemoglobin was doubtless to play an important part in the development of Barcroft's interest in physiology at high altitudes and, more generally, in the respiratory functions of the blood itself. At that point, however, I feel sure that I ought to stand down in favour of Krogh, Douglas, Hill and the others who are to follow me. Before I do so, I should like to make just passing mention of one of Barcroft's minor interests, growing out of his work on the salivary gland. His attention was attracted by the functional vasodilatation so conspicuously seen in that gland with stimulation of the chorda tympani. In more than one publication he made a tentative case for regarding all such vasodilator effects as due to the release of rather vaguely described ' metabolites '. That of course was before there was any precise knowledge of natural vasodilators such as histamine, and long before there was talk of cholinergic nerves. Although such more recent evidence has not supported Barcroft's idea that substances released by the functional activity of a gland or other organ may be the *sole* cause of the concomitant vasodilatation, I do not think that such a secondary effect has yet been by any means excluded as a possible contributory factor.

I have not had time to do more than touch on some of the main features of Barcroft's activities in his first period. Apart from publications in the *Journal of Physiology*, which I have mentioned, there is a great deal of personal observation by himself to be found

scattered among the Reports, from 1905 to 1912, of a number of Investigation Committees of the British Association, of which he acted as Secretary, as well as in communications to the International Physiological Congresses of Cambridge, Brussels and Heidelberg. There was also a most valuable review by him, on work up to 1908 on blood-gas exchanges in different organs, in *Ergebnisse der Physiologie*, Vol. 7. All those who are to follow me had to some extent the privilege, which I never enjoyed, of working at one time or another in close association with Barcroft and directly sharing his scientific activities. Most of the work of which I have spoken was done in fact after 1900, when I left Cambridge, and I saw him thereafter only occasionally.

There is a record as early as the beginning of 1895, in his second undergraduate year, of Barcroft's first public scientific utterance; in his Christmas vacation he addressed the Natural History Society of Belfast on surface tension. At that period he was enterprising in his scientific interests beyond the range of anything which could have been regarded as his special subject. It was he, for example, who borrowed a Crookes's tube from the Cavendish Laboratory almost as soon as the news of Röntgen's discovery reached Cambridge, and enabled a meeting of the Natural Science Club in his rooms to take what may have been the first x-ray photograph produced in this country. In later years, of course, he had to bring his interests to a focus, or a succession of such, but he never lost that air of youthful enthusiasm, the attitude which regarded research as an amusing adventure. In many ways he seemed to have the ideal research temperament, not over-elated by success or cast down by lack of it, or put out of countenance by the unexpected. He never lost his eagerness, but always tempered it with a humorous equanimity. And I know that you will all share my feeling of what physiology owed, in Cambridge, in Britain and in all the world, and what we who loved him owed individually to his buoyant comradeship, and to the solid goodness, the loyalty, the generosity and the unpretentious friendliness, which made it so easy for many to work with him and to follow where he led.

Professor A. S. Krogh

I HAVE only a few quite personal memories to relate about my old friend Barcroft. I met Barcroft for the first time at the International Congress of Physiology in Heidelberg in 1907 and that was, I believe,

the first congress for him and for me. It was a small congress ; so far as I know there were only about 400 people present, and it was a congress held in the most friendly spirit, which I am afraid cannot be said of all later congresses. I remember that on the second day of the congress we had a delightful river trip on the Neckar and in the evening we all gathered together in the Stadthalle. Then it was suggested that representatives of all the nations present should speak in their own language in praise of Heidelberg, and that was done. Barcroft presented several important papers on blood flow and metabolism of several organs, but what did create a sensation was his demonstration of the manometer for measuring the blood gases. It attracted the special attention of Zuntz, the professor of physiology in the Berlin Veterinary High School. He looked upon it with very great interest, and I remember his voice very clearly : ' Das ist ja für die Kliniker geradezu gefundenes Fressen.' I am afraid Zuntz was not right there ; it was not nearly simple enough for the clinic. Apart from that this manometer became of great significance in physiology and biochemistry by itself and through further developments.

Barcroft and I met again a few days later in Zuntz's laboratory in Berlin. We had to agree that Zuntz's methods were clumsy, but it could not be denied that he had made an outstanding contribution to the physiology of human work and that especially his book under the curious title *Höhenklima und Bergwanderungen*, which no one would suspect was a scientific book, was a beginning of the specific study of high altitudes and at the same time of the scientific study of muscular work. I believe it was on that occasion that the idea came of the high altitude expedition to Teneriffe which was undertaken, I think, in 1909–10 in which Barcroft took part with Zuntz. Barcroft told me somewhat later that this expedition was to a certain extent camouflage for attempts on the part of the German government to establish strategic points for use in war, but we were agreed that Zuntz could not have been a party to such devices. Zuntz was an unusually lovable man, but he was kept down severely by Rubner, who was the typical German ' Geheimrat '. High altitude work was taken up in Denmark a few years later by Hasselbalch and Lindhard and we had constructed in Copenhagen the first really large chamber for low pressure work. At that time we were in constant correspondence with Barcroft, and I remember a letter from Barcroft to my friend Lindhard who spent about a month in this chamber to study acclimatization to high altitudes. The address was given as Monte Rosa, Rosenvanget, Copenhagen, because the pressure in the chamber corresponded almost exactly to the pressure at Monte Rosa in the Alps. I am not going to try and characterize Barcroft's scientific work. That will be done much

better by others, but I must refer to the book of his which I consider his most important contribution to physiology, *The Architecture of Physiological Function*. The title is very characteristic of Barcroft, but perhaps not the best title to get people to read the book. I would suggest that it is a book which gives an integration of physiology of such a kind that it ought to be read by everybody who is going into experimental work in physiology. It gives the general ideas which cannot be obtained from any other book in existence.

Professor C. G. Douglas

PROFESSOR ROUGHTON has asked me to say something of my recollections of Barcroft between 1910 and the end of the First World War. I had, of course, known Barcroft before that date, for his brilliant elucidation of the problem of oxyhaemoglobin and its dissociation had a direct and fundamental bearing on the physiology of respiration with which Oxford had been so deeply concerned since the early years of this century. But it was not until 1910 that I became closely acquainted with him, for it was to his good offices that I owed an invitation to accompany him on an expedition to the Peak of Teneriffe, under the auspices of the International Tuberculosis Conference, to study the effects on man of exposure to high altitudes. This expedition, under the leadership of Professor Pannwitz of Berlin, included Zuntz and Neuberg (Berlin), Durig and von Schrötter (Vienna), and Mascart (Lyons), the French astronomer who was to observe Halley's comet.

Barcroft had already done his fundamental work on oxyhaemoglobin dissociation, and he now had the opportunity of testing whether the dissociation curve was modified in any way so as to assist in the adaptation of the respiration to the effects of high altitude and the resultant diminution of oxygen pressure in the atmosphere. He had already with great ingenuity modified his original differential blood-gas apparatus so that he could obtain accurate results using only one tenth of a cubic centimetre of blood, and when the time came the whole of his apparatus was very compact and of small bulk so that he could face transport difficulties with equanimity.

As a preliminary I visited Cambridge. As we left King's College after lunch I said to Barcroft: 'Now, I suppose, we go to the laboratory.' 'No', replied Barcroft, 'we visit the surgeon.' 'Why?' said I,

being somewhat mystified ; 'I want your blood', was the answer. And so I was duly venesected and then we walked to the laboratory, Barcroft carrying a small basin of my blood which he resolutely stirred with a bunch of feathers in order to defibrinate it. He thus began his investigation of the properties of my blood.

In Teneriffe we all acted as Barcroft's subjects. His experiments were made at sea-level at Orotava, at the German meteorological station at an altitude of 7,000 feet in the Cañadas or great crater of Teneriffe, from the centre of which the actual volcanic cone of the Peak arises, and at the Alta Vista hut at an altitude of 10,700 feet, 1,500 feet below the summit of the Peak. At the outset the experiments were nearly frustrated by the fact that the nitrogen which he had brought with him actually contained a small trace of carbon monoxide as an impurity, but the difficulty was overcome by absorbing all the oxygen from atmospheric air by means of a spare phosphorus pipette which had been brought by Durig for use with his gas analysis apparatus. At the Alta Vista hut Barcroft was slightly affected by the altitude which diminished his power of mental concentration and impaired his usual alertness and vigour, so he soon went back to the Cañadas observatory and blood samples were sent down to him for analysis.

The results which Barcroft obtained were clear-cut and showed that although the oxyhaemoglobin dissociation curve was shifted to the right if the determinations were made in the presence of a carbon dioxide pressure identical with that in the subject's alveolar air at sea-level, the curve was substantially unaltered if it was determined at the lowered carbon dioxide pressure characteristic of the same subject at the particular altitude at which the blood samples were taken.

Now at this date we were all, I think, under the impression that the hyperpnoea and reduced alveolar carbon dioxide pressure in the resting subject at a high altitude were due to an acidosis caused by the accumulation of excess lactic acid in the blood in consequence of the diminished concentration of oxygen in the atmosphere. Barcroft, too, shared this view, and on his return to England he showed how the facts which he had elicited in Teneriffe could be imitated by the addition of appropriate concentrations of lactic acid to human blood.

A year later, in 1911, Barcroft continued this work during an expedition in company with Camis, Mathison, Roberts and Ryffel to Monte Rosa, observations being made both at Col d'Olen (9,500 feet, barometer 542 millimetres) and the summit (15,000 feet, barometer 440 millimetres). The concentration of lactic acid required to cause any given shift of the oxyhaemoglobin dissociation curve of normal blood had already been ascertained, but when on this expedition the actual lactic acid concentration in the blood at a high altitude was

determined, it was found to be insufficient to account for the behaviour of the oxyhaemoglobin dissociation curve at that altitude. To explain this the assumption had to be made either that some other abnormal acid was present in the blood or that some new adjustment of the usual acid and basic radicals had been brought about by the kidneys.

While Barcroft and his colleagues were working on Monte Rosa another expedition under the leadership of Haldane, of which I was a member, was also studying acclimatization to high altitudes on Pike's Peak, U.S.A. While we were able in a few experiments to corroborate Barcroft's main conclusions in the Teneriffe expedition about the oxyhaemoglobin dissociation curve, we, too, found ourselves in difficulty in explaining the behaviour of the respiration in terms of the current theory based on excess lactic acid production.

In both instances it was not the facts which were wrong but the interpretation which was faulty. The analysis of the lactic acid concentration in the blood in the Monte Rosa expedition proved conclusively that the original idea about an acidosis due solely to lactic acid could no longer be maintained, and paved the way for a more rational explanation which was to be suggested after the end of the First World War.

Although the full report of the Monte Rosa expedition was not published until 1914, this did not check Barcroft's active research on other lines, and a succession of papers continued to appear on the gaseous metabolism of different organs and on further aspects of the dissociation of oxyhaemoglobin, as well as some studies in association with Lewis and others on the possible influence of acidosis in clinical cases of cardio-renal disease. In addition he published in 1914 the first edition of his great book *The Respiratory Function of the Blood* in which he reviewed the existing knowledge to which he had so signally contributed.

But the First World War had by now broken out and before long Barcroft was to be summoned to undertake a very different type of work. With the introduction of gas warfare by the Germans in 1915 various advisory committees were appointed to deal with the new situation, and a little later on Barcroft was assisting these committees by experimental work at Cambridge and at Porton, where a Government Experimental Station was established early in 1916. In January 1917 he was requested to take up duties as physiologist at Porton, and from that date until the end of the war he was resident at Porton and in charge of the Physiological Laboratory there. In April 1917 he was joined by Peters, who was brought back from France where he had been serving with distinction, and later by Boycott, Shaw Dunn and G. H. Hunt. Under Barcroft's inspired leadership this laboratory did an immense amount of experimental work, much of it having a direct

bearing on the medical aspects of this new form of warfare. Naturally much attention was given at first to the effects of such gases as chlorine, the first gas to be used for offensive purposes, which is highly irritant to the lungs and provokes acute pulmonary oedema. But in addition many other poisonous gases, which might or actually did serve as effective offensive agents, had to be investigated.

Let me quote but one instance to show the strength of Barcroft's scientific convictions and his personal courage. Hydrocyanic acid was naturally suggested as an offensive agent, but although it was actually employed in the field in the earlier days of gas warfare its effectiveness was uncertain. Tests on different animals of the toxicity by inhalation of this poison showed that there was a surprising difference of susceptibility in different species, the dog being particularly sensitive and the guinea-pig, goat and monkey being very much less sensitive. If, therefore, an assessment of the toxicity were to be based solely on experiments on a highly susceptible animal a false impression of the toxicity to man might be gained. Barcroft, feeling convinced that man must be much less susceptible than the dog, decided to settle this point. He therefore went, unprotected by any respirator, into a respiration chamber with a twelve-kilogram dog and released into the air in the chamber enough gaseous hydrocyanic acid to give a concentration of about one part in two thousand. In just over one minute the dog was unconscious, and at the end of one and a half minutes was in convulsions and appeared to be in extremis, when Barcroft left the chamber having felt neither breathlessness nor any symptoms. Directly afterwards the dog was pulled out apparently dead, but although the ' corpse ' was put aside for burial it was found walking about the next morning. But Barcroft had proved his point and shown conclusively that the toxicity of hydrocyanic acid to man was not nearly so great as is commonly supposed, and he thus prevented further futile efforts to use hydrocyanic acid as a chemical warfare agent.

I was myself serving in France in the R.A.M.C. during all this period, being seconded to the Directorate of Gas Services as Physiological Adviser, and Barcroft visited us a number of times for discussions about current problems or to take part in inter-Allied conferences in Paris on gas warfare. I have very happy memories of those visits, for the vigour and activity of his mind, his keen anxiety to appreciate the problems as we saw them in the field, the acuteness of his suggestions and his readiness at all times to help were a real stimulus to us all.

One recollection I have, of a rather different kind, which stands out in my memory. On one of his earlier visits I was ordered to show him something of conditions nearer the line. So, among other places, I took him to an advanced dressing station in the vicinity of Loos. This

was below ground but close to a cross-roads of dubious reputation since the range was accurately known to the Germans. When I got him there he insisted on standing in the middle of the cross-roads inquiring about points of interest around, while I, with one eye on German shells falling on some ruins further up the road and the other on the entrance to the dressing station, was trying to reply to his questions and at the same time to calculate our chances of reaching safety if the Germans should lift the range of their guns. Perhaps the fact that Barcroft had chosen on this occasion to wear, of all things, a bowler hat may have been our salvation, for the sight of a man standing calmly in a bowler hat at a point where no ordinary mortal would voluntarily linger may temporarily have paralyzed the Germans owing to the uncertainty and consternation created by such an apparition.

There you have an instance of Barcroft's calmness and my agitation, but my uneasiness was not unnatural for, after all, my orders were to return Barcroft to our headquarters safe and sound. And this leads me to re-echo some words of Sir Henry Dale in which he alluded to Barcroft's imperturbability in adverse or difficult circumstances. When things seemed likely to go amiss and others might well have been daunted, or when tempers were getting frayed, he merely tackled the difficulties and set them straight with calmness and a good humour which never failed him. Speaking for myself, I can only say this : that to have known Barcroft, to have worked with him, and above all to have been numbered among his friends, are privileges which I count very high indeed.

Professor A. V. Hill

We have had such admirable accounts of J. B.'s scientific activities that it will probably be best if I refer shortly to the more informal, familiar and intimate aspects of the friendship which existed between all of us and him. Some of my remarks might appear almost impudent —but Lady Barcroft has given me *carte blanche* to say what I like.

J. B. belonged to a unique class, a class which contains, far more than in proportion to its numbers, so many of the best of human kind. I refer to Irishmen educated in England. To this was added a deep affection for the sea, for sea-faring and adventurous folk, and we have heard from Douglas of that adventurousness which was natural to J. B.

I well remember taking a photograph on board Alex Forbes' schooner, the *Black Duck*, off the coast of Maine after the Physiological Congress in 1929 which shows J. B. in his element. With that in mind I should like to quote a few lines, which I am sure you know very well, from the preface of the first edition of *The Respiratory Function of the Blood*:—' At one time, which seems too long ago, most of my leisure was spent in boats. In them I learned what little I know of research, not of technique or of physiology, but of the qualities essential to those who would venture beyond the visible horizon. The story of my physiological " ventures " will be found in the following pages. Sometimes I have sailed single handed, sometimes I have been one of a crew, sometimes I have sent the ship's boat on some expedition without me. I should like to have called the book what it frankly is—a log.' He goes on to speak of his friends. ' The pleasantest memories of a cruise are those of the men with whom one has sailed. The debt which I owe to my colleagues, whether older or younger than myself, will be evident enough to any reader of the book ; it leaves me well-nigh bankrupt—a condition well known to most sailors.' All of us who have adventured with J. B., whether in boats or in research, beyond the visible horizon, will recognize that the bankruptcy of which he speaks is mutual ; and that J. B. should be bankrupt was inevitable in view of the extraordinary generosity which all of us have experienced who worked with him.

My first memory of J. B. was about 1908 or 1909. I had read of the work which he had done, or was doing, on blood and on the salivary gland and I remember asking him—I was a little astonished to find that this work, already so famous, was done by one so friendly and so young—I remember asking him whether he really was the author of those works and receiving his smiling confirmation. Thereafter I saw him continually in that old laboratory behind the green baize curtain, to which Sir Henry Dale referred, and I remember another accident that befell there, in addition to the one to Sir Henry Dale's trousers. This accident happened to an apparatus laboriously built up. Carelessness by another brought it all crashing to the floor. Instead of using sailor's language J. B. looked at it quietly and said : ' Oh, well, we'll just put it up again.' That was characteristic of the patience of his work. I witnessed in those days that unique capacity of his, to which others have referred, for getting other people on to a useful job of work—a capacity which remained with him all his life and was found in whatever he undertook. I well remember outside that green baize curtain various of his colleagues and pupils shaking blood gas apparatus endlessly in baths in the chemical laboratory just down the passage. The one I remember best was Camis, because of his short legs : these

required that he should stand on a stool by the bath. When I think of blood gas apparatus, the picture of Camis comes back to me.

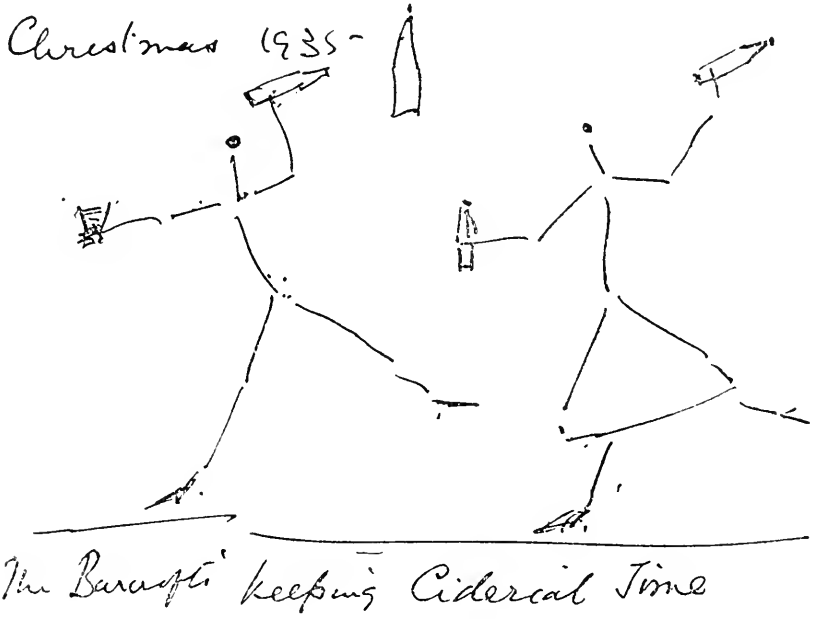
About 1912 I went to Carlingford to sail with him in a hired boat. Lady Barcroft may remember it. I think she was with us one day and I know that Henry was, when the weather turned bad on us and much of the tackle gave way. It had to be put right, but J. B. was a very resourceful sailor. We all know how much he was loved and admired in America. I crossed the Atlantic with him three times and witnessed another part of his planned economy—for he was also a great planner, inspired by Lady Barcroft—how he brought his oldest clothes with him, so that he might throw them out through the port-hole and thus save the trouble of collecting them from the laundry.

There is a picture in existence of J. B. many years ago smoking a pipe—I think at the Physiological Congress at Cambridge. Not many people will remember his smoking, but on those journeys to America he used to smoke one cigar after dinner in the evening.

One of the privileges of being an officer of the Royal Society—and Sir Henry Dale will confirm this—is the kind and unfailing help one gets from all the Fellows. For the last three years of his life J. B. was Chairman of the Physiological Committee of the Royal Society : he was extremely helpful to all the officers, especially the Biological Secretary. The work of the chairman of such a committee can be very heavy, particularly in connexion with the elections, and J. B. never spared any effort to help the Society and his colleagues there. His loyalty indeed to his friends and his loyalty to the institutions of any kind with which he was connected were among the most charming characteristics of his nature. I remember him—and the Provost of King's may remember also—proposing the toast ' Floreat Etona ' at Founder's Feast one year ; the theme of that speech was that each of us has his own Eton, his own loyalties and affections, that the toast of ' Floreat Etona ' really meant a toast to all those individual loyalties.

J. B. was quick, extraordinarily quick, in generous and effective repartee, never sarcastic or unkind. I corrected the proofs of the first edition of his book for him, and conceived my duty to J. B. to outweigh my obligation to the public, who might otherwise have been highly delighted had I left in some of the gems which occurred in the original: such phrases as ' The muscle is not a steam engine in which combustion takes place in the boiler ' and ' The chief error in Peters' experiments was the accurate measurement of 2 cc of blood.' When I pointed these out as being more suitable to conversation than to a learned treatise, he at once remarked that the chief virtue of the Irish bull was that it was always pregnant ; but he accepted my corrections. The humorous and tactful phrase is illustrated in a sentence that occurs

in the paper in the *Philosophical Transactions* on the Peru expedition, referring to the mental effects of high altitudes. He remarks : ' Meakins had a feeling akin to what he thought would be produced in him by excess of alcohol.' You can see it all revolving in J. B.'s mind. What Meakins had really said (in J. B.'s words) was that it made him feel squiffy ; but J. B. did not like to infer that Meakins had any personal experience of that condition. Of his quick and friendly wit, no better example perhaps can be given than his acknowledgment of a few bottles of cider I sent him in the winter of 1935.



In a personal record of J. B. those who knew and loved him would not wish to recall him—indeed they could not—without also recalling Lady Barcroft. As I wrote in the *Lancet* fifteen months ago, the laughter which like a nosegay decorated their joint lives made them the most perfect partners and the most perfect hosts. They realized that the most serious things can often be better said and done gaily and they said and did them so. Lady Barcroft is joint creditor with J. B. in our bankruptcy. I think he would like us, and I know she would like us, in all seriousness, to remember him not only with love but with gaiety.

Professor R. A. Peters

MUCH has been said so well in the preceding tributes that all I can possibly do is to fill in a few gaps, which may recall one or two points others have forgotten. I first came really into contact with Barcroft when I joined the advanced class in physiology for Part II of the Natural Sciences Tripos ; this was in 1910, and I knew him as a most inspiring teacher. I well remember now the interest of his lectures on controversies connected with the sub-maxillary gland, and they were always lectures which made us think. One of the subjects to which he devoted a great deal of thought himself was the question, so controversial then, of the secretion of oxygen in the lung. As has been said by others, he was first and foremost a biologist, and I feel that it was always a great disappointment to him that he could not get evidence for the secretion of oxygen himself ; he would have liked this. I spent two years over Part II, and that was how I came to go behind the well-known green baize curtain, which has been mentioned. Oddly enough I cannot remember positively how I came to start work on the specific oxygen capacity of the blood. Having two years to spend upon Part II, I suppose that it was considered that I could spare a little time for research ; so far as I remember I had even started to make a few observations with Hopkins on creatinine. But how that became diverted into research on blood is a mystery. I think Barcroft must have come along one day and said : ' Peters, can you help me ? ' The green baize curtain was very close to the advanced class room.

In that first chapter of his book to which Professor Hill has referred, altogether too much was devoted to myself. It was Barcroft's plan that the relation between iron and oxygen in haemoglobin should be settled chemically and that I tried to do for him. He was behind the work all the time and taught me to work with differential apparatus, with which I am still working in a different form. His capacity for knowing just when to help a research worker and when to leave him alone was extraordinary. He was there when you wanted him but when you wanted to be by yourself, he knew how to leave you. This rare quality is an important one. I can also recall coming back to the Physiological Laboratory in Cambridge after World War I ; there was someone trying to do an operation which I could do, taught by Barcroft, and I was getting irritated because I felt that I could do it better. He beckoned me out of the room and closed the door saying :

'No, Peters ; you must leave him because, you see, he has got to learn it for himself.'

When I got behind that green baize curtain, there was H. Hartridge working with four blood gas pumps at once, like a gnome. There were also Dr. Higgins, Dr. Verzar and others ; it was a heroic period for that laboratory. Among them all certainly one of the most active figures was Barcroft. Looking back on it now, when I have a laboratory of my own to direct, I find it difficult to know how he really carried through everything. I believe as one of his duties he always had to prepare the demonstration for Langley at the end of the Professor's lecture, as well as looking after all the research.

Then there was the expedition to Carlingford. There were two or three of us, including Dr. Ryffel of Guy's, and we were interested in how much lactic acid we could turn out in our urines, and what happened to the blood when you walked one or two thousand feet up a mountain. This expedition was great fun, as was anything you did with Barcroft. We worked in a small hotel bathroom, estimating alveolar air and so on ; he had an astonishing capacity for carrying things through under very crude conditions, and, as someone else has mentioned, he arrived at a final answer in that way. After one year's interlude with Professor Hill in 1912, I came back and worked with Barcroft again on the question of the *p*H of the blood. Characteristically he wanted to be ahead, and set me down to this estimation, making admirable suggestions upon the *p*H measurements. I suppose that we were among the earlier workers (it was 1913) to get estimations of blood *p*H in relation to carbon dioxide. Nowadays, I think it quite astonishing that we should have reached results as close as they were to some of the modern determinations.

During the latter part of World War I there was the Porton period, to which Professor Douglas has referred. I was intimately concerned with that, because I acted as Adjutant there through the period of eighteen or nineteen months to the end of the war, and therefore saw the whole Department of Physiology grow up under Barcroft. There is no doubt that he was the unquestioned leader of all the work in that Department. We had some little difficulties as he was the only civilian in an entirely service establishment ; but somehow we managed to build up a kind of aura round him as the mysterious 'Professor Barcroft', and he was accepted as a superior being. He was always making good suggestions, and was really behind the development of Shaw Dunn as an experimental pathologist. When we had done the work he collected the results and welded them into something worth while. During this time, Barcroft was instrumental in inviting down the two United States officers, Wright Wilson and Samuel Gold-

schmidt, to prove their point about the value of bleeding in gas poisoning, which was at that time so controversial. I can still see Barcroft trying to find out whether our tobacco smoke (a liquid particle) would go through the respirator then in use. It was at the time the irritant smokes were brought out by the enemy ; the smoke did go through that respirator. It was great fun acting as Adjutant, and one thing has not been said about the experiment with hydrocyanic acid ; he did it after we had all left one night, with the help of a corporal whom I had brought from the 60th Rifles, called Carlile, who was a grand fellow and had been a stretcher bearer. I think that Barcroft must have thought we might have tried to stop him. We were just a little upset that he had done it in this way, but quite understood why.

There were other things about him that were very characteristic—sometimes a little difficult. The head of the station was very orderly—Barcroft not always so. Sometimes the files from the Physiological Department would mysteriously disappear ; they would be off in the bag when he was examining in Ireland. I would be rung up by headquarters and asked for papers, and the file was not there. One had to hedge until the files came back, which they always did in the end.

As far as capacity for committee work was concerned I can remember him a year before World War II coming into my laboratory. At that time he was trying to get people to say they would be reserved for work in World War II. He told me incidentally that he was devoting much of his time to getting shells and fuses put together, because apparently things had got behind. This direct attention to the main point, whatever this might be, was a well marked characteristic of his mental elasticity.

Latterly there was his interest in the Nutrition Society, which owes an enormous amount to his drive and enterprise.

He disliked working in busy fields. This was connected with his originality ; if any subject got really busy, I think that he enjoyed going off into something new. I do not think that those of us who had the privilege of knowing him all this time, probably had anyone to whom they could go and be so certain that they would get honest advice. You knew that if you went to him and asked for an opinion, he would give his view whether you agreed with it or not. You always knew one thing also, that what you said to him would never get away. This was a very important point in having Barcroft as a friend. Then, of course, there was his capacity for keeping whole audiences intensely amused. I can remember one incident in Oxford. It must have been in 1927–28, when he came and talked to us about haemoglobin, and the ' span ' in Ångström units. I had the Regius Professor of Medicine, Sir Archibald Garrod, next to me. As happens in an after-dinner society

the whole audience wanted to be amused—and they were intensely amused at the stories which poured out. Sir Archibald at times seemed to think it was perhaps a little too much and then immediately afterwards there would be another story which set the whole room shaking, and I felt Garrod's shoulders shaking next to me !

I am afraid that these are only reminiscences. It is a little difficult to select things to say about someone of whom one has been so very fond.

G. S. Adair

I HAVE been asked to deal with Sir Joseph Barcroft's work on the oxygen dissociation curve, the expression of the degree of oxygenation of haemoglobin in equilibrium with gas mixtures containing known proportions of oxygen. This curve has been of fundamental importance in physiological and physicochemical investigations of haemoglobin, and recent work on methaemoglobinaemia has indicated one of its clinical applications.

This subject, the oxygen dissociation curve, provides a good example of Sir Joseph's quite remarkable talents as a writer as well as a research worker. His publications record new and important advances and at the same time convey a most vivid impression of the progress of the work.

Sir Joseph's first work on the dissociation curve was partly inspired by his interest in the oxygen tension in the blood in the capillaries. At that time Professor Krogh's direct determinations of the oxygen tension of blood were not published, and both Bohr in Copenhagen and Haldane at Oxford had concluded that oxygen was secreted into the blood in the lung capillaries. If the shape of the oxygen dissociation curve is accurately known, and if it can be assumed that the percentage oxygenation is a known function of oxygen tension, it should be possible to calculate the oxygen tension of blood from measurements of oxygen content and oxygen capacity, but when Sir Joseph began this work, the shape of the dissociation curve was uncertain, and, as he states in the first edition of his book *The Respiratory Function of the Blood*, ' Zuntz and Loewy had made the important observation that apparently no two samples of blood, whether from man or beast, could be relied upon to have the same dissociation curve.'

Much of the earlier work on the dissociation curve of blood had been hampered because the methods were elaborate and time-

consuming, and required large volumes of blood. Barcroft utilized the ferricyanide reaction, which had just been studied quantitatively by J. S. Haldane, to determine the oxygen capacity of blood samples, and he designed apparatus for blood gas analysis which required only small volumes of blood, dispensing with the elaborate vacuum pumps which had previously been necessary. For work on the dissociation curve, his differential manometer was the most elegant, and could be adapted for volumes of blood as small as a tenth of a cubic centimetre. This manometer has been very extensively used in many types of research, and is familiar to almost all biochemists.

With the differential apparatus it was possible to determine a fair number of points on the dissociation curve in a single day, a factor of great importance, since the oxygen capacity of blood and the properties of haemoglobin undergo changes with time.

In addition, he designed an apparatus for the equilibration of blood with gas mixtures. This is usually known as the Barcroft tonometer, and is essentially the same as that in general use at the present time. In this apparatus a small volume of blood was exposed in a thin film to a relatively large volume of gas, and equilibrium was reached with greater speed and certainty that had been possible with earlier methods.

To quote Barcroft's own words, when describing his early work with Camis: ' Our very early efforts seemed to augur speedy success in the construction of a uniform dissociation curve for the blood of various animals. When we came to the blood of man, however, we could never make the dissociation curve agree with that of the cat or rabbit. It was clear then that we had found our way into the morass in which our predecessors had floundered so hopelessly and our newer and more certain methods instead of saving us from their embarrassments only made the uncertainty of our position more certain.' Believing that experimental conditions would thereby be simplified, Barcroft decided to work with haemoglobin solutions instead of with whole blood, but he found more variable dissociation curves with haemoglobin than with blood. To obtain concentrated solutions of haemoglobin they had found it advisable to add ammonium carbonate, but on one occasion, whether by accident or design he could not remember, the ammonium carbonate was omitted. The curve obtained was unlike those determined in the presence of the salt and was reminiscent of curves which had previously been described by Bohr. This experiment suggested that the form of the curve was influenced by the salts present, and Barcroft therefore determined dissociation curves of haemoglobin dissolved in a salt solution based on an analysis of human red blood corpuscles, and, as he stated in his book: ' Great was our excitement and

delight when we found that point after point on the curve for human blood lay also on the curve of the haemoglobin solution.' Later work of Barcroft and Peters showed that variations of hydrogen ion concentration had much greater effects on the position of the curve than variations of salt content, and in the second edition of his book Barcroft said: 'I never know whether it was more fortunate or unfortunate that the observation [*i.e.* the effect of salts] was made at so early a stage. It was unfortunate, because at that time the effect of hydrogen ion concentration was not appreciated and therefore not controlled; moreover, the distilled water solution itself was not so free from salts as it would have been had the investigation been made a year later. The fortunate circumstance, however, was that had the discovery not been made at the time it was, it might have been long postponed, for the whole effect of salts on the dissociation curve might have been attributed to changes in the hydrogen ion concentration.'

This effect of salts suggested to Barcroft and Roberts that an investigation should be made of the dissociation curve of haemoglobin, which had been dialyzed against distilled water. The results were remarkable. The curve changed from a sigmoid type to one more like the rectangular hyperbola predicted by the Law of Mass Action, assuming that the haemoglobin molecule contains one atom of iron. Although this effect has now been known for almost forty years, and accounts of investigations of it are still being published, a complete explanation is not yet available. The first and most brilliant suggestion was due to A. V. Hill who was then working with Barcroft. He suggested that when dissolved in pure water, haemoglobin might exist in single molecules and in the presence of salts it might aggregate to form a polymer with the average number $n = 2{\cdot}5$. Before 1914 this aggregation theory was tested in many ways by Barcroft and his colleagues and the results obtained appeared to agree with the requirements of the theory. After the First World War, when I had the privilege of working with Barcroft, we planned to determine the value of n by independent methods on the same haemoglobin solution, namely by determinations of oxygen dissociation curves and measurements of osmotic pressure. As in so many investigations, early results seemed most encouraging, but more detailed studies did not confirm the theory. In dilute solutions a value of $n = 4$ was obtained by osmotic pressure measurements. About the same time Barcroft planned a systematic investigation of oxygen and carbon dioxide contents of the blood of a series of normal human subjects and a comparative study of the haemoglobins of the same subjects. In this work the most highly purified haemoglobins did not give hyperbolic curves.

Barcroft carried out work on the dissociation curve in connection with his investigations of respiration at high altitudes, at Teneriffe, at Monte Rosa and in the Andes. His work at sea level had shown that exercise caused a change of position of the dissociation curve, and this effect was found to be more pronounced at high altitudes.

The small and simple apparatus Barcroft had designed was, of course, a great convenience for work at the high altitudes, and his pioneer investigations in this field are of especial interest.

Barcroft was associated with the work published by Roughton and his colleagues on the influence of temperature on the dissociation curve. Then, as part of a very extensive contribution to foetal physiology, he organized a comparative investigation of the dissociation curves of foetal and maternal bloods and haemoglobins of a number of animals.

In this account I have dealt chiefly with the pioneer work of Barcroft. This represents only a part of his contributions to work on the dissociation curve. Through the work of his pupils carried out partly in his laboratory and continued in many different parts of the world, he was responsible for a very large part of modern knowledge of this subject.

Professor F. J. W. Roughton

I AM the last and youngest contributor to these tributes and therefore can hardly be expected to have known Joseph Barcroft as long as Sir Henry Dale. My own intimate friendship with J. B. goes back only as far as 1920, and thus covers little more than the last third of his life. But, as has been previously said, J. B. changed but little during his life, so that my briefer contact with him is not such a disadvantage as it would be in the case of some other men. I do, however, have one advantage which is not shared by any of his other friends, old or young. It was given to me to spend most of the last morning of his life with him in this very laboratory. Never have I known him in better form, fuller of interest in the past, present and future, fuller indeed of good fun, than he was on that memorable first day of Spring, 1947.

First, however, let us go back to 1919 and 1920. Those of us who were beginning to specialize in physiology at that time had many advantages. For me, among the most outstanding were those two great books—Bayliss' *Principles of General Physiology* and Barcroft's *Respiratory Function of the Blood.* Both had been published some five

years before, but since so little academic physiology was done in World War I, neither were seriously out of date in their subject matter and in their spirit both, I feel, are in many ways fully alive to-day. Bayliss ranged far and wide over physics, chemistry and biology ; his reading was tremendous and might well have overpowered any other man. Yet every one of his chapters was crammed, not only with interesting and varied data, but—more important—with critical opinions and fascinating ideas which linked them all together. It was no wonder that in the early twenties Bayliss Clubs sprang up in different parts of the United States for discussing and working out more fully the ideas he strewed so lavishly through his book.

Joseph Barcroft's *Respiratory Function of the Blood* was, in a different way, unique and equally priceless. Instead of ranging over many subjects and the work of many laboratories all over the world, Barcroft concentrated on one particular field, limiting himself very largely to the adventures of his friends and himself therein. As he says in that wonderful preface of his : ' I should like to have called the book, what it frankly is—a log ; did not such a title involve an air of flippancy quite out of place in the description of the serious work of a man's life.' The reader is indeed let into the secret of how ideas come to a great investigator, how he tries them out at the laboratory bench, how he prospers for a while, then retires discomfited by inexplicable rebuffs of nature, gets lost for a time in a mist and a morass, then suddenly sees a new light, makes his way out of the bog and the fog and emerges triumphant on to firm and higher ground, from which he sees the older knowledge with new eyes. It is the process which happens over and over again in scientific research, but the details of it are, and probably have to be, pruned so severely out of ordinary scientific papers, that from hundreds of these one could learn less about how actually to do scientific research than one did from one chapter of Barcroft's book. It was no wonder that almost every Cambridge physiologist who began research between 1905 and 1915, did his first job of work under Barcroft —I remember Professor Adrian telling me in 1919 that he was about the only exception to this rule. I remember, also, how one of J. B.'s first American associates after World War I, the late Cecil D. Murray, bounced into the lab. here with the words ' Mr. Barcroft, Lawrence J. Henderson tells me you've written a book which has enough unsolved problems in it to last for twenty-five years. Mr. Barcroft, I want you to know that that's why I've come to work with you.'

My own first research contact with Barcroft in the Spring of 1920 is only one of probably hundreds of examples of the way in which he delighted to help young scientists forward, but to me it is naturally a vivid and precious one. I had, at that time, to read a paper to the

Cambridge University Natural Science Club to which J. B. had demonstrated x-rays twenty years or so earlier. I had lately been thrilled to the marrow by the *Respiratory Function of the Blood* and so I chose one of its subjects—namely the acquisition of oxygen by the blood in the lung. The battle of Diffusion versus Secretion was then in full cry, the work of Copenhagen (as represented by Krogh) and of Cambridge (Hartridge) being pro Diffusion, whilst that of Oxford (Haldane and Douglas) was in favour of Secretion. Towards the end of World War I direct arterial puncture had come on the scene, and to Barcroft's concrete mind, seemed to offer the chance of settling the problem more conclusively in the case of man than was possible with what he considered the more indirect carbon monoxide methods hitherto used. In February 1920, as many will remember, he shut himself up for ten days in the Glass Box which is still upstairs in the Physiological laboratory, and with various persons on guard, including a series of undergraduates from his own College, King's, he had the oxygen percentage in the air of the box gradually reduced until he reached an equivalent altitude of 14,000–15,000 feet. The oxygen pressure in his lung alveolar air and his arterial blood were then compared, both at rest and at work, with a view to answering the two questions he posed at the beginning of his paper. *1.* Is the alveolar oxygen pressure greater than the arterial oxygen pressure ? *2.* If the answer to *1* is yes, is the gradient great enough for diffusion to account for the quantity of oxygen which is observed to pass into the blood in a given time ?

The answer to *1* was decisive if the principle of the arterial puncture method be accepted. In regard to *2*, more in a moment.

A month or two later I was busy reading up the literature for my paper at the end of May 1920, and was eagerly awaiting the details of the Glass Box experiment, but not daring to go direct to the great man to ask him for them in advance of publication. Fortunately the speed of publication was far faster after World War I than after World War II, and the full account of the work done in February actually appeared in the *Journal of Physiology* in mid-May, ten days before my own paper was due. You can imagine how I fell on the *Journal* like a famished dog on a bone. Everything was plain sailing until almost the end of the paper where question *2* was taken up. Here it at once struck me that a serious mistake had been made, as it must have struck any reader who was steeped at the time in the Copenhagen literature. I felt it great cheek to beard the great man on such a matter, especially as I had never met him personally before. However, I had already had engraved in me by Bayliss that ' the greatness of a scientific investigator does not rest on the fact of his never having made a mistake, but rather on his readiness to admit that he has done so, whenever the contrary

evidence is cogent enough', and so I bearded him on the stone stairs outside the lab. His response was instant: 'Yes, that's a valid point. Now will you join me as my colleague and set me back on the rails again?' Of course I jumped at this chance, and it had a great effect on my subsequent work, for it turned out to be one of the main factors which led me later into partnership with Hartridge and thus opened up our work on the measurement of the rate of very rapid reactions of haemoglobin with oxygen and carbon monoxide and to all sorts of other things. The point on which Barcroft and I had met opened up for him the problem of measuring the output of the heart per minute, and probably also the total volume of blood in the body. Two years later, in 1922, we find him setting out to the Andes at the head of an Anglo-American High Altitude Expedition, and on his way out there by boat he made an observation which was to have a great effect on his subsequent work. In practising up the blood volume method, he found there was a marked increase when the ship got into warmer weather, and he thought the increase too rapid to be explained by manufacture of new blood, but must have come about through the mobilization of blood, already existing, but in stagnant parts of the circulation. This led him, after the expedition was over, to his work on blood reservoirs —specially the spleen to begin with, but also the liver, skin and last and most important the uterus. For it was the contact in this connection with this last organ, I think, that brought him to the main scientific interest of his last fifteen years, foetal physiology. There is no time, nor would I be competent, to speak here of his great pioneering work in this field. Cambridge, a year ago, heard in this room a fine lecture on this part of his life work, by his enthusiastic partner in it, Dr. Donald H. Barron, now Professor at Yale University. Absorbed though he was in the physiology of the mother and foetus, particularly in regard to the differences in their haemoglobins, he never lost, or even abated in the slightest degree, his interest in the other problems of haemoglobin and of blood, whether old or new. He was always eager to hear and talk about them, and the only times I can think of in which he did not like to be interrupted were, either if he was in the middle of one of his hectic experiments when he was usually working flat out, or if he was on the way to catch a train, or on the way home to lunch with Lady Barcroft. At any other time he nearly always seemed to have most of the time in the world available, perhaps because he had, as a rule, already done much of his day's work early in the morning, before he ever got down to the laboratory, a salutary practice in which I believe Professor Krogh also indulges.

As illustrations of his abiding interest in older problems, I remember two remarks. First, a few months before his death he said to me:

' If I could only repeat at will the rectangular hyperbola dissociation curve that Roberts and I found in 1909, I would gladly order my coffin tomorrow '. Secondly, during World War II I wrote him from U.S.A. of our work with carbon monoxide which seemed to show that inhaled carbon monoxide not only combined with haemoglobin in the blood, but also migrated into the tissues and lingered there a long time. He at once wrote back : ' I wonder whether you have found the explanation of Haldane and Douglas' results on Pike's Peak in 1912.'

It would be easy to fill a whole volume with such incidents but space is limited, so we must now come to that last memorable morning of his life on the first day of spring, 1947. Once more there was, as in 1920, a meeting on the stone stairs at the entrance to this laboratory. This time, however, the great man was waiting for me, as he knew my usual time of arrival in the lab. (10.15 a.m.) was at least an hour later than his own. ' I've been waiting to waylay you about three things— (a) I want to settle with you about some research apparatus, which you may or may not be taking with you to your new lab., when you move at the end of the month, (b) I want to talk to you about the newly elected Fellows of the Royal Society of London, (c) I want to hear about the experiments you have been doing with the blood I've lately been giving you from my pregnant ewes.' After some amicable haggling in the dark room upstairs about the final destiny of old but useful apparatus which he and I had bought with personal grants before 1931, we got on to the Royal Society questions. He had just finished a two or three year term of office as Chairman of the Selection Committee in Physiology and Medical Sciences, and was full of the exploits of those who had just been chosen from this field, and likewise of the prospects of those who had not succeeded in 1947. I cannot, of course, go into any of the details of the extremely intimate personal comments he made. I would, however, like to tell you that on the last morning of his life, he was every bit as interested in all his younger colleagues— some as much as forty years younger, as if he had been one of them himself, and just as eager that full justice should be done to their claims.

' Now let's hear what you've been doing with my ewe blood, and why I have not heard about it already.' ' The only reason for the latter,' I replied, ' is that you have lately told me you have so much to do, and so little time to do it in, that I have not wanted to bother you with anything half-baked. Now I think it is definite enough not to waste your time.' So we went along to my old laboratory where my mathematical colleague Mrs. Nicolson, my experimental colleague Mr. Legge, and our assistant Alan Secker, were all busy preparing for the day's experiment. We showed and discussed with him a number of curves of the rate of carbon monoxide uptake by red blood corpuscles

of one of his own pregnant ewes, and likewise by the red blood corpuscle of a ram, which was markedly faster. I explained to him how from these data, by a combination of mathematics and physical chemistry it is possible to calculate the permeability of the red cell membrane to carbon monoxide and, from similar experiments, to oxygen which is, of course, the most important substance which passes in and out of the red cell during life. At the end of the explanation he gave a chuckle, and said : ' You know, this reminds me of an electric alarm clock which Hartridge invented many years ago, when he was a young man, which not only woke him up, but pulled him out of bed, made his tea and did all sorts of other things. One evening Hartridge came into dinner at King's, sat down next Milner-White, the theological Dean of the College, and proceeded to explain to him how this clock worked. Milner-White listened with a polite expression on his face, but how much he understood of it I do not know. I think, however, that I have understood at least as much of what you have just told me, as Milner-White did of Hartridge's electric clock.' He then proceeded to make some of his usual shrewd points, and finally concluded : ' Well, I'll take care of that particular ewe and when she is non-pregnant, we will see whether her blood gives the same curve as the ram, which I suppose we may assume—with a fair measure of confidence—not to have been pregnant.' Perhaps this was the very last scientific matter to occupy his attention.

By this time it was nearly noon, and he brought our time together to an end with some trivial, non-scientific matter as he often did on such occasions. ' When did you get that suit you're wearing ? ' ' Before the war.' ' Have you got many pre-war suits left ? ' ' Yes, several.' ' Lucky man ! I wish I'd bought more suits before the war. Those I buy now cost three times as much and only last about one-third as long. Well, let's hear more news later.' With that he backed towards the door with his characteristic gait, and a broad smile on his face. A moment later he was gone—gone for good—gone for good indeed !

When we got back from lunch we were told what had happened. It was, of course, a grievous shock though I still feel sure that no end could have been happier for him than to die suddenly right in the midst of his unceasing activity. Later that day a colleague said to me : ' I envy that man, Joseph Barcroft, going on doing first-class work right up to the last moment of his long life.' For him, as for his older friend, Starling, physiology was the greatest sport in the world.

II

REVERSIBLE REACTIONS WITH OXYGEN AND CARBON MONOXIDE

Aspects of the Oxygenation and Oxidation Functions

DAVID L. DRABKIN

Measurements are reported and their uncertainties discussed in the determination of the oxygen dissociation curve of the arterial blood of man in vivo. *The oxygen saturation (percentage of oxyhaemoglobin of total blood pigment) is determined by direct spectrophotometry, with a special cuvette of* 0·007 *cm depth. This container avoids the inaccuracy inherent in the delivery of a specified blood volume by means of pipettes, and permits the rapid measurement of the undiluted, fresh blood sample unexposed to the atmosphere. The saturation values are plotted against independent accurate measurements of the partial pressure of oxygen in the arterial blood sample, obtained from the subject, stabilized and breathing an appropriate gas mixture.*

In a separate investigation, involving oxygen utilization (the oxidation function) rather than oxygen transport (oxygenation function), cytochrome c metabolism has been under systematic study. An effective micro spectrophotometric technique has been designed for this purpose, and partial hepatectomy in the rat has been adapted as a metabolic procedure. Data are presented in support of the view that the thyroid gland (thyroxine) has a regulatory control on the concentration of cytochrome c in all tissues.

A RECENT writer[1], presumably thinking that haemoglobin is so wonderful that it is good enough to eat, has described the work on the crystalline structure of horse haemoglobin[2, 3] in these terms: 'The general picture of the molecule is thus two hydrophobic sandwiches pressed together and capable of being divided along the mid-hydrophilic plane'. I suppose that when the latest studies of the Cambridge investigators[4] are reviewed by this commentator he might be tempted to extend his simile, and, in the American idiom, describe the haemoglobin of man as a 'double decker' or, perhaps, a 'club sandwich'. It seems to me that such culinary descriptions, no doubt intended to stimulate the popular appetite, overshoot the mark, and miss the intent of Lord Kelvin's cogently expressed view that it isn't science unless 'you can measure what you are speaking about, and express it in numbers'.

For many years past in our laboratory we have attempted to measure haemoglobin and related substances more accurately by means of the

precise tool of spectrophotometry[5], with the hope that it would permit us to speak about these functional entities in more exact and satisfactory terms. By suitable extensions in the optical method[6-10], we have secured quantitative information upon certain details, not possible or not attainable with equal accuracy by other techniques. And occasionally we have been doubly repaid by the measurements themselves in the disclosure of new facts.

Biological position of oxygen— Though in this new world the slogan ' need for energy ', even in the affairs of the cell, has replaced that of ' need for oxygen ', the stature of this gas has not shrunk. Oxygen ' spark-plugs ' the oxidative, energy-yielding processes in aerobic organsisms and tissues. The use of oxygen creates the need, and the familiar formula of ' balance of demand and supply ' applies in the concept of *oxygen homeostasis*, which remains of imperative importance. It may be said without hesitation that *oxygen homeostasis* is one of the most beautiful examples in biological economy of the balanced integration of a number of complicated separate processes. Particularly striking, from the biochemical viewpoint, in this integrated activity are the intracellular agents (' respiratory chemicals '), so similar and yet so different, and each apparently so suitable functionally, as haemoglobin in the erythrocytes, poised to resist *oxidation* and to favour oxygenation, and cytochrome *c* in tissues, poised to favour oxidation[11].

I shall confine myself to two of several aspects which have recently claimed our attention, in the respective phases of ' supply ' and ' demand ', of the broad problem of oxygen homeostasis : *1* the collection of data required for the construction of an oxygen dissociation curve which would reflect conditions in the arterial blood of man *in vivo*, and *2* the study of cytochrome *c* metabolism, which suggests that oxygen consumption is under hormonal control, operative through its effect on the concentration in tissues of the haemin protein.

OXYGEN DISSOCIATION OF ARTERIAL BLOOD IN VIVO

It appears particularly appropriate that the first presentation of our work upon the establishment of the oxygen dissociation curve of the arterial blood of man *in vivo* should be made at the Conference in honour of Barcroft, who pioneered this very field[12] and long maintained an interest in it[13]. These experiments were a collaborative effort with C. J. LAMBERTSEN and P. L. BUNCE. My share was to furnish the data on the percentage of oxyhaemoglobin in the samples. I was happy to leave in the capable hands of Lambertsen and Bunce what seemed to me the major job of accurately determining the corresponding oxygen tensions.

Oxygenation and Oxidation

Several participants in this Conference (J. WYMAN, L. C. PAULING and F. J. W. ROUGHTON) deal with the oxygen dissociation curve of haemoglobin. It seems that expert tailoring is still required to fit well the garment of theory to the peculiar shape of the experimentally obtained curve, and the concern has been both here and in the past[13] to supply fitting explanations, consonant with advancing knowledge of molecular structure, to the experimental data. I do not wish to imply that this objective is not important. It is. But our concern has been with the value of the measurements themselves, with the securing of accurate points from which the curve is constructed, and especially with obtaining reliably the necessary data *in vivo*, rather than as in most past work upon blood or haemoglobin solutions equilibrated *in vitro*.

Definitions and Methods

Statement of the problem— The construction of an oxygen dissociation curve requires information upon at least two quantities, percentage oxygen saturation and oxygen partial pressure, pO_2. In the past the problem was simplified. A blood sample was equilibrated in a tonometer with gas mixtures of varying composition of oxygen and nitrogen, but of constant carbon dioxide content. The latter precaution removed a second variable. The pO_2 was determined in the gas mixture and not in the blood. Upon the latter, after equilibration, the percentage saturation was determined by an indirect, standard gasometric procedure[14, 15], the reliability of which for this purpose has come to be seriously questioned[10, 16]. The classical oxygen dissociation curves have been plotted from such data, and show the percentage of oxyhaemoglobin, inferred to be synonymous with the percentage saturation, against corresponding pO_2 (and at constant pCO_2). It has been assumed that these curves represented what would have been obtained if the determinations had been done upon blood ' equilibrated ' *in vivo* rather than *in vitro*. And, owing to the then unavailable micro method for the direct accurate determination of the blood pO_2, the practice originated of analyzing for percentage saturation and ' determining ' the corresponding pO_2 by reading if off from the previously constructed dissociation curves. As the curves were asymptotic at higher pO_2 values (*Figure* 4) it should have been obvious that, at least in this region, the procedure was scarcely precise.

This practice of indirectly obtaining the pO_2 has had an important consequence upon physiological thought in this field. Gasometric determinations of the degree of saturation (per cent of oxyhaemoglobin) of the arterial blood of man at sea level yielded values as low as 93 per cent, with a mean of 95 per cent[17-19]. On typical dissociation curves[20]

such values for per cent HbO_2 corresponded respectively to 65 and 80 mm of Hg for the arterial pO_2, whereas the pO_2 of the alveolar air was known to be of the order of 100 mm. Herein lay the origin of the concept, accepted and regarded as authoritative for many years, that an appreciable difference (ΔpO_2) existed between the tensions of oxygen in alveolar air and arterial blood. This ΔpO_2 was accorded the status of a ' physiological ' phenomenon and was explained by the hypothesis that ' oxygen equilibrium is not attained until after passage through the lung capillaries '[21].

During the late war I had the pleasure of participating with Roughton in a memorable session of our Philadelphia Physiological Society (meeting of 16 May 1944) at which three independent investigations, with mutually concordant results, were simultaneously reported. The validity of the measurements, upon which the ΔpO_2 was based, was challenged, and the existence of the sacred cow (or at least its size) was questioned. Roughton and his colleagues[16] had analyzed the sources of error in the indirect gasometric determination of the oxygen saturation of blood, and concluded that the results were about 2 per cent too low. J. H. COMROE JR. and R. D. DRIPPS JR.[22] had found by direct measurement (with an adaptation of the Scholander micro gas analyzer[23]) that the pO_2 of arterial human blood was of the order of 97 mm of Hg, or only slightly lower than that of alveolar air. Of both historic and present interest is the fact that the magnitude of the newly reported arterial pO_2 agreed with two early and, at that time, exceptionally high values published by Barcroft and his team[24, 25]. Finally, D. L. DRABKIN and C. F. SCHMIDT[10] had succeeded in measuring directly by means of spectrophotometry the percentage of oxyhaemoglobin in the arterial blood of dog and man. In both species the mean values for this quantity were 98·5 per cent, or 3 to 3·5 per cent higher than that deduced from the indirect gasometric measurements.

The simplest way to suggest the potential errors to which older determinations of percentage saturation are subject appears to be to define this quantity in terms of the analytical procedures used to obtain it.

$$1.\ \textit{Percentage saturation} = \frac{(O_2\ \textit{content} - \textit{dissolved}\ O_2) \times 100}{(O_2\ \textit{capacity} - \textit{dissolved}\ O_2)}$$

This is the percentage saturation obtained by the indirect gasometric technique. The analysis for O_2 content is usually performed promptly upon the fresh blood sample, collected anaerobically. The analysis for O_2 capacity is carried out upon an aliquot, equilibrated with air or an oxygen mixture to attain full saturation. This consumes time, and the changes which may occur in the processing of the blood, which is not

an inert system, rather than the manometry *per se*, are responsible for the errors of this method[16]. For example, if met- or ferrihaemoglobin, which does not bind O_2, is present in the original blood, it may revert to Hb and thence to HbO_2 in the time consumed before and during equilibration for O_2 capacity[26, 27]. Such a reversion of methaemoglobin will result in obtaining a value for the denominator too high in respect to the numerator in the above equation, and the percentage saturation will be underestimated.

$$2.\ \textit{Percentage saturation} = \frac{(O_2\ \textit{content} - \textit{dissolved}\ O_2) \times 100}{\textit{Total pigment}}$$

This is the percentage saturation determined by a combination of gasometry for O_2 content and ' colorimetry ' or photometry for total haemoglobin pigments. The combined method[28] has advantages over the purely gasometric, in that the interpretation of the total pigment value is unambiguous and the determination may be performed promptly and simultaneously with that of the O_2 content. O_2 capacity as a measure of total blood pigment is strictly valid only if no other haemin derivatives besides Hb and HbO_2 are present in the sample. However, this method suffers from the uncertainty inherent in the use of two independent and different techniques (gasometry and photometry) whose exactness of alignment with each other may be questionable. Interestingly enough, the presence of methaemoglobin in the original blood may still result in an underestimation of percentage saturation by the combined method, just as in the first procedure. In the present case the numerator will be too low for the denominator.

$$3.\ \textit{Percentage saturation, or Percentage}\ HbO_2 = \frac{[HbO_2 \times 100]}{[\textit{Total pigment}]}$$

This is the percentage saturation defined by the direct spectrophotometric analytical procedure of Drabkin and Schmidt[10]. It is the only method in which the percentage saturation is unequivocally equal to what it is regarded to represent, the percentage of oxyhaemoglobin, since the fraction of each component (HbO_2 and Hb) in the sample is determined directly. For the most accurate results the total pigment concentration as cyanmethaemoglobin or ferrihaemoglobin cyanide, MHbCN, is independently measured spectrophotometrically[29]. We prefer this procedure, though it may be side-stepped in the method. If the total pigment concentration in blood samples from normal dogs or from normal young men (non-smokers) is determined spectrophotometrically either as HbO_2 (fully oxygenated), Hb (after deoxygenation with $Na_2S_2O_4$), or MHbCN (after conversion with ferricyanide and cyanide), the results agree within 0·5 per cent, the analytical error. This has led to the conclusion[10] that the concentration of methaemo-

globin, if present in normal blood, is of the order of 0·5 per cent or less, an amount too low to be identified in the spectrophotometry of the mixture. That the concentration of ferrihaemoglobin in normal blood does not exceed 0·5 per cent also in carefully conducted gasometric analyses has recently been reported[30]. (The present status of the question of the existence of methaemoglobin in blood is ably presented in the paper by W. N. M. RAMSAY, p. 231.) Since the amount of the oxidized form of haemoglobin was negligible, it permitted the assumption that ' total pigment ' was comprised of HbO_2 + Hb, thus allowing a simple treatment of our spectrophotometric data as representing a two component mixture. The presence of a measurable amount of a third component would not have precluded the use of this technique, but would have made the analysis of the measurements more troublesome.

The appropriateness of the spectrophotometric technique— The extension of spectrophotometry to the direct measurement of the oxygen saturation of arterial blood was made possible by the earlier introduction of the Drabkin and Austin special cuvette of 0·007 cm depth[7], described in *Figure* 1. Within several minutes after its collection, the measurements upon the undiluted blood sample, introduced into the cuvette without exposure to the atmosphere, can be completed. Electively the measurements are performed, as in the present work, on samples haemolyzed with saponin, but accurate results may also be obtained on non-haemolyzed whole blood[8]. At this stage, however, the precision is greater on haemolyzed samples. The 0·007 cm depth cuvette has also been successfully introduced in the vascular system, thus permitting the continuous observation of circulating blood *in vivo* in the completely anaesthetized dog (*Figure* 2).

Barcroft was correctly concerned with what he regarded as one of the least accurate of the analytical steps in such studies of the blood and haemoglobin—the delivery of a specified blood volume by means of a pipette (Memorial Address by A. V. HILL, p. 18). An essential and important feature of our spectrophotometric cuvette (of calibrated depth) is that the volumetric measurement of the blood sample is circumvented.

Figure 3 and its accompanying legend should suffice as a description of the spectrophotometric procedure for the direct determination of the percentage HbO_2 (oxygen saturation), and provide an example of the simple calculations involved in handling data obtained on a two component system. The possibility of accurately deducing the concentration of individual components (of known spectroscopic character) in a mixture, without the need for their separation, is one of the peculiar advantages of the spectrophotometric technique. Under the best conditions two components in a mixture can be measured with an

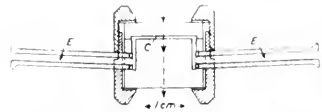


Figure 1. Drabkin and Austin cuvette[7]. The drawing is a vertical section through the centre of the cuvette and is to scale, except the chamber C whose actual depth is only 0·007 cm; E, entry and exit capillary tubes. The broken arrow indicates the direction of passage of radiant flux; the direction is horizontal when in position for reading in the spectrophotometer. The photograph is of the unassembled cuvette, lying on its side.

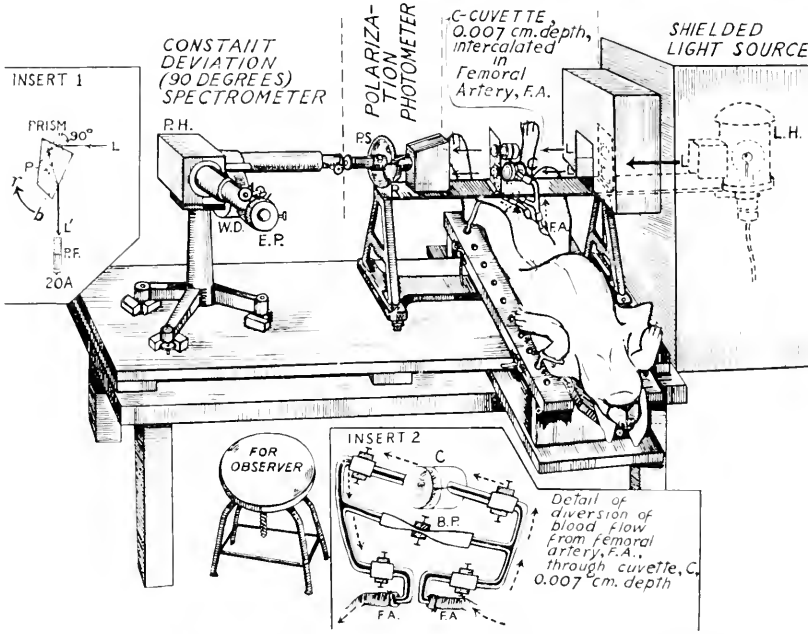


Figure 2. Arrangement for continuous direct spectrophotometric observation of circulating blood in vivo[10]. *The alignment of the polarization spectrophotometer assembly (Bausch & Lomb), permanently set up in a dark room, is shown. The observer, reading through the eyepiece E. P. of the spectrometer, is completely shielded from the light L as the lamp housing L. H. and the accessory biprisms which split the light into two parallel, optical paths are enclosed in an additional large box shielding. Insert 1—a rectangular monochromatic L' photometric field of two halves P. F. of 20 Å in width is defined by a diaphragm in E. P. Different wave-lengths are brought into position by rotation of a drum W. D. translated into rotation of the prism housed within P. H. from red r to blue b or vice versa, about the pivotal point P. The photometer scale P. S., after matching the half fields, is read at R. Insert 2 illustrates the introduction of the 0·007 cm depth cuvette into the femoral artery F. A. The arrangement of clamps is shown. The passage of blood through the cuvette C. may be arrested and the circulation by-passed by closing the clamps at the entry and exit capillaries of the cuvette and opening the clamp B. P. in the by-passing channel. The connections at the clamp sites between the glass capillaries are of small bore, pure gum tubing.*

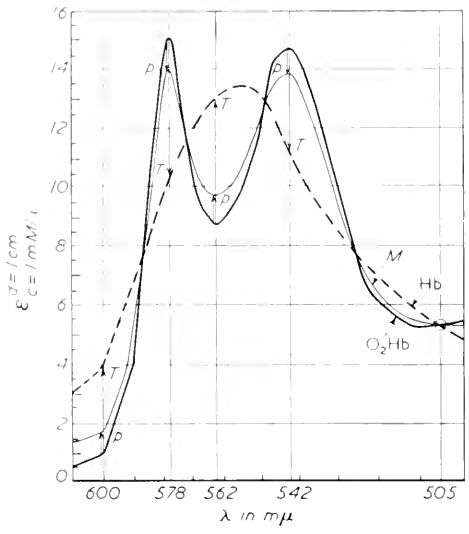


Figure 3. *Absorption spectrum curves obtained from measurements on haemolyzed (saponized) dog blood in the* 0·007 *cm cuvette, illustrating the method used in the direct spectrophotometric determination of percentage saturation*[10]. *Curve* HbO_2 (*heavy, solid line*), *based on mean* ε *values for fully oxygenated blood. The absorption constants (mean* ε *values for a depth of* 1 *cm and for a concentration of* 1 *mM/l, where M refers to the equivalent weight, on the iron basis, of* 16,700) *for* HbO_2, *at the indicated characteristic wavelengths used in the estimation of saturation, are given in the column headed by* 0·96. *Curve* Hb (*broken line*), *based on mean* ε *values upon aliquots of the samples used for curve* HbO_2, *after deoxygenation by means of solid hydrosulphite or dithionite,* $Na_2S_2O_4$ (0·1 *mg per ml*). *The mean* ε *values for* Hb, *used in the estimation of saturation, appear in the column headed by* 4·03. *Curve* M (*light solid line*), *drawn from data upon a sample (used as an example) of a mixture of* HbO_2 *and* Hb, *with total pigment concentration determined independently as cyanmethaemoglobin,* MHbCN. *Appended data supply the calculations of the fraction of each component in* M, *and the percentage saturation of the sample.* Σ_T *is the summation of the total change in absorption,* $\Sigma\Delta\varepsilon$, *at the specified wavelengths, between* HbO_2 *and* Hb, *while* Σ_p *is the summation of the partial change in absorption, at the same wavelengths, between* HbO_2 *and* M, *the particular mixture of the two components,* HbO_2 *and* Hb, *measured*[6]. *For the blood of man the values of* Σ_T *and the absorption constants are slightly different than for dog blood. For man,* Σ_T = 16·45, *and at the respective wavelengths of* 600, 578, 562 and 542 *mμ,* ε_{HbO_2} = 0·75, 15·34, 8·50 *and* 14·61, *and* ε_{Hb} = 4·05, 9·96, 12·75 *and* 11·09[32].

Data :

$\Sigma_T = \Sigma\Delta\varepsilon_{HbO_2} - \varepsilon_{Hb}$

0·96 — 4·03 = 3·07 *at* λ 600
15·08 — 10·46 = 4·62 *at* λ 578
8·62 — 13·16 = 4·54 *at* λ 562
14·75 — 11·09 = 3·66 *at* λ 542

$\Sigma_T = 15{\cdot}89$

$\Sigma_p = \Sigma\Delta\varepsilon_{HbO_2} - \varepsilon_M$ *at same* λ

$\Sigma_p = 0{\cdot}77 + 1{\cdot}10 + 1{\cdot}10 + 0{\cdot}91 = 3{\cdot}88$

$r = \Sigma_p/\Sigma_T =$ *Fraction of* Hb $= \dfrac{3{\cdot}88}{15{\cdot}89} = 0{\cdot}244$

$1 - r =$ *Fraction of* $HbO_2 = 0{\cdot}756$

$(1 - r) \times 100 = \%\ 75{\cdot}6$

accuracy of $\pm$ 0·5 per cent, or equal to that attained in the determination of a single species[6].

For the present, we regard as most unequivocal the description of haemoglobin derivatives in terms of their characteristic and at times highly selective absorption spectra (*Figure* 3). ' Oxyhaemoglobin ' is hence defined by us as the substance capable of yielding established absorption constants at specified wave-lengths, before and after appropriate chemical treatment. Since *oxygen capacity*, determined gasometrically, may be equivocal, I have taken a step towards the much-needed ultimate standardization of the haemoglobins, based on several independent measurements[31]. For this purpose the oxyhaemoglobin and metmyoglobin of man were originally crystallized [31, 32]. Summarized data upon such crystallized haemoglobins are presented in *Table I*. They suggest that the spectrophotometric constant for cyan-

Table I

Standardization data on crystallized haemoglobins[31]

*Preparation**	*Species*	MHbCN *constant used*	*Iron analyses*† *(averages)*	*Nitrogen analyses (averages)*
		$\epsilon^{\ddagger}_{\downarrow}$	*per cent*	*per cent*
Oxyhaemoglobin	*Man*	11·5§	0·338	17·08
		11·5	0·340‖	17·13‖
	Dog	11·5	0·340	17·35
	Horse	11·5	0·337	16·88
Metmyoglobin	*Man*	11·5	0·342	16·92
	Dog	11·5	0·338	
	Horse	11·5	0·341	16·95
Average for haemoglobins			0·339	17·11
Average for myoglobins			0·340	16·94

* All preparations were crystallized, with the exception of dog myoglobin.

† Orthophenanthroline method[33].

‡ ϵ, extinction at depth = 1 cm and concentration = 1 mM per l (as in legend to *Figure* 3).

§ Value originally based on concentrations determined from O_2 capacities of 15 dog bloods[29]. Averages of 0·339 and 0·340 per cent for haemoglobin and myoglobin iron (above) correspond respectively to ϵ values for MHbCN, on the iron basis, of 11·37 and 11·33.

‖ Lyophilized (dried in the frozen state) preparation.

methaemoglobin may be used interchangeably for the haemoglobins and myoglobins of man, dog and horse. The iron content of the different haemoglobins is identical, within the error of the method, and the nitrogen content closely similar. The spectrophotometric determination of MHbCN (total pigment) is actually equivalent to a determination of the haemin iron.

The direct determination of pO_2 *and* pCO_2 *of arterial blood*— R. L. RILEY *et al*[34] had adapted the Roughton and Scholander syringe analyzer[35] for the direct micro volumetric analysis of blood pO_2 and pCO_2. C. J. LAMBERTSEN and P. L. BUNCE have greatly increased the precision of this technique by (1) use of a larger syringe, which accommodates a 5 ml blood sample instead of 1 ml, and (2) attachment of a 200 mm long capillary (with 200 scale divisions) to the syringe, thereby materially reducing the reading error. The analyses are performed in duplicate upon whole arterial blood at 37°C. Meticulous attention to details, which cannot be discussed here, in the handling and preparation of the samples is necessary. The error of the Lambertsen and Bunce modified technique is, in terms of the standard deviation, $\pm$ 2 mm of Hg for both pO_2 and pCO_2.

Experimental and Results

The oxygen dissociation data (solid circles in *Figure* 4) were obtained by the application of the above methods for percentage of HbO_2 (direct spectrophotometric) and for the tensions of O_2 and CO_2 to 44 separate arterial samples. The latter were drawn by femoral puncture from 16 healthy, young adult males, non-smokers, and from one older subject, comfortably recumbent and who had subjectively come to a steady state of relaxation while breathing appropriate gas mixtures, containing different amounts of oxygen. A 15-minute period was allowed for adjustment before drawing the blood. Obviously nervous individuals were not used as subjects. Other determinations, including *p*H, were also done on the blood samples.

In *Figure* 4 each solid circle (and one open circle) represent a point, whose position was determined by our raw, uncorrected data for percentage HbO_2 against corresponding pO_2. Correction to constant pCO_2 or to constant specified *p*H was purposely not attempted, although data for pCO_2 and *p*H were available. Such corrections, as well as others which might have been applied, would not materially alter the picture, and were thought to be inadvisable for a number of reasons. They would imply, for pCO_2, a ' baseline ' not existent *in vivo*. The order of magnitude of the various corrections was well within the margin of uncertainty imposed by the error range of $\pm$ 2 mm of Hg in the pO_2 and pCO_2 determinations. It may be mentioned, at this point,

that Riley, Lilienthal, Proemmel and Franke[28] had applied far larger corrections (in our opinion, with doubtful justification) to the data from which their oxygen dissociation curve of arterial blood *in vivo* was constructed.

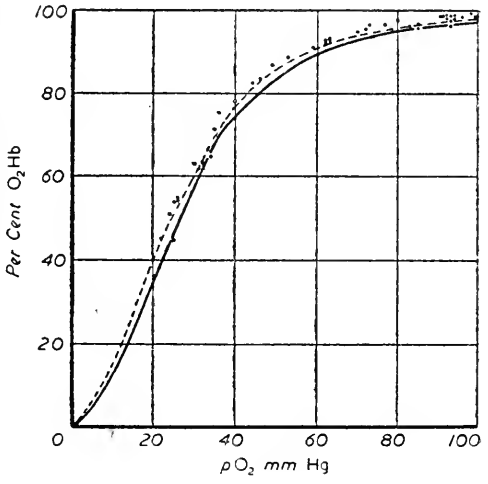


Figure 4. *The percentage of oxyhaemoglobin (oxygen saturation) of the arterial blood of man. Solid curve, based on composite gasometric data of* D. B. DILL[36] *on blood samples equilibrated* in vitro, *with* $pCO_2 = 40$ *mm and* $pH = 7{\cdot}4$. *The broken curve, based on gasometric measurements upon* A.V. BOCK'S *blood,*[20] *equilibrated* in vitro *with* $pCO_2 = 40$ *mm and* $pH = 7{\cdot}4$. *The* 44 *individual points, based on our direct spectrophotometric analyses for per cent* HbO_2 *and corresponding determinations of* pO_2 *on arterial blood 'equilibrated'* in vivo. *These data have not been 'corrected' (see the text).*

Attention may be directed to the following in our data.

1 Under conditions equivalent to breathing air at sea level, both percentage of HbO_2 and arterial pO_2 are high. Thus, the findings of Drabkin and Schmidt[10] are confirmed and extended, since sufficient corresponding values are now available for both per cent HbO_2 and pO_2 (see data in region of 94 to 95 mm pO_2, *Figure* 4).

2 It may be seen (*Figure* 4) that data were not obtained below 22 mm of pO_2 (corresponding to about 45 per cent HbO_2). Somewhere in the region of 30 mm pO_2 (with approximately 60 per cent HbO_2 in the arterial blood), unconsciousness set in after breathing the experimental gas mixture for only one minute. Upon revival, the subjects stoutly denied that they had been unconscious. In spite of these protestations, it was not deemed advisable either to prolong the exposure to such moderately low levels of oxygen, or to attempt to attain still lower levels of arterial pO_2. Riley *et al*[28] have carried their curve for 'arterial' oxygen saturation farther, but only by applying appreciable corrections of questionable accuracy to data upon venous blood.

3 An examination of our data, in comparison with classical dissociation curves obtained *in vitro* by means of gasometry, discloses that they agree more closely with the earlier results of Bock, Field and Adair[20] upon the blood of one subject, Bock (open curve, *Figure* 4), than with the more recent composite data (closed curve, *Figure* 4) of Dill[36]. If we had chosen to draw a curve through our points, it is obvious that it would fall consistently slightly above the curve of Bock and his colleagues.

Many years ago L. J. HENDERSON[37] pointed out that it was not possible to represent correctly the alterations which occur in the body in the change from arterial to venous blood by conventionally plotting two oxygen dissociation curves, each at different pCO_2 (arterial and venous), on the same two-dimensional graph. The difficulty was clear ; physiologically, pO_2 and pCO_2 were interdependent. I have tried to meet this need for an adequate graphic portrayal of the changes in oxygen saturation *in vivo*. The result is embodied in *Figure* 5, a ' three-dimensional ' graph, in which the percentage of HbO_2 is treated as a function of the two variables, pO_2 and pCO_2. In constructing the figure, ' separate ' graphs of oxygen dissociation for each pCO_2 have been appropriately aligned. Definitive values for percentage of HbO_2, pO_2 and pCO_2 in normal arterial and venous blood are given in the legend.

Discussion and Criticism

Towards the completion of this phase of the work we began to be assailed by grave doubts as to how well we had met our primary objective of establishing the dissociation curve of arterial blood *in vivo*. Our concern with the value of the measurements had led us, as already stated, to choose haemolyzed blood for the analysis of oxygen saturation, whereas pO_2 and pCO_2 had been determined on non-haemolyzed blood. Furthermore, the spectrophotometric measurements were done at a temperature of 25 to 27°C, while the gas tensions were measured at 37°C, the temperature of the body. The consequences of having done one set of determinations upon a ' one phase ' system (haemolyzed), the other upon a ' two phase ' system (non-haemolyzed) were many and disturbing. Consideration made it evident that haemolysis (producing a mixture of the cellular and plasma phases) would induce alterations in pO_2 and pCO_2, and consequently in percentage saturation and in *p*H. The difference in temperature would also operate (and in the same direction). In effect, our measurements of percentage of HbO_2 were not precisely at the pO_2 and pCO_2 which we had determined. It was clear from the flatness of the dissociation curve at high pO_2, where little change in per cent HbO_2 accompanies relatively large changes in pO_2, that in this region there was no real need for doubt. However,

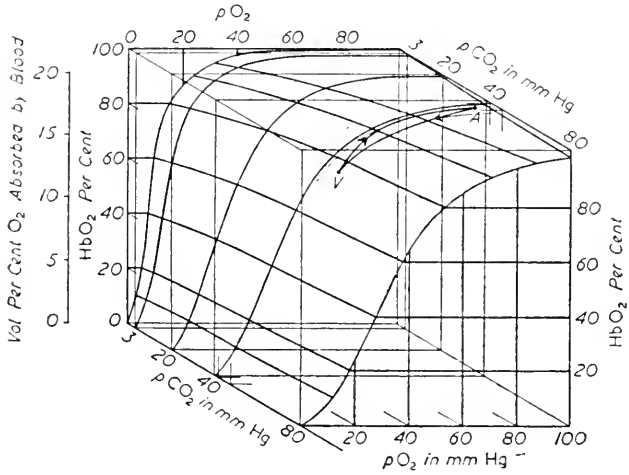


Figure 5. *The percentage saturation of haemoglobin with oxygen (or the percentage of oxyhaemoglobin of total pigment), or the volumes per cent of oxygen absorbed by blood, as a function of two variables, the* pO_2 *and the* pCO_2. *A three-dimensional effect is attained by originally plotting the oxygen dissociation curves on separate graphs for each* pCO_2. *The curves of oxygen saturation at different* pCO_2 *and the grids connecting the curves at corresponding percentages of saturation together form a complex curvilinear surface on which the conditions in arterial A and venous V blood can be adequately represented by the area between A and V. The 'point' A for arterial blood is defined by* 98 *per cent of oxyhaemoglobin at* $pO_2 = 95$ *mm and* $pCO_2 = 41{\cdot}5$ *mm, while V for venous blood is defined by* 77 *per cent of oxyhaemoglobin at* $pO_2 = 40$ *mm and* $pCO_2 = 47{\cdot}5$ *mm (from the data of Lambertsen, Bunce and Drabkin; see Figure* 4). *The data of Bock* et al[29] *were used to plot the curves at* 3, 20, 40, *and* 80 *mm* pCO_2. *The curve at* $pCO_2 = 0$ *was obtained by extrapolation. The scale of volumes per cent* O_2 *absorbed was based on the fact that in blood containing* 16 *gm of haemoglobin per* 100 *ml (Drabkin*[33]*), the volumes per cent* O_2 *absorbed at full saturation would be* 21·74 (21·44 *volumes per cent in oxygenated haemoglobin,* 0·3 *volume per cent dissolved).*

particularly for the values of saturation along the steep part of the curve it became essential to evaluate the magnitude of the various changes owing to temperature differences and haemolysis.

Our analyses indicated that the change in temperature form 37 to 27°C had only a negligible effect on the percentage of HbO_2. At a level of 98·4 per cent saturation, no change could be demonstrated at 38·0, 34·0 and 28·0°C ; at lower levels of saturation, such values as 74·6 per cent, at 37·8°C, and 74·8 per cent, at 27·2°C, were obtained.

When normal arterial blood was haemolyzed in a closed system (tonometer), the following characteristic changes were found: pO_2 decreased about 4 mm, pCO_2 increased about 8 mm, and pH fell 0·07 to 0·1 pH unit. A superficial view of the pO_2 and pCO_2 changes could lead into a morass of inconsistencies unless it is remembered that we were dealing with a closed gas space. Since we were interested in the effect of the changes on the oxygen saturation, only the change in pO_2 was pertinent. A decrease in pO_2 could only mean here that oxygen had been taken up by the haemoglobin, freed from cellular confinement. This was the thing we had feared. We were, therefore, relieved to find that a change (decrease) in 5 mm of pO_2 (equivalent to 0·015 volumes per cent of the dissolved gas) corresponds to only a surprisingly small change (and increase of 0·07 per cent) in the percentage of HbO_2 at high levels of saturation.

Though not pertinent in the present considerations, the change in pH in haemolysis is interesting. The pH of the new, one phase system is lower, but haemoglobin is now at a higher pH than it had been intracellularly. Also, our dependence on a special reference point leads us into descriptive inaccuracy. Classical dissociation curves are referred to a pH of 7·4, the pH of whole blood, which is really the pH of the plasma, but haemoglobin, responsible for the oxygen saturation, functions in a cellular environment of appreciably lower pH. The futility of empirically applying certain corrections becomes obvious.

The human subject— With the above issues out of the way, an uncertainty of a different character arose. Lambertsen and Bunce discovered that we had been over-sanguine in the subjective judgement of the steady state of relaxation of our subjects. They used the Pauling oxygen meter[39] as a ' lie detector ' of the steady respiratory state. This ingenious instrument, which affords a continuous record of pO_2 through the unique paramagnetism of oxygen, was introduced into the respiratory equipment, permitting periodic sampling at ' end expiration '. At five minutes of accommodation to a gas mixture with the composition of air, when overbreathing (owing to excitement) was visually not evident, but detected by the meter, arterial pO_2 was up, higher than 100 mm, pCO_2 was down. Full adjustment was not reached objectively until about 30 minutes, when arterial pO_2 fell to a steady 96 mm and pCO_2 rose, at times to as high as 45 mm. Again, we had been fortunate in our experiments to use an adjustment period of at least 15 minutes. With a similar gas mixture our subjects had yielded a mean value of 95 mm for arterial pO_2, or only 1 mm below that found at complete relaxation, judged objectively.

Thus, although our measurements were not made under the conditions which exist in the circulation, we believe that our data permit

a close approximation of the oxygen dissociation curve of the arterial blood of man *in vivo*.

CYTOCHROME *c* AND METABOLISM

Without haemoglobin life would not be as we know it. The same may be said for cytochrome *c*, functioning in the other phase of oxygen homeostasis. Indeed, without the cytochrome system, haemoglobin function would be an extravagance.

The flexibility of the spectrophotometric technique is well illustrated by its extension to such diverse measurements as those upon the very concentrated solutions of haemoglobin or whole undiluted blood with the 0·007 cm cuvette, discussed above, and the direct spectrophotometric determination of cytochrome *c*, isolated in micro quantities from small amounts of tissue, as individual rat organs. For the latter purpose we have developed an isolation procedure and designed a capillary cuvette-diaphragm technique[9]. The latter is shown diagrammatically in *Figure* 6. The small volume needed to fill this cuvette permits more concentrated extracts to be measured, while small volume and long depth of layer cooperate to increase sensitivity.

With these means, combined with partial hepatectomy, adapted as a metabolic procedure[40], we have undertaken a systematic investigation of cytochrome *c* metabolism. The influence of such factors as diet (high and no protein)[41], anoxia, and parenteral cytochrome *c* administration[42] were studied. This work encouraged the conclusion that ' certain cellular components, like cytochrome *c* and PNA [ribose nucleic acid], are preferentially produced or deposited in tissues, and are important or essential in growth and proliferative processes, which appear to depend on intrinsic (tissue) as well as extrinsic (dietary) factors [41].'

Most recently I have turned to a study of hormonal influences, particularly thyroxine, on cytochrome *c*. I was directed towards this path by a number of suggestive observations[11], which are summarized in *Tables II* and *III*. The concentration of cytochrome *c* in tissues was proportional to their ' respiratory ' (oxidative) activity (*Table II*). In a way this was curious, since actually cytochrome *c* is a substrate for the enzyme, cytochrome oxidase. The concentrations of both substrate and enzyme therefore have a relationship of proportionality, or, what seems more likely, the concentration of the substrate is a limiting factor in the activity. The other observation was derived from our accumulated data on the total concentrations, in the bodies of several species, of the three chromoproteins, haemoglobin, myoglobin, and cytochrome *c* (*Table III*). Total haemoglobin was strictly proportional

in the different species to their body mass. The quantity of cytochrome *c*, on the other hand, in rat, man and cow, was proportional to a fractional exponent of their body weight, or, if you prefer to express it that way, to their surface area. The total cytochrome *c* of the horse (*Table III*) does not fit this relationship for reasons which need not be discussed here, but it may be seriously suggested that this discrepancy has something to do with the difference expressed by the phrases 'excitable horse' and 'placid cow'.

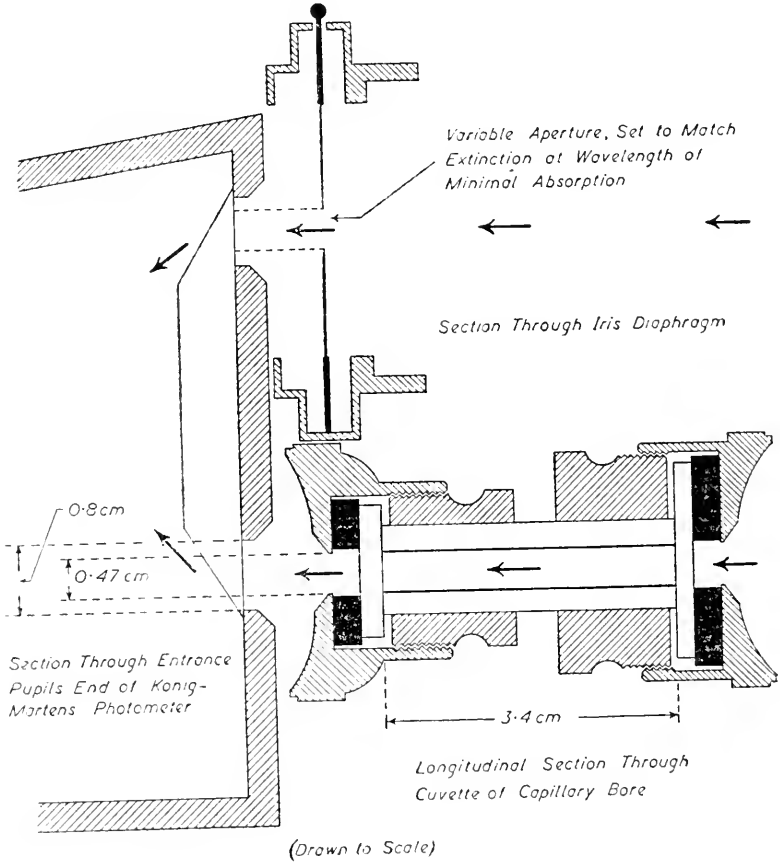


Figure 6. An exact diagrammatic representation of the alignment of the photometer, capillary cuvette, and iris diaphragm in the micro spectrophotometric determination of cytochrome c in tissues (*Rosenthal and Drabkin*[9]).

These relationships of cytochrome *c* to oxygen consumption in tissues (*Table II*) and to metabolism (*Table III*) naturally led to thyroxine, which somehow regulates oxygen consumption and metabolic rate. There was some pertinent, though scanty and inconclusive literature in this field. S. R. TIPTON[44, 45] had reported evidence for the influence of the adrenal cortical hormones, particularly on cytochrome oxidase activity. A. TISSIÈRES[46] had studied the influence of the thyroid gland on cytochrome *c*, but his results were confined to a single tissue, muscle, and his analytical values for cytochrome *c* in the normal tissue appeared unacceptably low. I am glad to state that Tissières (now working in Professor Keilin's laboratory) has now informed me that he has more recently[47] applied our technique[9] and has obtained much higher values for muscle cytochrome *c*. To be of conclusive significance, a hormonal effect upon cytochrome *c*, contained in all aerobic cells, must be demonstrated for all tissues. *Table IV* is a summary of mean values[11, 48] for the concentration of cytochrome *c* in liver, kidney, heart and skeletal muscle in groups of rats, subjected to thyroidectomy, thiouracil thyrotoxicosis, and acute hyperthyroidism induced by injection of thyroxine, in comparison with normal controls. After both thyroidectomy and thiouracil there was a marked reduction in total body cytochrome *c*, reflected in statistically significant decreases (*Table IV*) in the cytochrome *c* concentration (and content) of all the tissues examined. After the administration of thyroxine conclusive results in the opposite direction were obtained.

These data consistently support the view of a relationship of thyroid function to cytochrome *c* concentration in tissues, and permit a tentative thesis : Thyroxine exerts its effect through the agency of cytochrome *c*.

Table II. Proportionality of Cytochrome c Concentration[9, 40, 43] *and Oxygen Consumption (Oxidase Activity) in the Tissues of the Rat*

Tissue	Q_{O_2} *	*Cytochrome c*†
Red blood corpuscles	0·1	8
Skin	1 to 2	51
Muscle	6	381
Brain	10	375
Liver	10+	607
Kidney	20	1433
Heart	20+	1940
Retina	30	

* μl of O_2 consumed per mg of dry weight of tissue per hour ; approximate magnitudes.
† γ or μgm of cytochrome *c* per gm of dry weight of tissue.

Table III. Relationships of Total Chromoproteins to Body Mass, W, and Surface Area, S[11]

Species	Chromoprotein	Chromoprotein			
		Total	Per kg	Per 1 m^2	Per $W^{0.75}$
		gm	gm	gm	gm
Rat	*Haemoglobin*	3·19	12·76	88·3	9·01
$W = 0{\cdot}250$ *kg*	*Myoglobin*	0·101	0·404	2·8	0·286
$S = 0{\cdot}0361$ m^2	*Cytochrome c**	0·0144	0·056	0·399	0·041
Man	*Haemoglobin*	912·8	13·04	488·1	37·7
$W = 70$ *kg*	*Myoglobin*	40·0	0·57	21·4	1·65
$S = 1{\cdot}87$ m^2	*Cytochrome c**	0·780	0·011	0·417	0·032
Horse	*Haemoglobin*	5,800·0	11·60	876·0	55·4
$W = 500$ *kg*	*Myoglobin*	1,868·0	3.74	282·0	17·83
$S = 6{\cdot}62$ m^2	*Cytochrome c*†	16·6	0·033	2·51	0·159
Horse‡					
$W = 455$ *kg*	*Myoglobin*	1,347·0	2·96	216·0	13·70
$S = 6{\cdot}23$ m^2	*Cytochrome c*†	24·4	0·054	3·92	0·248
Cow, heifer	*Haemoglobin*	2,215·0	12·17	758·6	44·8
$W = 182$ *kilos*	*Myoglobin*	307·0	1·69	105·1	6·20
$S = 2{\cdot}92$ m^2	*Cytochrome c*†	1·24	0·0068	0·424	0·025

* Values based on complete analyses of individual organs.
† Values based on (total muscle cytochrome *c*) /0·8.
‡ Steeplechase thoroughbred, out-of-training.

I would like to add a disturbing point about cytochrome *c*. The isolated and relatively pure pigment can be shown to possess biological activity, but the activity is far smaller than that deduced from such measurements of tissue oxygen consumption and cytochrome *c* concentration, as presented in *Table II*. Have we gone too far in insisting that the goal is to work with pure enzyme systems ? Is not the real goal the activity of the living cellular structure ? Are we missing something ? Barcroft[13] had this to say about the early work on cytochrome, before component *c* was isolated or its structure known : ' I will at once concede to the organic chemist of the purist school that such a material is less satisfactory than if it had been isolated, but I claim for those who are prepared to study " life as a whole " that a man places an undue limitation on his intellect if he is not prepared to look at living things as they are, but will merely study artifacts about which he can obtain more precise information '.

Table IV. Effect of Thyroidectomy, Thiouracil and Thyroxine on Cytochrome c (Drabkin 1948[11,48])

Rats of 200 to 250 gm body weight on high protein diet;[10] thiouracil, 50 mg per day ; thyroxine subcutaneously, 1 mg every other day.

Experiment	Cytochrome c				Cytochrome c in restored liver		Liver restoration	Liver PNA*	Liver DNA†
	Liver	Kidney	Heart	Muscle	Total	New pigment			
	γ per gm‡	γ per gm	γ per gm	γ per gm	γ	per cent	per cent	mg per gm	mg per gm
Thyroidectomized 35 days before liver lobectomy §	138 ±5‖							7.19 ±0.10	2.80 ±0.05
14 days after liver lobectomy	181 ±5	248 ±12	316 ±16	57 ±3	780	68.9	65.0	8.35 ±0.11	2.79 ±0.06
Thiouracilized 45 days before liver lobectomy §	145 ±3							8.35 ±0.21	2.70 ±0.03
14 days after liver lobectomy	165 ±3	250 ±5	331 ±4	55 ±3	776	66.1	80.2	9.82 ±0.40	2.74 ±0.05
Thyroxinized, for 14 to 22 days	247 ±6	422 ±8	618 ±11	133 ±6				11.08 ±0.13	2.60 ±0.08
Controls	178 ±4							8.50 ±0.25	2.46 ±0.02
14 days before liver lobectomy §	210 ±5	352 ±21	447 ±16	98 ±6	1325	67.6	73.9	6.96 ±0.24	3.53 ±0.04

* Ribose nucleic acid
† Desoxyribose nucleic acid
‡ Wet weight of tissue
§ 68.4 per cent of liver excised
‖ Values after ± are standard errors

Most of the work reported here was carried out under contract between the Office of Naval Research and the University of Pennsylvania.

Received August 1948

REFERENCES

[1] BULL, H. B. in SAHYUN, M. *Outline of the Amino Acids and Proteins* New York (1944) 82
[2] PERUTZ, M. F. *Nature, Lond.* 149 (1942) 491 ; 150 (1942) 324
[3] BOYES-WATSON, J. and PERUTZ, M. F. *Nature, Lond.* 151 (1943) 714
[4] PERUTZ, M. F. and WEISZ, O. *Nature, Lond.* 160 (1947) 786
[5] DRABKIN, D. L. in GLASSER, O. *Medical Physics* Chicago (1944) 967
[6] AUSTIN, J. H. and DRABKIN, D. L. *J. biol. Chem.* 112 (1935–36) 67
[7] DRABKIN, D. L. and AUSTIN, J. H. *J. biol. Chem.* 112 (1935–36) 105
[8] — and etc. and SINGER, R. B. *J. biol. Chem.* 129 (1939) 739
[9] ROSENTHAL, O. and DRABKIN, D. L. *J. biol. Chem.* 149 (1943) 437
[10] DRABKIN, D. L. and SCHMIDT, C. F. *J. biol. Chem.* 157 (1945) 69
[11] — in Biochemical Symposium on ' Hemin Pigments and Chromoproteins ' *Federation Proc.* 7 (1948) 483
[12] BARCROFT, H. and CAMIS, M. *J. Physiol.* 39 (1909–10) 118
— and ROBERTS, F. *J. Physiol.* 39 (1909–10) 143
[13] — *The Respiratory Function of the Blood, Part II, Haemoglobin, 2nd Edition, chapters X and XI* Cambridge (1928)

[11] VAN SLYKE, D. D. and NEILL, J. M. *J. Biol. Chem.* 61 (1924) 523
[15] — *J. biol. Chem.* 73 (1927) 121
[16] ROUGHTON, F. J. W., DARLING, R. C. and ROOT, W. S. *Amer. J. Physiol.* 142 (1944) 708
[17] LENNOX, W. G. and GIBBS, E. L. *J. clin. Invest.* 11 (1932) 1155
[18] KEYS, A. and SNELL, A. M. *J. clin. Invest.* 17 (1938) 59
[19] CULLEN, S. C. and COOK, E. V. *Amer. J. Physiol.* 137 (1942) 238
[20] BOCK, A. V., FIELD, H. Jr. and ADAIR, G. S. *J. biol. Chem.* 59 (1924) 353
[21] —, DILL, D. B., EDWARDS, H. T., HENDERSON, L. J. and TALBOTT, J. H. *J. Physiol.* 68 (1929–30) 277
[22] COMROE, J. H. Jr. and DRIPPS, R. D. Jr. *Amer. J. Physiol.* 142 (1944) 700
[23] SCHOLANDER, P. F. *Rev. Sci. Instrum.* 13 (1942) 264
[24] BARCROFT, J. and NAGAHASHI, M. *J. Physiol.* 55 (1921) 339
[25] —, BINGER, C. A., BOCK, A. V., DOGGART, J. H., FORBES, H. S., HARROP, G., MEAKINS, J. C. and REDFIELD, A. C. *Trans. Philos.* 211 (1923) 351
[26] COX, W. W. and WENDEL, W. B. *J. biol. Chem.* 143 (1942) 331
[27] DRABKIN, D. L. *Federation Proc.* 5 (1946) 132
[28] RILEY, R. L., LILIENTHAL, J. L. Jr., PROEMMEL, D. D. and FRANKE, R. E. *J. clin. Invest.* 25 (1946) 139
[29] DRABKIN, D. L. and AUSTIN, J. H. *J. biol. Chem.* 112 (1935–36) 51
[30] VAN SLYKE, D. D., HILLER, A., WEISIGER, J. R. and CRUZ, W. O. *J. biol. Chem.* 166 (1946) 121
[31] DRABKIN, D. L. *Amer. J. med. Sci.* 209 (1945) 268; *Federation Proc.* 4 (1945) 88
[32] — *J. biol. Chem.* 164 (1946) 703
[33] — *J. biol. Chem.* 140 (1941) 387
[34] RILEY, R. L., PROEMMEL, D. D. and FRANKE, R. E. *J. biol. Chem.* 161 (1945) 621
[35] ROUGHTON, F. J. W. and SCHOLANDER, P. F. *J. biol. Chem.* 148 (1943) 541
[36] DILL, D. B. *Handbook of Respiratory Data in Aviation* Washington (1944)
[37] HENDERSON, L. J. *Blood, A Study in General Physiology* New Haven (1928) 77
[38] DRABKIN, D. L. *Amer. J. med. Sci.* 215 (1948) 110
[39] PAULING, L., WOOD, R. E. and STURDIVANT, J. H. *J. Amer. chem. Soc.* 68 (1946) 795
[40] CRANDALL, M. W. and DRABKIN, D. L. *J. biol. Chem.* 166 (1946) 653
[41] DRABKIN, D. L. *J. biol. Chem.* 171 (1947) 395
[42] — *J. biol. Chem.* 171 (1947) 409
[43] ROSENTHAL, O. and DRABKIN, D. L. *J. biol. Chem.* 150 (1943) 131
[44] TIPTON, S. R. *Endocrinology* 34 (1944) 181
[45] —, LEATH, M. J., TIPTON, I. H. and NIXON, W. L. *Amer. J. Physiol.* 145 (1945–46) 693
[46] TISSIÈRES, A. *Arch. int. physiol.* 54 (1946) 305
[47] — *Arch. int. physiol.* 55 (1947) 252
[48] DRABKIN, D. L. *Federation Proc.* 7 (1948) 151

The Bond between Haem and Globin

FELIX HAUROWITZ*

1 The iron atom of reduced haemoglobin is saturated coordinatively by a water molecule instead of O_2. *Haemoglobin is an aquo-compound.*

2 Oxyhaemoglobin cannot dissociate into haemoglobin and oxygen in the absence of water. The equilibrium between haemoglobin and oxygen is represented by the equation: $Hb(H_2O) + O_2 \rightleftharpoons HbO_2 + H_2O$.

3 The influence of salts, hydrogen ions and of the globin component on the equilibrium is attributed to the action of these substances on the iron-linked water molecule.

THE most important reaction of haemoglobin is certainly its combination with oxygen to form oxyhaemoglobin. This reaction is represented by the well-known equation $Hb + O_2 \rightleftharpoons HbO_2$. Although this equation is accepted quite generally, it does not correspond to the actual reaction taking place between haemoglobin and oxygen. This can be shown by two simple experiments.

The first of them consists in drying oxyhaemoglobin cautiously and then exposing the dry preparation in a vacuum. It is found, that the typical colour and the absorption spectrum of dry oxyhaemoglobin do not undergo any alteration, even if the pressure is reduced to 0·1 mm of Hg. Under the same conditions wet oxyhaemoglobin crystals or oxyhaemoglobin dissolved in water pass instantaneously into haemoglobin.

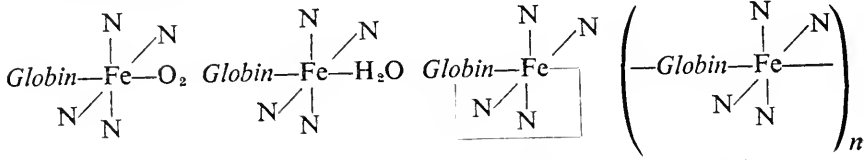
Layers of dry oxyhaemoglobin were obtained by the evaporation of 0·5 ml of a 5 per cent solution of beef oxyhaemoglobin. The solution was placed in a glass dish and kept in a desiccator under slightly reduced pressure over large amounts of phosphorus pentoxide, so that drying was achieved within few minutes. Only traces of methaemoglobin were formed under these conditions.

The conclusion from this experiment is that the oxygen molecule cannot be detached from haemoglobin in the absence of water. This is in agreement with our view, advanced several years ago[1], that haemoglobin is an aquo-compound, in which one water molecule is bound coordinatively to the iron atom (Formula II).

This view is supported by a second series of experiments, in which we attempted to detach the water molecule from reduced haemoglobin. It had been shown, previously, by R. VON ZEYNEK[2] that reduced

* Present address: Department of Chemistry, Indiana University, Bloomington, Ind., U.S.A. The experiments were carried out in the Department of Medical Chemistry, University of Istanbul (Turkey) with the assistance of Radiye Cindi and Saide Tunç.

haemoglobin, upon drying *in vacuo*, undergoes a marked alteration of its absorption spectrum. The broad absorption band of haemoglobin is replaced by two narrow bands of a typical haemochromogen spectrum. The reaction is completely reversible when water is added and it can be repeated several times without any apparent denaturation of the haemoglobin.



I. *Oxyhaemoglobin* — *II.* *Haemoglobin* (*Aquo–Hb*) — *III.* — *IV.* *Dry Haemoglobin* (*Anhydro–Hb*)

We have attempted to record the spectral alterations by a quantitative method. The usual spectrophotometric method could not be applied, because dry layers of haemoglobin had to be measured. The height of these layers is not quite uniform. Good results were obtained, however, by making use of spectrography. *Figure* 1 shows the visible absorption spectra of beef oxyhaemoglobin (a), haemoglobin (b), dry haemoglobin (c), globin-haemochromogen (d) and oxyhaemoglobin obtained by dissolving the dry haemoglobin in water (e).

It is evident from *Figure* 1 that the absorption spectrum of dry haemoglobin (c) is very similar to the true haemochromogen spectrum (d). But, on dissolving dry haemoglobin in water and saturating with air a typical oxyhaemoglobin spectrum (e) is obtained. There is no doubt, therefore, that the reaction is completely reversible.

Similar results were obtained with myoglobin from beef heart and with synthetic haemoglobins. The latter were prepared by coupling native beef globin with protohaemin, mesohaemin or the dimethyl ester of mesohaemin. All of these preparations gave typical haemoglobin spectra, when reduced by small amounts of dithionite $Na_2S_2O_4$, and haemochromogen spectra upon drying *in vacuo* at 40°C. Dissolving of the dry haemoglobins in water and saturation with air furnished in all cases typical oxyhaemoglobin spectra. It is evident from these experiments that the observed alteration of the haemoglobin spectrum upon drying is independent of the molecular weight of the globin component (which is lower in myoglobin than in haemoglobin) and that the side chains of the haem component are not involved in this alteration.

Since all known haemochromogens contain two substituents, linked coordinatively to the iron atom, the same may be assumed for the haemochromogen-like substance formed from haemoglobin on drying.

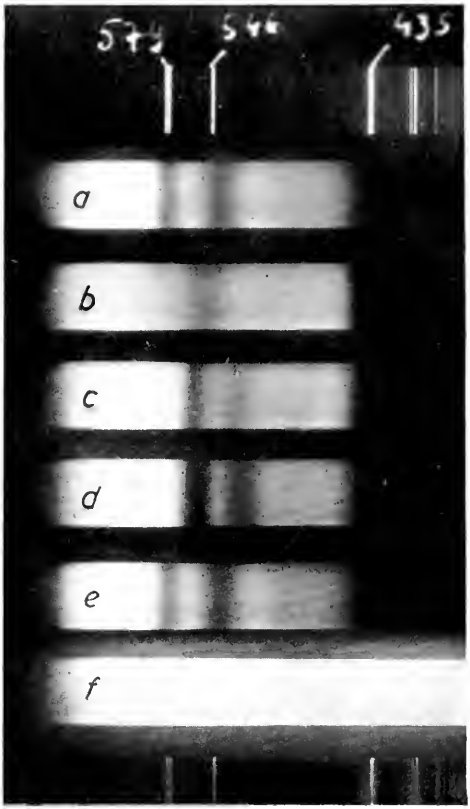


Figure 1. *Visible absorption spectra of beef oxyhaemoglobin.*

The concentration of the oxyhaemoglobin solution was 0·2 per cent, the depth of the absorption vessel 18 mm. A Schmidt and Haensch spectrograph with grating lattice was used. The width of the slit was 0·1 mm, the time of exposure was 3 seconds. As source of light for the spectra (a)–(f) a bulb with tungsten filament was used. It was replaced by a mercury arc for the wavelength spectra (*Figure* 1). Spectrum (b) was obtained by reducing the solution (a) with a trace of solid sodium dithionite. About 1 ml of a 15 per cent solution of the beef oxyhaemoglobin was brought into a Kjeldahl flask and dried by reducing the pressure to 10–15 mm of Hg and dipping the flask into a water bath of 35–40 C. A tube containing dry calcium chloride was inserted between the neck of the flask and the pump, so that the water vapours did not condense in the neck of the flask. The spectrum of oxyhaemoglobin was replaced very quickly by that of reduced haemoglobin and then upon drying of the wet substance, by the spectrum of dry haemoglobin (c). Spectrum (d) was obtained by mixing solution (a) with one tenth of its volume of 10 per cent NaOH solution, keeping the alkaline mixture in a boiling water bath for two minutes, cooling and adding a trace of solid sodium dithionite. Spectrum (e) is the absorption spectrum of oxyhaemoglobin obtained by dissolving the dry haemoglobin (c) in 75 ml of 0·1 per cent sodium carbonate solution. The emission spectrum of the electric bulb is shown by (f), the absorption vessel in this case being filled with distilled water. Panchromatic films were used for the spectra shown in *Figure* 1. The alterations of the absorption spectra could be demonstrated particularly distinctly by using coloured Ansco films as demonstrated at the Conference.

The two substituents linked to the iron atom are either atomic groups of the same globin molecule (Formula III) or atomic groups of two different globin molecules (Formula IV). Since it has been demonstrated by S. GRANICK[3] that the haem group is situated on the surface of the globin component and is accessible even to very large foreign molecules, we prefer formula IV to formula III. This is also in better agreement with X-ray measurements of M. F. PERUTZ[4], who has shown that water molecules coat the surface of the haemoglobin molecule. The correctness of formula IV was tested experimentally by drying haemoglobin in the presence of a large excess of glucose. It was expected that glucose owing to its multiple polar groups would be attached to the surface of the haemoglobin molecules and would, thus, prevent the mutual association of these molecules to form the polymeric substance IV. Actually we did not observe any haemochromogen spectrum when 1 ml of a 15 per cent solution of haemoglobin was dried in the presence of 1·5 gm of glucose under the same conditions as those which had brought about the absorption spectrum (c) in the absence of glucose.

We conclude from our experiments that the reversible reaction taking place during the drying of haemoglobin must be represented by the following equation:

$$\underset{\substack{\textit{Aquo-Haemoglobin}\\(\textit{Spectrum b})}}{n\ \mathrm{Hb(H_2O)}} \rightleftharpoons \underset{\substack{\textit{Anhydro-Haemoglobin}\\(\textit{Spectrum c})}}{n\ \mathrm{H_2O} + (\mathrm{Hb})_n}$$

Or, in other words, monomeric haemoglobin molecules can exist only in aquous solutions or in wet crystals (Formula II). As soon as the iron-linked water molecule is detached, polymerization of the haemoglobin molecules occurs (Formula IV). On the other hand, oxyhaemoglobin and carboxyhaemoglobin can exist as true molecules in solutions as well as in the dry state (Formula I).

It has been shown by L. PAULING and C. CORYELL[5] and by H. THEORELL[6] that haemochromogens are devoid of paramagnetic susceptibility. This is due to the deep penetration of coordinative substituents into the electron shell of the iron atom. The magnetic momentum of the 3d electrons is lost as soon as they become involved in the bonding of the coordinative substituents. We suppose, therefore, that haemoglobin loses its paramagnetic susceptibility on drying. One has to assume, moreover, that the iron-bound water molecule of haemoglobin is bound less firmly than the oxygen molecule of oxyhaemoglobin and that the 3d electrons are not involved in the bonding of the water molecule.

Discussion— The main result of the presented experiments is the revelation that oxyhaemoglobin does not dissociate into haemoglobin

and oxygen according to the classical monomolecular equation

$$HbO_2 \rightleftharpoons Hb + O_2,$$

but that this reaction takes place according to the bimolecular equation

$$HbO_2 + H_2O \rightleftharpoons Hb(H_2O) + O_2.$$

It is obvious that both equations lead to the same results in dilute aqueous solutions, because in such solutions the excess of water molecules is so large that the water concentration remains practically constant during the reaction. Differences may arise, however, in very concentrated solutions of oxyhaemoglobin or in wet haemoglobin crystals.

The new equation renders understandable the large influence of salts, hydrogen ions and proteins on the equilibrium between haemoglobin and oxygen. For all these substances are polar hydrophylic substances and are able to act by means of Coulomb forces on the iron-linked water molecule of haemoglobin. It is evident that such an action will modify the affinity of oxygen for the haemoglobin molecule. For the same reason the globins of different species, containing different hydrophylic groups in the environment of the iron-bound water molecule, will have a different influence on this water molecule and thus will alter the equilibria between haemoglobin and oxygen. Indeed it has been demonstrated by Barcroft[7] that the affinity of O_2 to the haemoglobin molecule varies with the species specificity of globin. For the same reason this affinity is different in maternal and in foetal haemoglobin[8, 9] and undergoes an alteration upon haemolysis of the red blood corpuscles[8, 9]. It is hardly possible to explain this latter phenomenon by the classical equation. But it is understandable according to the new equation and Formula II that the affinity of the iron-linked water molecule to the Fe atom is altered as soon as the electrostatic influence of closely adjacent protein or lipid molecules on the iron-linked water molecule is abolished by haemolysis.

Received July 1948

REFERENCES

[1] HAUROWITZ, F. *Hoppe-Seyl. Z.* 232 (1935) 146
[2] V. ZEYNEK, R. *Nowiny Lekarske Poznan* 38 (1926) 10
[3] GRANICK, S. *J. gen. Physiol.* 25 (1942) 571
[4] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. *Proc. roy. Soc. A* 191 (1947) 83
[5] PAULING, L. and CORYELL, C. *Proc. nat. Acad. Sci. Wash.* 22 (1936) 159
[6] THEORELL, H. *J. Amer. chem. Soc.* 63 (1941) 1820
[7] BARCROFT, J. *The Respiratory Function of the Blood* Cambridge (1928) p. 38
[8] HAUROWITZ, F. *Hoppe-Seyl. Z.* 183 (1929) 78 ; 186 (1930) 141
[9] MCCARTHY, E. *J. Physiol.* 102 (1943) 55

The Electronic Structure of Haemoglobin

LINUS PAULING

It is possible to explain the power of specific combination with oxygen and carbon monoxide possessed by ferrohaemoglobin, by means of the postulate of the approximate electrical neutrality of all atoms in stable compounds. It is shown that the iron atom in ferrohaemoglobin itself is made approximately neutral by the bonds to the nitrogen atom of the porphyrin group, and that accordingly only a molecule that can form covalent bonds with the iron atom without transferring a large amount of electrical charge to this atom would be expected to combine with ferrohaemoglobin. Oxygen, carbon monoxide, cyanide ion, and the alkyl isocyanides have such structure as to permit them to combine with ferrohaemoglobin without a large change in the electrical charge of the iron atom, and accordingly these molecules, and not substances such as water molecules, chloride ions, hydroxide ions, etc, are expected to form ferrohaemoglobin compounds.

DURING the past twenty-five years great progress has been made in the development of a detailed theory of molecular structure, both by the application of experimental methods of determining the arrangement of atoms in molecules and crystals (the methods of spectroscopy and of X-ray diffraction and electron diffraction) and by the application of quantum mechanics to the problem of the electronic structure of molecules and the nature of the chemical bond. In addition, much new experimental information has been obtained about haemoglobin and haemoglobin compounds, which has been combined with the theory of molecular structure in an effort to explain the chemical properties of haemoglobin in terms of its structure. Let us consider to what extent this effort has been successful.

THE ENVIRONMENT OF THE IRON ATOMS

Each of the iron atoms in haemoglobin occupies a position at the centre of a square formed by the four nitrogen atoms in the porphyrin ring system. It is accordingly in a position to complete its Werner coordination complex by ligating to itself two more atoms, one above and the other below the plane of the porphyrin molecule, as was suggested by J. B. CONANT[1]. Investigation of the magnetic properties of haemoglobin and haemoglobin derivatives has verified this structure. It was found[2] that haemoglobin (ferrohaemoglobin) is paramagnetic and has approximately the same magnetic moment as the hydrated

ferrous ion, $[Fe(OH_2)_6]^{++}$, whereas oxyhaemoglobin and carbonmonoxyhaemoglobin are diamagnetic, and are thus similar in magnetic properties to the ferrocyanide ion, $[Fe(CN)_6]^{----}$. The bipositive iron ion Fe^{++} has 24 extranuclear electrons, permitting it to form a completed argon shell plus six outer electrons. There are nine stable orbitals outside of the argon shell, the five 3*d* orbitals, one 4*s* orbital, and three 4*p* orbitals, of which the 3*d* orbitals are somewhat more stable for unshared electrons than the other two. In the isolated ferrous ion the six electrons occupy the five 3*d* orbitals, and give rise to a normal state in which four of the electron spins are unpaired. It is assumed that in the hydrated ferrous ion and in ferrohaemoglobin the bonds between the iron atom and the ligated atoms do not make use of more than four of the nine outer orbitals, permitting the six electrons to occupy the remaining five orbitals, with four electron spins unpaired. The magnetic moment expected for four electron spins, without contribution from orbital moments, is 4·90 Bohr magnetons, and the values observed for the hydrated ferrous ion, 5·3 magnetons, and the ferrohaemoglobin molecule, 5·44 magnetons per iron atom (assuming that the moments of the four iron atoms in the molecule orient themselves independently in the applied magnetic field), are in approximate agreement with this value. In the ferrocyanide ion, on the other hand, all of the nine outer orbitals are indicated by the observed diamagnetism to be used in the formation of covalent bonds or for occupancy by unshared pairs of electrons. The same sort of structure was proposed for oxyhaemoglobin and carbonmonoxyhaemoglobin.

It has been customary to refer to the structure indicated by the large magnetic moment of ferrohaemoglobin as involving essentially ionic bonds between the iron atoms and surrounding atoms, and to the structure in oxyhaemoglobin and carbonmonoxyhaemoglobin as involving octahedral coordination with essentially covalent bonds.

The magnetic properties of ferrihaemoglobin (methaemoglobin) and its derivatives show that a similar change in electronic structure occurs on chemical reaction of this molecule. Ferrihaemoglobin itself has magnetic moment 5·46 in acid solutions (below *p*H 5), and the moment changes to 5·77 when the solution is made more basic (*p*H 6·0–7·0). These values are slightly smaller than the value 5·92 corresponding to five unpaired electron spins, which would be expected in case that the five 3*d* orbitals were all available for occupancy by five outer electrons of the Fe^{+++} ion. Values close to the theoretical are observed for ferrihaemoglobin fluoride (5·90) and the ferrihaemoglobin-ethanol complex (5·89).[3,4] Other compounds of ferrihaemoglobin have a magnetic moment not far from that given by the spin of a single

unpaired electron, 1·73 magnetons, and accordingly are considered to contain essentially covalent bonds, which utilize six of the outer orbitals. These compounds include ferrihaemoglobin cyanide (2·49), ferrihaemoglobin azide (2·84), ferrihaemoglobin hydrosulphide (2·26), ferrihaemoglobin imidazole (approximately 2), and the ferrihaemoglobin hydroxide-ammonia complex (2·98).[3,4,5]

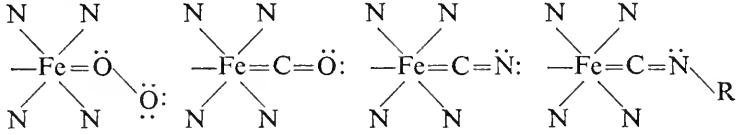
The moment of ferrihaemoglobin hydroxide (alkaline methaemoglobin) is observed to be 4·45. This value is approximately equal to the spin moment for three unpaired electrons, 3·88 magnetons, and the structure of this complex has been considered to be one in which five of the nine outer orbitals of the iron atom are involved in covalent bond formation, leaving only four orbitals for occupancy by the five unpaired electrons, corresponding to three unpaired electron spins in the complex.

THE POWER OF COMBINING WITH OXYGEN AND CARBON MONOXIDE

It has recently become possible to formulate a structural explanation of the power of specific combination of oxygen and carbon monoxide that is possessed by haemoglobin.[6] This explanation is based on a new postulate, the postulate of the approximate electrical neutrality of all atoms in stable compounds.[7] We assume that in a stable molecule, complex ion, or crystal the electronic structure is such as to associate with each atom the number of electrons that makes the residual electrical charge of the atom zero or, at the most, a small fraction of an electronic charge. Thus in the hexahydrated ferrous ion, $[Fe(OH_2)_6]^{++}$, each iron atom forms six bonds with the six oxygen atoms of the surrounding water molecules, and each of the six bonds has about one-third covalent character and two-thirds ionic character, as given by the electronegativity values of iron and oxygen, iron having a value about 1·5 on the electronegativity scale.[8] The formation of six bonds with one-third covalent character, using electron pairs originally belonging to the water molecules, transfers a total negative charge 2 − to the iron atom, which is just sufficient to neutralize the positive charge originally present on the ferrous ion. Similarly the ionic character of the oxygen-hydrogen bonds then transfers the total positive charge to the twelve hydrogen atoms on the periphery of the complex. In the ferrohaem group of ferrohaemoglobin the bonds between the iron atom and the four nitrogen atoms of the porphyrin ring have about 50 per cent covalent character, corresponding to the difference in electronegativity of iron and nitrogen. These four bonds between iron and nitrogen accordingly transfer two negative charges to the ferrous atom, neutralizing its charge exactly, in the same way

as six iron-oxygen bonds. It is interesting to note that the structure of the porphyrin molecule is such as to involve resonance among a number of structures, each of which places a double bond and a single bond with adjacent carbon atoms for two of the nitrogen atoms, and two single bonds to carbon for the other two nitrogen atoms ; each nitrogen atom is then given zero charge by forming a bond with 50 per cent covalent character with the iron atom.

Inasmuch as the iron atom of the ferrohaem group has achieved electrical neutrality through its formation of the four bonds with nitrogen, it will tend to resist the formation of further bonds in which electron pairs are donated to it by groups to be attached to it, even though there are two coordination positions, above and below the plane of the porphyrin group, available for additional ligands. Water molecules, chloride ions, hydroxide ions, and similar groups would in this way be kept from combining with the iron atoms of ferrohaemoglobin. Certain molecules, however, including oxygen, carbon monoxide, cyanide ion, and the alkylisocyanides, have such a structure as to permit them to combine with this group without destroying the electrical neutrality of the iron atom. These molecules can form a double bond with the iron atoms, using two electrons of their own and two electrons of the iron atom. The electronic structures that would be written for the complexes of these molecules with the ferrohaem group are the following :



THE HAEM-LINKED ACID GROUPS

Our knowledge of the haem-linked acid groups of haemoglobin is summarized in *Table I* (from Ref. 10). The explanation given above of the ability of ferrohaemoglobin to form compounds with only a few molecules also provides an explanation of the linking between the haem group and an acidic group, the imidazole ring of a histidine side chain, which has been suggested as the important haem-linked group[1,9,10] leading to $pK_2 = 7{\cdot}83$ for ferrohaemoglobin and 6·80 for oxyhaemoglobin and carbonmonoxyhaemoglobin. In ferrohaemoglobin itself there would be only a small tendency of an iron atom to form a covalent bond with a nitrogen atom of the imidazole ring, because its electrical charge has been made zero by the formation of the four bonds in the ferrohaem complex. However, when a new double bond is formed, with carbon monoxide or the oxygen molecule,

a positive charge is transferred to the iron atom as a result of the attraction of the strongly electronegative atom oxygen for the electrons in the double bond, this attraction being transmitted through the carbon atom in the case of carbonmonoxyhaemoglobin. The iron atom would then be enabled to form a bond with partial covalent character with another nitrogen atom, receiving from this bond enough negative charge to neutralize the positive charge given to it by the partial ionic character of the double bond. The positive charge transferred to the nitrogen atom would then be shifted to the other nitrogen atom of the imidazole ring, through the imidazole system of conjugated double bonds, and would have the effect of increasing the acidity of the imidazole group.[10]

Table I

Haem-linked Acid Groups in pH *Range* 4·5 *to* 9[a]

Hb^+	$pK_1 = 5{\cdot}3$ *Mo*	$pK_2 = 6{\cdot}65$ *Si, Mi, Po*	$pK_3 = 8{\cdot}10$ *Si, Mo*
Hb	$pK_1 = 5{\cdot}25$ *Mi*		$pK_2 = 7{\cdot}83$ *Si, Mi*
HbO_2	$pK_1 = 5{\cdot}75$ *Mi, Po*	$pK_2 = 6{\cdot}80$ *Si, Mi, Po*	
HbCO			

[a] *S, M, P mean spectrophotometrically, magnetically, and potentiometrically, respectively ; o means operative, i inoperative. All acid groups are of course potentiometrically operative, except when cancellation of the effects of two groups occurs, as indicated by Pi for bracketed pairs.*

There is another haem-linked acid group in haemoglobin, with acid constant $pK_1 = 5{\cdot}25$ for ferrohaemoglobin, 5·3 for ferrihaemoglobin, and 5·75 for oxyhaemoglobin. It has been suggested[1,9,10] that this group also is an imidazole group of a histidine residue, held by the structure of the globin molecule in a position somewhat removed from the iron atom ; but the nature of the interaction between the haem group and this acid group has not yet been determined. No explanation has been offered of the fact that ionization of this group changes the magnetic moment of ferrihaemoglobin significantly, but does not have an observable magnetic effect on ferrohaemoglobin and its compounds.

The acid group giving $pK_3 = 8{\cdot}10$ for ferrihaemoglobin presumably is a coordinated water molecule, which is converted into a hydroxide ion by loss of a proton.

THE HAEM–HAEM INTERACTIONS

Reliable evidence about the nature of the structural mechanism of the haem–haem interactions that are responsible for the sigmoid character of the oxygen equilibrium curve of haemoglobin and that affect other haemoglobin equilibria[11] has not yet been obtained. The possibility that the interactions take place through the conjugated system of double bonds, and involve the vinyl side chains of the protoporphyrin molecules, is made unlikely by the absence of detectable spectroscopic or magnetic effects.[12] A more likely mechanism may be suggested : steric hindrance (possibly by the imidazole ring responsible for pK_1), which interferes with the apposition of a diatomic molecule to the iron atom of a haem group, and which is decreased in its effect for a second haem by the loosening of the structure caused by the conversion of a first haem to oxyhaem or carbonmonoxyhaem.

THE MAGNETIC MOMENTS OF HAEMOGLOBIN AND ITS DERIVATIVES

The magnetic moments found by experiment for haemoglobin and its derivatives correspond roughly to the values of the spin moment for the numbers of unpaired electrons described in the foregoing discussion of their electronic structure, but the quantitative agreement is poor, and no explanation of the discrepancy has previously been advanced, except the general one that the total magnetic moment is due in part to the orbital motion of the electrons.

It may be expected that the theoretical interpretation of the observed moments would throw further light on the structure of these molecules.

Let us first consider the compounds of haemoglobin that are usually thought to contain one odd electron per haem group. These include ferrihaemoglobin cyanide, with magnetic moment 2·49 magnetons, ferrihaemoglobin azide, with magnetic moment 2·84, ferrihaemoglobin hydrosulphide, with moment 2·26, imidazole-ferrimaemoglobin, with moment approximately 2, and probably also ferrihaemoglobin hydroxide-ammonia, with moment 2·98. All of these moments are considerably larger than the value 1·73 that corresponds to the spin of one electron ; the only haem compound giving good agreement with this value is nitric oxide haemoglobin, for which the reported moment is 1·7 magnetons. Let us assume that in the approximately octahedral field of the complex the orbital moment is not quenched, but is combined with the spin moment to a resultant total angular momentum, given by a total quantum number J. We also assume that J is a constant of the motion of the system, but that the orbital quantum number, L, need not be a constant of the motion ; that is,

we assume that it is possible for the actual state of the system to be a hybrid of two or more Russell-Saunders states of the atom, with the same J value, and presumably the same value of the spin quantum number, S.

For a complex such as that in the ferrihaemoglobin cyanide molecule the odd electron might be expected to occupy one of the 3*d* orbitals, the other eight outer orbitals of the iron atom being used in the formation of covalent bonds or occupied by electron pairs. The normal spectroscopic state would then be $^2D_{5/2}$, with magnetic moment 3·55. The maximum value of J would be expected, according to Hund's rule, because the electronic configuration corresponds to the second half of a partially filled 3*d* subshell. If, however, the odd electron were promoted to a 4*p* orbital, the 3*d* orbital being made available for use in bond formation, the spectroscopic state would be $^2P_{3/2}$, with magnetic moment 2·58 Bohr magnetons, and hybridization would be expected between this state and the state 3*d* $^2D_{3/2}$. The magnetic moment for $^2D_{3/2}$ is 1·55. (It seems likely that the *s* orbital would be utilized entirely for bond orbitals ; if, however, the odd electron were to occupy the 4*s* orbital, the spectroscopic state would be $^2S_{1/2}$, with moment 1·732, which might hybridize with $^2P_{1/2}$ and $^2D_{1/2}$ to produce states with smaller moments.) The two reasonable alternatives thus are $^2D_{5/2}$, with moment 3·55, and a hybrid of $^2P_{3/2}$ and $^2D_{3/2}$, with moment between 2·58 and 1·55, depending upon the relative amounts of *p* and *d* character of the orbital occupied by the odd electron. We see that the state with $J = 5/2$ is ruled out by the lack of agreement of the predicted moment and the observed moment for the ferrihaemoglobin compounds, and it seems reasonable to assume accordingly that ferrihaemoglobin cyanide and similar compounds are in states with total angular momentum quantum number $J = 3/2$. The reported magnetic moments of ferrihaemoglobin cyanide and ferrihaemoglobin hydrosulphide lie within the predicted range, and indicate that the odd electron is mainly in the 4*p* orbital. The value for ferrihaemoglobin azide is high, although possibly the discrepancy is within experimental error. That for the ferrihaemoglobin hydroxide-ammonia complex involves an extrapolation, and hence may be unreliable.

It is interesting to mention that the moment of the ferricyanide ion, which has been carefully determined, is 2·33, which corresponds to a resonating structure with 24 per cent *d* character and 76 per cent *p* character for the odd electron.

The moment for ferrihaemoglobin itself is 5·44 magnetons.[13] In this structure there are six outer electrons. If they all occupied 3*d* orbitals, the configuration being d^6, the allowed spectroscopic state would be ^5D, presumably with $J = 4$, leading to moment 6·72. If one odd

electron were promoted to a p orbital, giving configuration d^5p (with one d orbital used for bonding), there might occur hybridization between the states $^5P_3$, $^5D_3$, and $^5F_3$, with moments 5·78, 5·19, and 4·32 respectively. The observed moment indicates that this hybrid does represent the structure of the iron complex in ferrihaemoglobin, and also the structure of the hexahydrated ferrous ion, which has reported moment 5·2.

In ferric compounds the configuration d^5 leads to a $^6S_{5/2}$ state, with moment 5·92. This structure might interact with $^6P_{5/2}$ and $^6D_{5/2}$ from the configuration d^4p, their moments being 5·58 and 4·90 respectively. The observed moments 5·92 for the hexahydrated ferric ion, 5·90 for ferrihaemoglobin fluoride, and 5·89 for the ethanol-ferrihaemoglobin complex indicate that for these complexes the configuration is very close to d^5. For ferrihaemoglobin itself, with moment 5·77 in solutions of moderate acidity and 5·46 in very acid solution, an increasing contribution of the configuration d^4p for the odd electrons is indicated by the change in magnetic properties.

The magnetic moment of ferrihaemoglobin hydroxide, 4·45, suggests that there are three unpaired electrons in the complex. This situation would result in case that five orbitals were used for bonds, leaving four orbitals for occupancy by the five outer electrons of the ferric atom. The configuration d^7 leads to $^4P_{5/2}$ as the normal state, with moment 4·73. Resonance with $d^7{}^4F_{5/2}$ (moment 3·04), or with other states based on promotion of an odd electron to a p orbital, might explain the observed moment.

It is evident from the foregoing discussion that the possibilities of progress in the understanding of the structure of haemoglobin through the investigation of its magnetic properties have not been exhausted. Haemoglobin is one of the most interesting and important of all substances, and even the great amount of work that would be needed for a complete determination of its structure, involving the exact location of each of the many thousands of atoms in its molecule, would be justified.

Received November 1948

REFERENCES

[1] CONANT, J. B. *Harvey Lect.* 28 (1932) 159
[2] PAULING, L. and CORYELL, C. D. *Proc. nat. Acad. Sci., Wash.* 22 (1936) 210
[3] CORYELL, C. D., STITT, F. and PAULING, L. *J. Amer. chem. Soc.* 59 (1937) 633
[4] — and STITT, F. *J. Amer. chem. Soc.* 62 (1940) 2942
[5] RUSSELL, C. D. and PAULING, L. *Proc. nat. Acad. Sci., Wash.* 25 (1939) 517
[6] PAULING, L. *Stanford Med. Bull.* 6 (1948) 215
[7] — *The Valences of the Transition Elements, Victor Henri Memorial Volume* Desoer, Liège, 1948

[8] PAULING, L. *The Nature of the Chemical Bond* Cornell University Press, 2nd edition, 1940, Chapter II
[9] WYMAN, J., Jr. *J. biol. Chem.* 127 (1939) 581
[10] CORYELL, C. D. and PAULING, L. *J. biol. Chem.* 132 (1940) 769
[11] — *J. phys. Chem.* 43 (1939) 841
[12] — PAULING, L. and DODSON, R. W. *J. phys. Chem.* 43 (1939) 825
[13] TAYLOR, D. S. and CORYELL, C. D. *J. Amer. chem. Soc.* 60 (1938) 1177

The Kinetics of Haemoglobin in Solution and in the Red Blood Corpuscle

F. J. W. ROUGHTON, J. W. LEGGE* and P. NICOLSON

1 The rate of reaction of X with haemoglobin, where X = O_2 or CO, has been expressed by the equation d [X][Hb] /dt = k' [X][Hb] − k [X Hb]. At high [X Hb] *the equation fails, for k' is now found to rise and k to fall. The discrepancy is explained on the intermediate compound hypothesis. Discrepancies might also occur if haemoglobin at zero time is partially saturated, instead of fully saturated or fully reduced as in most previous work. None were, however, found except in the dissociation of* O_2Hb *at neutral* pH. *Here the reaction seems to occur in two phases, the first fast, the second slow. Apparently this effect is largely due to formation of choleglobin and other secondary products, presence of which interferes with the photo-electric estimation of haemoglobin saturation.*

2 Mathematical procedures are outlined for investigating the rate of passage of O_2 *and* CO *in and out of a layer of haemoglobin solution, of the same thickness and concentration as the red blood corpuscle, and bounded by a membrane containing no haemoglobin. By these methods, and with new experimental data, it is possible to estimate the permeability of the corpuscle membrane to* O_2 *and* CO. *The further scope of the work is discussed.*

DURING the sixteen years before World War II, a large number of observations were reported by H. HARTRIDGE, F. J. W. ROUGHTON and G. A. MILLIKAN[1–11] on the kinetics of the rapid reactions of haemoglobin with oxygen and carbon monoxide. SIR JOSEPH BARCROFT was always deeply interested in this work and, indeed, spent an appreciable part of the last morning of his life in discussing certain aspects of it with the three authors of the present paper. For this reason, as well as for its interest in regard both to special and general problems of haemoglobin, we have felt it not inappropriate to contribute this article to the Barcroft Memorial Conference and Volume. A preliminary account of this work was given at the International Congress of Physiology at Oxford in July 1947.

Work on the kinetics of haemoglobin was almost completely interrupted by World War II, and it was not till January 1947 that we were able to take up the subject again in Cambridge. A review of the pre-war work showed that there were many points in the kinetics of haemoglobin, both in solution and in the red blood corpuscle, which had not been fully worked out or understood. During the past 18 months we

* This work was carried out during the tenure of a Fellowship from the Trustees of the Estate of the late Sir Henry S. Wellcome, to whom J. W. L. wishes to express his thanks.

have tackled some of these outstanding matters both by means of new experimental work (J. W. L. and F. J. W. R.) and also by further theoretical investigation (P. N. and F. J. W. R.). In this paper we shall summarize the advances which we have recently made in the kinetics of haemoglobin, first in homogeneous solution and secondly in the intact red blood corpuscle. All the work has been done with a modification of the Millikan[8] photo-colorimetric method, and the haemoglobin solutions and corpuscle suspensions have all been prepared from sheep blood, since this was the main species used in the previous kinetic work and in the case of adult sheep the haemoglobin is believed not to split into sub-units at the dilutions used (*i.e.* 1 part of blood to 60 parts buffer solution).

THE KINETICS OF HAEMOGLOBIN IN SOLUTION

The earlier work[1-11] seemed to show that the rates of the reactions could be expressed, within the limits of the rather large experimental errors, by the equation put forward by Hartridge and Roughton, namely

$$\frac{d[\mathrm{XHb}]}{dt} = k'[\mathrm{X}][\mathrm{Hb}] - k[\mathrm{XHb}] \qquad \ldots.(1)$$

where [X] = concentration of dissolved O_2 or CO

[XHb] = concentration of oxyhaemoglobin or carboxyhaemoglobin expressed in g mil combined O_2 or CO per litre

[Hb] = concentration of reduced haemoglobin expressed in same units as [XHb]

and k', k are the velocity constants of the reaction.

There are several points about equation (1) which call for further thought and inquiry.

Reconciliation with the intermediate compound hypothesis— Equation (1) suggests that the molecule of mammalian haemoglobin combines reversibly with 1 molecule of X, whereas it has been known for 25 years that the haemoglobin molecule actually combines with 4 such molecules. How does (1) fit in with Adair's intermediate compound hypothesis, which is now widely, if not generally, accepted ? On this theory, the reaction proceeds in four stages :—

$$\mathrm{Hb_4} + \mathrm{X} \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} \mathrm{Hb_4X}, \quad \frac{k_1'}{k_1} = \mathrm{K_1} = \textit{equilibrium constant of reaction} \qquad \ldots.(2.1)$$

$$\mathrm{Hb_4X} + \mathrm{X} \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} \mathrm{Hb_4X_2}, \quad \frac{k_2'}{k_2} = \mathrm{K_2} = \textit{equilibrium constant of reaction} \qquad \ldots.(2.2)$$

$$Hb_4X_2 + X \underset{k_3}{\overset{k_3'}{\rightleftharpoons}} Hb_4X_3, \quad \frac{k_3'}{k_3} = K_3 = \textit{equilibrium constant of reaction} \qquad \ldots.(2.3)$$

$$Hb_4X_3 + X \underset{k_4}{\overset{k_4'}{\rightleftharpoons}} Hb_4X_4, \quad \frac{k_4'}{k_4} = K_4 = \textit{equilibrium constant of reaction} \qquad \ldots.(2.4)$$

k_1', k_2', k_3', k_4' are the respective combination velocity constants of the four successive reactions, and k_1, k_2, k_3, k_4 the corresponding dissociation velocity constants. These equations lead to the equilibrium equation

$$\frac{y\ (\textit{percentage saturation})}{100} = \frac{[XHb]}{[XHb] + [Hb]}$$

$$= \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4\,(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)} \qquad \ldots.(3)$$

where p = partial pressure of X.

In an adjoining paper, one of us (F. J. W. R.) makes an attempt to describe *both* the kinetics and equilibria of the haemoglobin reactions in terms of the intermediate compound hypothesis.

The range of validity of equation (1)— Equation (1) has been most thoroughly tested as regards the dissociation kinetics by MILLIKAN[8, 9], who has shown that over the range 75 per cent O_2Hb to 0 per cent O_2Hb the experimental data yield constant values of k. The range above 75 per cent O_2Hb has not, however, been properly considered (see below). As regards the combination kinetics, the reactions of haemoglobin with CO are more suited for study than the reactions with O_2, since the velocities of the former are much slower, and larger variations in concentrations of the reagents are feasible. ROUGHTON's kinetic data[7] conform very satisfactorily with equation (1) for the first *half* of the process of combination with CO, but the agreement was not seriously tested over the second half of the reaction.

Unfortunately the kinetic data in the missing ranges are few and far between, but the analysis of them now to be given points to the need of important modifications of equation (1) at higher values of [XHb], both as regards the dissociation and the combination kinetics.

The rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ is known to show a lag period at the beginning of the reaction[2], but this has hitherto been attributed entirely to the fact that the dissociation is a two-stage process,

viz.

$$O_2Hb \rightleftharpoons O_2 + Hb \qquad \ldots.(4.1)$$

$$O_2 + Na_2S_2O_4 \rightarrow \textit{oxidation products of } Na_2S_2O_4 \qquad \ldots.(4.2)$$

Until the $Na_2S_2O_4$ has reduced the dissolved O_2 concentration to a low enough value, the dissociation of O_2Hb in reaction (4.1) will be opposed by the back reaction. On this theory, the lag period (at *p*h 9–10, temp. 10–20°C) should certainly be over by the time the percentage O_2Hb has dropped to 90 per cent, for at this percentage saturation the maximum amount of oxygen which could exist in physical solution would be very small compared with that in chemical combination (detailed calculations are given by Roughton, 1949). *Table I* shows the calculated value of k for various saturation ranges in two recent experiments on sheep haemoglobin solution at *p*H 10·0. It is clear that k does not attain its constant plateau value until about one-third of the reaction is completed. This point was missed by Hartridge and Roughton[2], because their method of observation was not available above 70 per cent saturation. Some of Millikan's results show a similar trend to that of *Table I*, but unfortunately he only recorded very few data in the upper range.

Table I

Relation of velocity constant, k, to range of saturation used for calculation

Experiment A (13.3.47)				
*p*H 10·0, temperature 10·7°C.				
Saturation range	87% to 71% O_2Hb	71% to 35%	35% to 20%	60% to 14%
Time in seconds	0·11	0·16	0·14	0·31
Calculated value of k	1·8	4·5	4·0	4·7
Experiment B (27.3.47)				
*p*H 10·0, *temperature* 16·8°C				
Saturation range	85% to 68%	68% to 53%	53% to 31%	31% to 15%
Time in seconds	0·07	0·035	0·048	0·07
Calculated value of k	4·2	7·2	11·0	10·5

Let us turn now to the kinetics of combination of CO with haemoglobin. Re-examination of Roughton's data[7] shows that in some cases the COHb concentration rises faster after the half-way stage has been passed than would be expected on the basis of a constant value of k'

in equation (1). None of his data are nearly as adequate, however, for quantitative tests in this respect as those subsequently obtained by Bateman and Roughton in a control experiment[11], carried out with Millikan. In this experiment the speed of the reaction was measured both thermally and photo-colorimetrically with exactly the same solutions, and concordant results were obtained over the whole range studied, which amounted to about 85 per cent of the course of the reaction. The validity of equation (1) in respect of these data is tested by plotting

$$\frac{1}{\alpha - \beta} \ln \frac{\alpha - y}{\beta - y} \textit{ against time, } t$$

where α = total concentration of CO dissolved and combined

β = total concentration of haemoglobin } expressed in same
y = concentration of COHb at time t } units as α.

For, if the back reaction term in equation (1), *i.e.* $- k$ XHb, be neglected (as is legitimate in the case of the CO + Hb reaction) integration of (1) leads to the equation $k't = \frac{1}{\alpha - \beta} \ln \frac{\alpha - y}{\beta - y} + \textit{constant}$ (Roughton[7])

and the data, if plotted as in *Figure* 1, should give a straight line inclined to the time-axes at angle whose tangent is proportional to k'. *Figure 1*, however, shows that this is only true for the six earliest

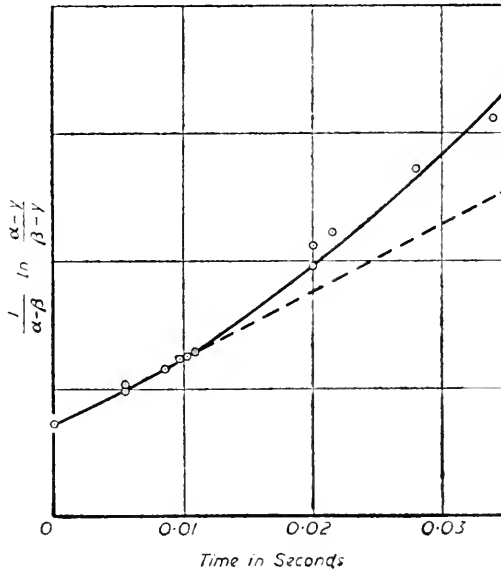


Figure 1. *Test of equation* d [COHb]$/dt = k'$ [CO] [Hb] *on data of Bateman and Roughton.*

points, which in fact cover the first half of the reaction. The later points all fall above the line, and indicate that the calculated value of k' tends to rise to 30 per cent or 40 per cent above its initial value.

Although more data are certainly necessary, those at present available leave little doubt that equation (1) needs to be modified at high values of [XHb], both in respect of the term involving k and of that involving k'. Later it will be shown that the effects so far observed are just what would be expected on a simple extension of the intermediate compound hypothesis.

The effect of partial saturation at time zero on the kinetics of the reaction— In all the previous kinetic work of Hartridge, Roughton and Millikan the haemoglobin at the start of the reaction was either as completely reduced or as completely saturated (with O_2 or CO) as possible. No tests have hitherto been made as to whether the reaction velocity curves obtained with partially saturated haemoglobin at the starting point agree with those found with the fully saturated or fully reduced haemoglobin. A discrepancy would be of much interest, especially from the standpoint of the intermediate compound hypothesis. For it would imply that the concentration of the various intermediates for a given percentage saturation differ appreciably according to whether the system is at equilibrium or in a state of flux at the time at which the particular saturation in question is reached. The matter is also of physiological interest, for *in vivo* the state of combination of the haemoglobin varies cyclically during the circulation, but never reaches either complete saturation or complete reduction. Finally, work of this kind might throw light on some of the anomalies observed by Roughton in the reactivity of freshly-formed haemoglobin compounds[7]. In 1947 we therefore carried out the following tests :—

1 The rate of combination of CO with fully reduced sheep haemoglobin (concentration 0·2–0·3 gm/100 ml) was compared with the rate of combination of CO with the same haemoglobin already 20–30 per cent saturated with CO at the start. Neither at *p*H 10 nor at *p*H 6·8 could any difference be observed in the rate of combination, provided proper allowance was made for slight variations in the concentrations of the reagents.

2 The rate of dissociation of O_2Hb in presence of $Na_2S_2O_4$ was measured at (a) 99–100 per cent initial saturation, (b) 50–60 per cent initial saturation. At *p*H 10·0, the points obtained in case (b) fell within experimental error on the curve for case (a), but at *p*H 6·8 the initial rate of dissociation in the case of the partially saturated haemoglobin was somewhat faster than was to be expected from the curve with the fully saturated haemoglobin (*Figure 2*). The effect is, however, overshadowed by, and probably

connected with, the fact that the dissociation appears to proceed in two phases, a rapid one from 100 to 25 per cent O_2Hb taking about 0·07 seconds and then a slow one from 25 to 0 per cent O_2Hb lasting several seconds. This remarkable effect is discussed in the next section.

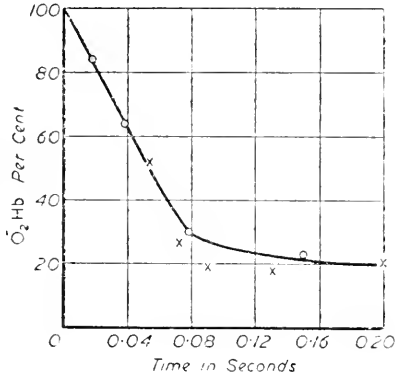


Figure 2. *Rate of dissociation of* O_2Hb *in presence of* $Na_2S_2O_4$ *at p*H 6·8, 15·8° C o = 100% O_2Hb *at zero time*, x=51% O_2Hb *at zero time.*

*The diphasic dissociation of oxyhaemoglobin at neutral p*H— We were at first led to suppose that this effect was due to one of the haems of the molecule behaving in a quite different way from the others at neutral *p*H. A much fuller investigation, however, showed great variability in the percentage saturation at which the break occurred, the ' plateau ' (as we have called it) varying from 33 per cent O_2Hb to 0 per cent O_2Hb, in the *p*H range 6·1 to 7·4, temperature 10–20°C. In the case of the corpuscle suspensions, the plateau in three cases out of four was non-existent and in the fourth case was only at *ca* 9 per cent O_2Hb. This led us to the view that the apparent ' plateau ' must be associated with the formation of abnormal pigments due to the action of $Na_2S_2O_4$, or more probably its oxidation products, upon the haemoglobin. Such compounds would clearly upset the photocolorimetric determination of the per cent O_2Hb in the running fluids in the rapid reaction observation tube.

Two pieces of definite evidence were obtained in favour of this view. In cases where a low ' plateau ' was observed it was found that the photo-colorimetric reading obtained with a mixture of the oxyhaemoglobin and buffered $Na_2S_2O_4$, which had been allowed to stand for 30 seconds, agreed with that obtained with a similar mixture of the fully reduced haemoglobin (prepared by shaking *in vacuo*) and the $Na_2S_2O_4$. On the other hand, in cases of a high ' plateau ', the reading with the O_2Hb-$Na_2S_2O_4$ mixture differed from that of the reduced Hb-$Na_2S_2O_4$ mixture, the former appearing to be even more ' reduced ' than the latter, and in one control experiment showing appreciable

amounts of choleglobin[12] or cruoralbin[13]. The reduced Hb-$Na_2S_2O_4$ mixture, in the same experiment, showed no choleglobin (as judged by the appearance of a band in the red at 620–630 mμ, when the mixtures were treated with CO-saturated, buffered $Na_2S_2O_4$). We have no explanation as to why this reaction occurred to such variable extents, but the correlation between it and the apparent ' plateau ' was striking. In our final pair of experiments (9 and 10 March 1948) choleglobin formation and a ' plateau ' at *ca* 18 per cent O_2Hb were seen on the first day, but on storing the blood overnight both these effects were found next day practically to have disappeared. The apparent high ' plateaux ' may thus be, in large measure, artefacts due to the effect on the photo-colorimetric readings of the development of pigments other than O_2Hb or reduced Hb. At present, therefore, we do not feel that we can be sure of the presence of appreciable amounts of oxyhaemoglobin during the apparently high ' plateau ' periods, and unless independent evidence in favour of it is secured, preferably by gasometric rather than by optical methods, it would not be profitable to discuss further the possible bearing of the apparent diphasic dissociation at neutral *p*H on other properties of haemoglobin. Although this work, on which we have spent much time and energy, has been disappointing, we have thought it well to report it in some detail in view of the wide use of $Na_2S_2O_4$ for studying the dissociation of oxyhaemoglobin and other oxygen-carrying pigments. It is a pity that no alternative O_2-absorbing agent has been, or is at present, available with which to compare the results obtained with $Na_2S_2O_4$ at neutral *p*H, valuable and in many ways satisfactory though this reagent has been.

THE KINETICS OF HAEMOGLOBIN IN THE RED BLOOD CORPUSCLE

The first measurements on the rate of combination of haemoglobin in the intact red blood corpuscle with O_2 and CO were made by Hartridge and Roughton[4]. Later and rather more accurate results, including also data on the rate of dissociation of carboxyhaemoglobin and oxyhaemoglobin in the corpuscle, were subsequently obtained[7, 14, 15]. *Table II* gives a summarized comparison of the various reactions, measured under comparable conditions for adult sheep haemoglobin, in the red blood corpuscle and in homogeneous solution (blood 1 in 60 about). It will at once be noted that the reactions in the corpuscle take longer than in solution, except the very slow dissociation of carboxyhaemoglobin.

This difference in speed might be due to one or more of several possible factors :—

1 Presence of gradients of dissolved gas in the fluid surrounding the corpuscles, thus leading to a retardation owing to the limiting effect of diffusion.

2 Delay caused by the time of diffusion through the membrane surrounding the corpuscle.

3 Delay due to the finite thickness of the corpuscle, the first dissolved gas to enter combining with haemoglobin on the periphery of the corpuscle, and succeeding dissolving gas having to penetrate a finite distance into the corpuscle before combination occurs.

4 Differences between the chemical kinetics of the reactions of haemoglobin in dilute solution and its concentrated condition inside the red blood corpuscle.

Table II

Comparison of speed of various reactions for adult sheep haemoglobin in the red blood corpuscle and in homogeneous solution

	Average time for half reaction in seconds (10–20°C)		
	(a) *In corpuscle*	(b) *In solution*	*Ratio* (a)/(b)
$O_2 + Hb \rightarrow O_2Hb$ (at $pO_2 = 75$ mm)	0·05	0·004	12·5
$CO + Hb \rightarrow COHb$ (at $pCO = 75$ mm)	0·15	0·06	2·5
$O_2Hb \rightarrow O_2 + Hb$ (pH 6·8)	0·21	0·034	6·2
$COHb \rightarrow CO + Hb$	60·0	60·0	1·0

Hartridge and Roughton's control experiments[4] seemed to show conclusively that, under the conditions of their reaction velocity experiments, the fluid between the corpuscles must have remained so well stirred that no appreciable concentration gradients of dissolved gas could have existed therein (*see also Roughton*[6] see pp 3 and 4). If these be accepted, factor *1* is ruled out. The fact that the difference between the observed overall rate in the corpuscle and in solution disappears when the reaction is slow enough, *i.e.* in the very slow dissociation of carboxyhaemoglobin, suggests that there is no appreciable difference in the chemical kinetics *per se*, and that the observed discrepancy must be conditioned by factors *2* and *3*, in both of which the rate of diffusion has a limiting effect. Another rather slow reaction, *viz.* the rate of displacement of O_2 from combination with Hb by CO, was also found[14] to proceed at the same rate in the corpuscle (blood

of man and dog) and this provides further evidence in favour of our present contentions.

Roughton investigated[6] the importance of factor *3* by itself, by calculating the rate at which O_2 or CO would penetrate into a layer of haemoglobin solution of the same haemoglobin concentration and average thickness as pertain to the red blood corpuscle, but without any bounding membrane to the layer. His approximate calculations indicated that factor *3* would be expected to cut down the rate of the reaction $O_2 + Hb \rightarrow O_2Hb$ to about one quarter of its rate in homogeneous solution, whereas the slower reaction $CO + Hb \rightarrow COHb$ should only be cut down to about two-thirds of its solution rate. Factor *3* should thus be responsible for a significant part, but by no means the whole, of the discrepancy between the rates in the corpuscle and in solution. The remainder was attributed to factor *2*, *i.e.* the limiting effect of diffusion through the membrane surrounding the red blood corpuscle, but the mathematical technique available at the time was insufficient for factors *2* and *3* to be tackled jointly.

During the past two years newer methods of calculation based on the calculus of finite differences[16], [17] have been applied by one of us (P. N.) to the problem. Sufficiently exact numerical solutions have been obtained both for the particular conditions under which the experimental data have so far been obtained, and also for more general cases. The success of this theoretical investigation has encouraged two of us (J. W. L. and F. J. W. R.) to secure new and more accurate comparisons on the rate of combination of CO with sheep haemoglobin in homogeneous solutions and in corpuscle suspensions, prepared from the same blood. No new data have as yet been obtained on the more physiologically interesting reaction of O_2 with haemoglobin, since the latter proved, both on technical and on theoretical grounds, to be less suitable for exact work than the CO + Hb reactions. We have also obtained new experimental and theoretical data on the comparative rates of dissociation of O_2Hb in the corpuscle and in solution. As a result it has become possible to put forward tentative calculations as to the actual permeability of the corpuscle membrane to CO and O_2, the latter of which is of course the most important substance that passes through the corpuscle membrane *in vivo*.

Our recent experimental data have been obtained essentially by the method of Hartridge and Roughton[4] but with the use of the Millikan photo-colorimeter[8], [9] for measuring the proportions of the various haemoglobin compounds in place of the Hartridge reversion spectroscope. The details of the theoretical assumptions, the mathematical equations and their numerical solutions are given in full in a current paper by Nicolson and Roughton[18] to which reference should be made

by those interested, since limitations of present space prevent any detailed description of them. All that can be done here is to give an outline of the assumptions, upon which the new mathematical investigation has been based, and of the more important results which it has yielded, together with a brief discussion of its future prospects.

In working out the rate of entrance of dissolved CO (or O_2) into the corpuscle, it is assumed that

1 The concentration of dissolved gas remains constant at the surface of the corpuscle membrane.

2 The corpuscle membrane is devoid of haemoglobin, and no chemical reaction, but only diffusion, takes place therein.

3 The diffusion of dissolved gas both through the membrane and the interior of the corpuscle obeys Fick's Law, according to which the quantity diffusing in unit time = concentration gradient × diffusion coefficient × area surface through which diffusion occurs.

4 Diffusion of haemoglobin within the corpuscle can be neglected owing to the size of the molecule and also (especially) to its close packing[19]. The haemoglobin is assumed to be uniformly distributed throughout the interior of the corpuscle.

5 The kinetics of the reaction in the corpuscle are given by $d[\mathrm{XHb}]/dt = k'\,[\mathrm{X}][\mathrm{Hb}] - k\,[\mathrm{XHb}]$, the values of the velocity constants k', k being the same as in solution.

6 The corpuscle is assumed to be equivalent to an infinitely extended parallel layer of haemoglobin, bounded by a haemoglobin-free membrane (for justification of this model see Roughton[6]). The thickness and concentration of the haemoglobin layer are supposed to be the same as the average thickness and concentration in the red blood corpuscle.

The results of the mathematical treatment based on these assumptions will now be given briefly.

Figures 3 and *4* show the calculated rate of uptake of CO and of O_2 by the haemoglobin layer without membrane, as compared with the observed rates in the case of homogeneous solutions. The dotted curves in the two figures show the maximum and minimum solutions previously obtained by Roughton[6]. All he could then say was that the true solutions must lie somewhere between the dotted curves, and it is interesting to find, with the modern methods of calculation, that they do in fact lie almost exactly half way between his extremes.

Figure 5A, B shows the observed results for the rate of uptake of CO by a haemoglobin solution and corpuscle suspension prepared from the blood of a pregnant ewe, kindly supplied by the late Sir Joseph Barcroft. *Figures 5C, 5D* show the calculated results, assuming

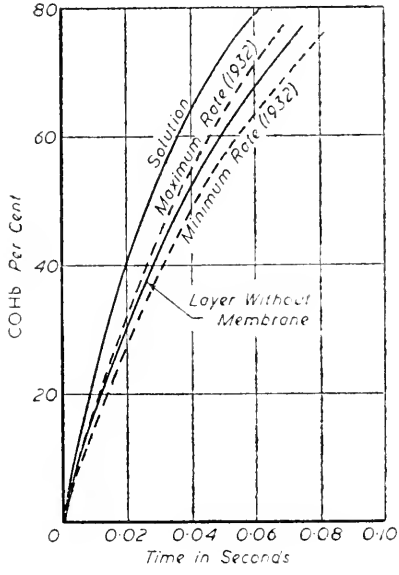


Figure 3. Calculated rate of CO *uptake by layer of haemoglobin solution of same thickness and concentration as in red blood corpuscle, compared with rate of* CO + Hb ⟶ CO Hb *in homogeneous solution (full lines). Dotted lines represent maximum and minimum solutions previously obtained by Roughton*[6].

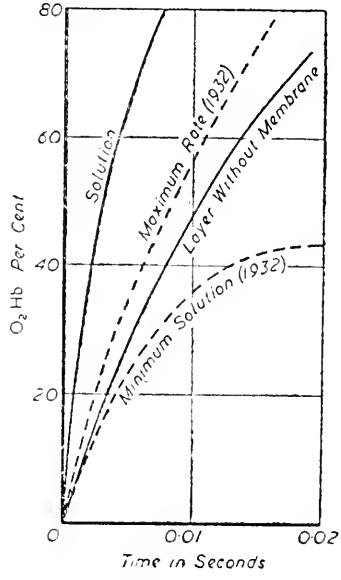


Figure 4. Calculated rate of O_2 *uptake by layer of haemoglobin solution of same thickness and concentration as in red blood corpuscle, compared with rate of* O_2 + Hb ⟶ O_2Hb *in homogeneous solution (full lines). Dotted lines represent maximum and minimum solutions previously obtained by Roughton*[6].

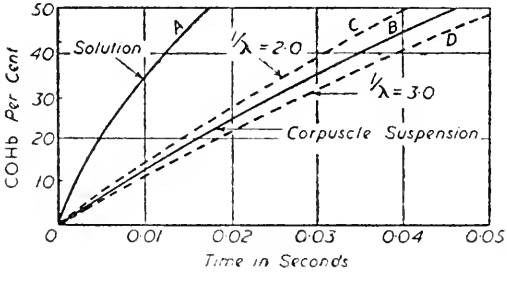


Figure 5. Observed rates of uptake of CO *by haemoglobin solution and corpuscle suspension of blood of pregnant ewe (full lines). Dotted lines represent calculated rates of uptake by layer of haemoglobin solution (of same thickness and concentration as in red blood corpuscle) enclosed by membranes with* $1/\lambda$ *values of* 2·0 *and* 3·0 *respectively.*

that the layer of concentrated haemoglobin is bounded by a membrane of two different permeabilities, indicated by $1/\lambda = 2{\cdot}0$ and $3{\cdot}0$ respectively. The observed results are fitted by assuming that $1/\lambda = 2{\cdot}6$. $1/\lambda$ is defined as equal to $D_1/b_1 \div D_2/b_2$

where D_1 = diffusion coefficient of interior of corpuscle to CO
D_2 = diffusion coefficient of corpuscle membrane to CO
$2b_1$ = thickness of interior of corpuscle
b_2 = thickness of corpuscle membrane.

b_1 for sheep red blood corpuscles may be taken as 7×10^{-5} cm. The value of b_2 is uncertain, but according to various biophysical estimates is of the order of 10^{-6} cms.

On this basis it follows that $D_1/7\times10^{-5} \div D_2/10^{-6} = 2{\cdot}6$ whence $D_2/D_1 = {\cdot}0055\ D_1$

The value of D_1 is not known, but is probably appreciably less than that for pure water. According to A. S. KROGH[20], the diffusion constant of D_2 through a 20 per cent gelatine gel = about 80 per cent of D_{O2} for water. We have provisionally assumed a similar value for D_1 inside the corpuscle. It is hoped later to test the validity of this postulate by direct determinations of D_{O2} and D_{CO} in 36 per cent solution of haemoglobin. The value of D_2, if our assumptions are correct, is thus not more that 1/200 the value of D_{CO} in water.

Figure 6 shows the calculated relations between the half time of the reaction θ and $1/\lambda$ (defined as above) for three reactions of widely varying rate, *viz.* $k' = 3{\cdot}3$ (corresponding to $O_2 + Hb \rightarrow O_2Hb$, with O_2 measured in partial pressures of dissolved gas in mm Hg),

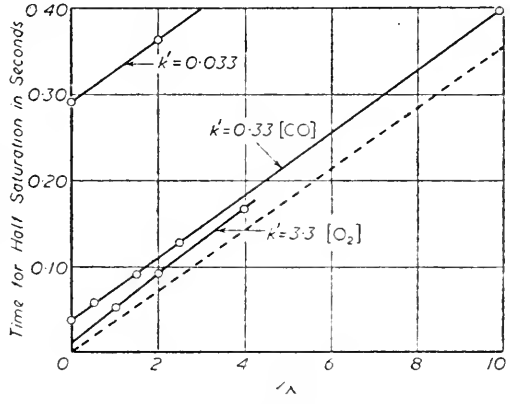


Figure 6. *Relation between calculated value of half time of saturation, θ, and $1/\lambda$, for three reactions of widely varying rate.*

$k' = 0{\cdot}33$ (corresponding to $CO + Hb \rightarrow COHb$) and $k' = 0{\cdot}033$. There is an almost linear relation in all three cases: the slope of the line is practically independent of the value of k', and is approximately

equal to $0{\cdot}5\ y_o b_1^2/D_1 p_b$ as can be shown theoretically should be the case[18]. In the latter expression y_o = concentration of haemoglobin in the interior of the corpuscle, expressed in the same units as p_b, the constant partial pressure of dissolved gas at the surface of the corpuscle. *Figure 6* applies to the special case of $b_1 = 7 \times 10^{-5}$ cm. The more general case (*i.e.* for a wide range of values of b_1 and k_1) has been worked out by Nicolson and Roughton (*q.v.*).

The detailed calculations for the rate of dissociation of oxyhaemoglobin in the corpuscle are more involved than for the rates of combination, but fortunately it is again found that a practically linear relation exists between $1/\lambda$ and the half time of the reaction. This simplification makes it possible for the procedure to be applied to many experimental data, without need of performing or knowing how to perform the finite difference calculations on which the treatment is based. With further developments of the rapid reaction velocity technique it is therefore hoped to determine the permeability of the red blood corpuscle to O_2 and CO under a wide variety of conditions—normal and pathological—and with only occasional expert mathematical help.

Table III

Summary of estimates of permeability of red blood corpuscle membrane (sheep) in CO_2*-bicarbonate-Ringer-Locke solution* (*p*H 6·8–7·1, *temp. ca* 15°C.)

		Corpuscle interior	*Corpuscle membrane*
Thickness		$1{\cdot}4 \times 10^{-4}$ *cm*	*ca* 1×10^{-6} *cm*
Diffusion coefficient		D_1	D_2
Reaction	*Blood species*	*Half reaction time in solution (sec)*	*Calculated value of* D_2/D_1
$CO + Hb \rightarrow COHb$	*Pregnant ewe A*	0·016	0·0055
(*p*CO=*ca* 260 *mm Hg*)	*Ram A′*	0·016	0·014
	Pregnant ewe B	0·020	0·005
	Ram B′	0·020	0·014
$O_2Hb \rightarrow O_2 + Hb$	*Ram I*	0·03	0·032
	Ram II	0·04	0·032
	Ram III	0·03	0·032

The results we have so far obtained are summarized in *Table III*. The difference between the pregnant ewe blood corpuscles and the

(non-pregnant [8]) ram corpuscles has already been referred to in the description of Sir Joseph Barcroft's last morning (p. 30). Clearly there is opportunity for much further work in this direction. The permeability to O_2, as estimated from the dissociation velocity experiments, is apparently distinctly higher but is of the same order as the rough average value (*i.e.* $D_2/D_1 = 0{\cdot}024$) which we have calculated from the early experiments on O_2 uptake by sheep haemoglobin solutions and corpuscle suspensions[6]. In these cases, however, the same blood was not used for the suspensions as for the solutions, and hence the calculated value of D_2/D_1 is much less certain.

In concluding this section, we should call attention to two points, wherein caution is required :—

1 In studying the kinetics of corpuscle suspensions outside the body, it is of course necessary to apply some anti-clotting measure to the drawn blood. In our work, this has usually consisted of defibrination. This probably alters the permeability of the membrane from its value *in vivo*, and at present there is no obvious way of gauging the degree of alteration. It will be a challenge for the future to devise some means of settling this matter, if possible.

2 Elsewhere in this paper it is shown that the kinetic equation $d[\mathrm{XHb}]/dt = k'[\mathrm{X}][\mathrm{Hb}] - k[\mathrm{XHb}]$ requires modification at high values of [XHb], since the calculated value of k' rises, and that of k, on the other hand, falls. As regards the combination velocity studies, the error due to a rise in k' in the later stages of the reaction must be to some extent compensated by the back reaction term, $k[\mathrm{XHb}]$, becoming appreciable. As the latter has been neglected in most of our calculations, it is not improbable that the results obtained on the simpler basis may be reasonably correct. In the dissociation velocity experiments and calculations, the saturation of the haemoglobin should not exceed 80 per cent at the outset, for at saturations below this figure k appears to be sensibly constant.

Received September 1948

REFERENCES

[1] HARTRIDGE, H. and ROUGHTON, F. J. W. *Proc. roy. Soc. B* 94 (1923) 336
[2] — — *Proc. roy. Soc. A* 104 (1923) 376, 395
[3] — — *Proc. roy. Soc. A* 107 (1925) 654
[4] — — *J. Physiol.* 62 (1927) 232
[5] ROUGHTON, F. J. W. *Proc. roy. Soc. A* 126 (1930) 470
[6] — *Proc. roy. Soc. B* 111 (1932) 1
[7] — *Proc. roy. Soc. B* 115 (1934) 451, 464, 473, 495
[8] MILLIKAN, G. A. *Ph.D. Thesis.* Cambridge University (1932)
[9] — *J. Physiol.* 79 (1933) 152, 158
[10] — *Proc. roy. Soc. B* 120 (1936) 366

[11] BATEMAN, J. B. and ROUGHTON, F. J. W. *Bio-chem. J.* 29 (1935) 2630
[12] LEGGE, J. W. and LEMBERG, R. *Bio-chem. J.* 35 (1941) 353
[13] HOLDEN, H. F. *Aust. J. exp. Biol. med. Sci.* 21 (1943) 159
[14] ROUGHTON, F. J. W. *Amer. J. Physiol.* 143 (1945) 609
[15] LEGGE, J. W. and ROUGHTON, F. J. W. In course of publication (1949)
[16] HARTREE, D. R. and WOMERSLEY, J. R. *Proc. roy. Soc. A* 161 (1937) 353
[17] CRANK, J. and NICOLSON, P. *Proc. Camb. phil. Soc.* 43 (1947) 50
[18] NICOLSON, P. and ROUGHTON, F. J. W. In course of publication (1949)
[19] PERUTZ, M. F. *Nature, Lond.* 161 (1948) 204
[20] KROGH, A. S. *J. Physiol.* 52 (1919) 391

The Intermediate Compound Hypothesis in Relation to the Equilibria and Kinetics of the Reactions of Haemoglobin with Oxygen and Carbon Monoxide

F. J. W. ROUGHTON

The various forms of the intermediate compound hypothesis which have appeared in the literature during the past twenty-five years are reviewed in light of the most satisfactory experimental data on the oxyhaemoglobin dissociation curve. Several of the special forms prove to be unserviceable and have to be rejected. A common feature of the remaining is that in each case very little ' interaction ' has to be assumed until three out of four of the O_2 or CO molecules have combined. It is then shown how in one such special case, the kinetics of the reactions of combination and dissociation can be rather successfully explained. Further work is, however, needed to see whether the selection of this special case is justified.

THE present paper is devoted to two main objects:

1 To review the existing position of the intermediate compound hypothesis in regard to the best available dissociation curve data;

2 To study further the relation between the empiric equations to which the kinetic data on the haemoglobin reactions conform and the requirements of the intermediate compound hypothesis.

Not until the kinetics of the reactions as well as the equilibrium are fully interpreted on the intermediate compound hypothesis can the position of the latter be regarded as satisfactory.

THE INTERMEDIATE COMPOUND HYPOTHESIS IN RELATION TO THE DISSOCIATION CURVE DATA

According to this hypothesis the general equation for the equilibrium between O_2 (or CO) and haemoglobin is given by

$$\frac{y}{100} = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4\,(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)} \quad \ldots.(1)$$

where y is the percentage saturation

p is the partial pressure of O_2 (or CO) in mm Hg

$K_1, K_2 \ldots$ are the equilibrium constants of the successive reactions $Hb_4 + X \rightleftharpoons Hb_4X_1$, $Hb_4X_1 + X \rightleftharpoons Hb_4X_2$ etc. where $X = O_2$ or CO.

The literature contains numerous examples, which will be summarized later, of different sets of values of the unknown constants K_1, K_2, K_3 and K_4, all of which fit the observed data quite well provided that the values of y are allowed a fairly generous margin of experimental error (*e.g.* $\pm$ 3 per cent). For a more rigorous physico-chemical test of the theory, the following conditions should be met :

1 The haemoglobin concentration should not exceed 4 gm/100 cm³, otherwise its osmotic pressure will not be proportional to the concentration, and the law of mass action will therefore be inapplicable without elaborate corrections.

2 The estimates of y should be accurate to $\pm$ 1·5 per cent saturation over the whole of the curve, and especially at the top and bottom, since these regions turn out to be of critical importance. Sufficient points must therefore be available in both these regions.

3 The CO_2 content of the solution should be minimal, otherwise there will be complications from the presence of carbamino compounds which are thought to affect the O_2–Hb equilibrium[1].

4 To reduce the chances of inactivation of the haemoglobin, the temperature should not exceed 20°C, and the *p*H should not be below 7·0. Actually a *p*H of *ca* 9·0 is very favourable, since in this range the haemoglobin is still quite stable, and the dissociation curve is not sensitive to slight variations in *p*H, as is the case at neutral *p*H.

5 The species of haemoglobin used should if possible be homogeneous and not split into sub-units on dilution.

Many of these conditions are far from ' physiological ', and since much of the work on the oxyhaemoglobin dissociation curve has been done with physiological applications in mind, it is not surprising that it fails to meet most of the requirements just listed, especially *1*, *2* and *3*. The data of W. H. FORBES and F. J. W. ROUGHTON (both published and unpublished, 1931) comply, however, satisfactorily with conditions *1* to *4*, and in part with *5*. Their experiments were mostly done on dilute solutions of adult sheep haemoglobin (0·3 to 0·5 gm/100 cm³) in borate buffer, *p*H 9·3, temperature 15–20°C. Two quite independent, but concordant, methods of gasometric analysis were used. *Figure* 1 summarizes their best data, all reduced to the same scale of abscissae, with $pO_2 = 3$ mm Hg at 50 per cent saturation (*i.e.* if in the case of a

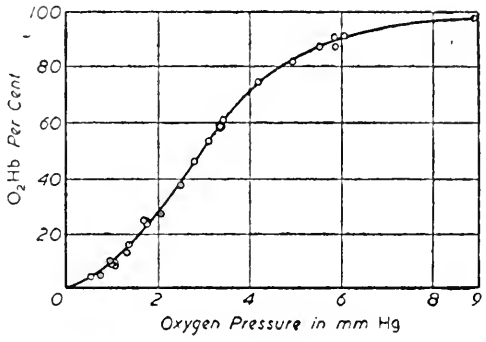


Figure 1. *Oxyhaemoglobin dissociation curve of dilute sheep blood in borate buffer, p*H 9·3, 17–19°C, *plotted from data of Forbes and Roughton*[2] *and Forbes*[5].

particular sample, $pO_2 = 3{\cdot}6$ mm at 50 per cent saturation, all the oxygen pressures for the corresponding curve are multiplied by 3·0/3·6 before being plotted in *Figure* 1*). The smooth curve is based not only on the actual points plotted therein (22 out of 25 of these fall within $\pm$ 1·5 per cent O_2Hb of the curve), but also on numerous other points, which though technically less satisfactory, tally well on the average with the preferred data. *Table I* shows the relation between pO_2 and y, as read off from the points and the curve of *Figure* 1. It is believed

Table I

Relation between oxygen pressure, p, and percentage saturation, y, as given by data of Forbes and Roughton[2] *on oxyhaemoglobin dissociations of diluted sheep blood at p*H 9·3

p	0·3	0·6	0·9	1·2	1·5	1·8	2·1	2·4	2·7	3·0	3·6
y	2·0	4·5	7·5	11·0	16·5	22·5	28·0	34·5	42·5	50·0	64·0
p	4·2	4·8	5·4	6·0	7·5	9·0					
y	73·5	80·5	86·0	90·0	95·0	97·5					

that the values of y are reliable to within $\pm$ 1·5 per cent saturation over the whole range, and that the accuracy of p ranges from $\pm$ ·01 mm Hg at the lower values of y to $\pm$ ·03 mm Hg at the higher values.

We shall now test the various sets of values of K_1, K_2, K_3, K_4, which have appeared in the literature, against the data of *Table I*. In each

* This procedure assumes that individual blood variations within the same species only affect the scale of oxygen pressures but not the shape of the dissociation curve. This assumption has been shown to be true *within* experimental error for the effects of *p*H and temperature on the dissociation curve (*cp* Roughton, 1936), but, as stressed in that paper, there is no proof that the assumption is *mathematically* exact.

particular equation the ratios of K_1 to K_2 to K_3 to K_4 are those put forward by the author but the absolute value of K_1 is, in each case, so chosen that at $p = 3$ mm, $y = 50 \pm 0{\cdot}05$ per cent saturation.

Figure 2 *A* to *J* shows graphically the size of the discrepancies between the observed data of *Table I*, and the figures calculated according to the respective equations, over the whole range of the dissociation curve.

A W. H. FORBES and F. J. W. ROUGHTON assumed[2] that the first three intermediate reactions take place on statistical principles, the velocity constants of combination being proportional to the number of uncombined Fe atoms, and the velocity constants of dissociation to the number of combined Fe atoms. It then follows that $K_1 : K_2 : K_3 :: 4 : {}^3/_2 : {}^2/_3$. In the fourth and final reaction ($Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8$) it is assumed that there is a great increase in K_4, *i.e.* a large 'interaction' in Pauling's sense[4]. With these assumptions equation (1) can be shown to reduce to

$$\frac{y}{100} = \frac{K_1 p\left(1 + \frac{K_1 p}{4}\right)^3 + \lambda p^4}{4\left(1 + \frac{K_1 p}{4}\right)^4 + \lambda p^4}$$

where $\lambda = K_1{}^3 K_4/4$ approximately.

With $K_1 = 0{\cdot}32$ and $K_4 = 8{\cdot}8$, *Figure* 2*A* shows agreement of calculated and observed values to within $\pm$ 1·5 per cent over the whole range.

B Forbes[5] tried the effect of assuming the concentrations of Hb_4O_4 and Hb_4O_6 were so slight as to be negligible. This, as he pointed out, is an impossible assumption from the kinetic point of view, but it leads to the equation

$$\frac{y}{100} = \frac{K_1 p + \mu p^4}{4(1 + K_1 p) + \mu p^4}$$

which, with $K_1 = 0{\cdot}544$, and $\mu = 0{\cdot}0896$, gives good agreement except at the bottom of the curve, where the discrepancies exceed experimental error. The equation must therefore be rejected both on theoretical and experimental grounds.

C is based on the well-known equation of L. C. PAULING[4]

$$\frac{y}{100} = \frac{K_1 p + (2\alpha + 1)K_1{}^2 p^2 + 3\alpha^2 K_1{}^3 p^3 + \alpha_4 K_1{}^4 p^4}{1 + 4K_1 p + (4\alpha + 2)K_1{}^2 p^2 + 4\alpha^2 K_1{}^3 p^3 + \alpha_4 K_1{}^4 p^4}.$$

In deriving this equation, Pauling assumed that when an O_2 molecule combines with a Fe atom adjacent to one already combined, there is an 'interaction' resulting in an increase of the equilibrium constant

from K_1 to αK_1, α being the ' interaction constant '. Similarly if an O_2 molecule combines with a Fe atom adjacent to two Fe atoms already combined the equilibrium constant is $\alpha^2 K_1$.

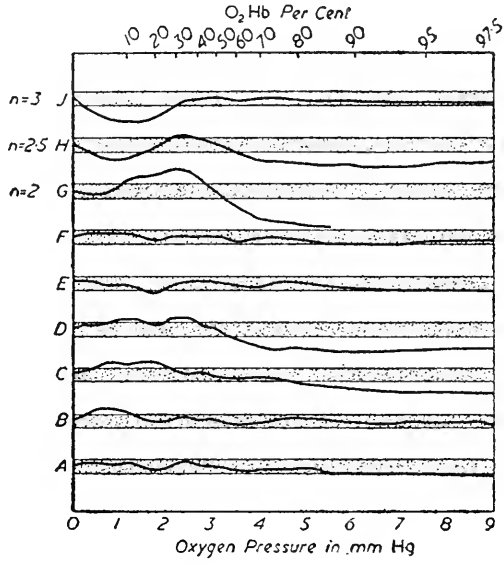


Figure 2. Charts showing agreement in per cent oxyhaemoglobin between observed data (Figure 1 and Table I) and values calculated from equations A, B, C, D, E, F, G, H, J listed in text. The zones of permissible discrepancy are all 3 per cent O_2Hb deep, corresponding to an experimental error of ± 1·5 per cent O_2Hb.

Figure 2C shows that with $K_1 = 0{\cdot}0278$ and $\alpha = 12$, there is good agreement over the middle of the curve, but serious failure at the top and bottom. Decrease of α below 12 leads to better agreement at the bottom, but only at the cost of worse disagreement at the top—*vice versa* with increase of α. It seems therefore that Pauling's stimulating theory must be abandoned. It was originally based on the more rugged experimental data of R. M. FERRY and A. A. GREEN[6].

D G. S. ADAIR in an early paper on the intermediate compound theory[7] put forward tentatively, and indeed mainly as an example, the set of ratios of K_1 to K_2 to K_3 to K_4 which form the basis of *Figure 2D*. The actual values for the present data are

$K_1 = 0{\cdot}359$, $K_1K_2 = 0{\cdot}0646$, $K_1K_2K_3 = 0{\cdot}0155$, $K_1K_2K_3K_4 = 0{\cdot}0167$.

There is considerable disagreement between the calculated and observed data, especially in the upper half of the curve.

E FERRY and GREEN[6] gave a set of ratios which they found very satisfactory for describing their crystalline horse haemoglobin dissociation curve data. If their ratios are applied to the data of *Table I*, if is found that with

$K_1 = 0{\cdot}28$, $K_1K_2 = 0{\cdot}0251$, $K_1K_2K_3 = 0{\cdot}00562$, $K_1K_2K_3K_4 = 0{\cdot}0166$

there is good agreement except in the neighbourhood of 20 per cent

O_2Hb and 95 per cent O_2Hb. It is of interest that the experimental data for crystalline horse haemoglobin and diluted sheep haemoglobin agree closely when the respective scales of O_2 pressure are suitably adjusted.

F Another set of ratios put forward by Roughton[2] also give a good fit (*Figure* 2*F*). In this case

$$K_1 = 0{\cdot}408,\ K_1K_2 = 0.031,\ K_1K_2K_3 = 0{\cdot}0022,\ K_1K_2K_3K_4 = 0{\cdot}0195.$$

G, *H* and *J*. For many years Hill's equation $y/100 = Kx^n/(1+Kx^n)$ has been used for empiric description of the dissociation curve, n being usually taken to be between 2 and 3 for mammalian blood. The equation lost its theoretical basis 25 years ago, when the molecule of haemoglobin was shown to contain 4 atoms of iron. *Figure* 2*G*, *H* and *J* show that the discrepancy between the calculated and experimental results, alike for $n = 2{\cdot}0$, $2{\cdot}5$ and $3{\cdot}0$, much exceeds experimental error. It is impossible to get a good fit whatever value of n be chosen. It is questionable therefore whether the continued use of Hill's equation, even for empiric purposes, is justifiable.

The present position may now be summarized. With more accurate data, especially at the top and the bottom of the dissociation curve, than those mostly used in previous tests, it has been shown that some of the equations which have appeared in the literature must be discarded. There still, however, remain several rather widely varying sets of intermediate constants, all of which give an almost equally satisfactory fit between calculated and observed results. At present we have no grounds for choosing between them, nor can we be sure there are not many other sets of constants which would give as good a fit. All we can be at all sure of, at present, is the range within which K_1 and $K_1K_2K_3K_4$ must lie. Numerical trial and error when applied to the data of *Table I* show that K_1 must lie between $0{\cdot}2$ and $0{\cdot}6$, and once it is fixed there is little choice in the range of values that may be assigned to $K_1K_2K_3K_4$. There still remains, however, considerable liberty in regard to K_1K_2 and $K_1K_2K_3$. The only way that suggests itself at present, of cutting the Gordian knot caused by such a large number of arbitrary constants, is to work out to a finish the very accurate study of the extreme bottom of the curve (which should give K_1 independently) and the extreme top of the curve (which should give K_4 independently). This suggestion was put forward by Forbes and Roughton[2] and is now being carried out in our laboratory, with Dr W. Paul. With two of the unknown constants independently settled, it should then be feasible to fix the remaining two and thus get a decisive test of agreement between calculation and experiment. Such procedure should be applied to haemoglobin under as wide a variation of conditions as possible, *e.g.* of species, *p*H, salt content, *etc*, since all too

often in the past a particular theory of haemoglobin has been put forward on the basis of data obtained under too restricted conditions. It may, however, be noted that Forbes and Roughton[2] showed that the curve of Figure 1, if the pressure scale was suitably adjusted, held good over the *p*H range 7·3–9·3 (borate buffer) and for sheep haemoglobin in distilled water (*p*H *ca* 8·2). It also agreed quite closely with that of whole sheep blood at *p*H *ca* 8·1. Furthermore the recent data of F. C. COURTICE and C. G. DOUGLAS[8] on whole human blood, in presence of CO_2, though they do not comply in several respects with the conditions laid down at the beginning of this section, nevertheless agree very closely with the sheep data except perhaps at the top of the dissociation curve.

AN INTERPRETATION OF THE KINETICS OF THE HAEMOGLOBIN REACTIONS ON THE BASIS OF THE INTERMEDIATE COMPOUND HYPOTHESIS

This matter was considered in a preliminary way by Forbes and Roughton[2], Millikan[9] and in somewhat more detail by Roughton[11] On the intermediate compound hypothesis the kinetics of the chain of four intermediate reactions may be formulated as follows :—

$$Hb_4 + X \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} Hb_4X_1,\ Hb_4X_1 + X \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} Hb_4X_2,\ etc$$

where k_1', k_2' *etc* are the velocity constants of the four combination reactions

k_1, k_2 *etc* are the velocity constants of the four dissociation reactions

and $[Hb_4] = C_0$, $[Hb_4X_1] = C_1$, $[Hb_4X_2] = C_2$ *etc*

C = total concentration of various haemoglobin compounds
$= C_0 + C_1 + C_2 + C_3 + C_4$

y = percentage saturation

p = partial pressure of dissolved X (either O_2 or CO).

$$\text{Then } [XHb] = \frac{4Cy}{100} = C_1 + 2C_2 + 3C_3 + 4C_4$$

$$\text{and } [Hb] = 4C\frac{(100-y)}{100} = 4C_0 + 3C_1 + 2C_2 + C_3$$

On this basis the velocity of the n^{th} intermediate reaction

$$= k_n'pC_{n-1} - k_nC_n$$

In the case of the dissociation of O_2Hb in presence of $Na_2S_2O_4$, the concentration of X remains zero, and the back reaction velocity

terms can thus all be neglected. The kinetic equations for dissociation then reduce to

$$\frac{dC_4}{dt} = -k_4C_4, \frac{dC_3}{dt} = k_4C_4 - k_3C_3, \frac{dC_2}{dt} = k_3C_3 - k_2C_2,$$

$$\frac{dC_1}{dt} = k_2C_2 - k_1C_1, \frac{dC_0}{dt} = k_1C_1 \qquad \ldots.(2)$$

This set of equations is mathematically identical with those obtaining in a chain of radio-active changes, the general solution for which has been given by H. BATEMAN[11]. He has shown that the concentration of the n^{th} intermediary product, C_n is given by

$$C_n = C(P_1e^{-k1t} + P_2e^{-k2t} + P_3e^{-k3t} + P_4e^{-k4t}) \qquad \ldots.(3)$$

where the coefficients P_1, P_2 . . . are each functions of k_1, k_2, k_3, k_4 of given form, so that numerical values of P_1 *etc* can at once be worked out when k_1, k_2 *etc* are known.

The corresponding equations for the combination velocities are only mathematically tractable, if *1*, the back reaction velocity terms (*i.e.* $-k_nC_n$ *etc.*) can be neglected (as in the case of the CO + Hb reaction over almost its whole range) and 2, X is present in such large excess that its concentration may be taken as constant. These simplifications lead to a set of equations similar to (3), but with k_1 *etc* replaced by k_1' *etc.* For the exact form of the equations and of the coefficients P_1, P_1 *etc* and P_1', P_2' *etc* (for the combination velocity), reference should be made to the current paper by Roughton[12].

In attempting to apply the intermediate compound hypothesis to the kinetics of the haemoglobin reactions with the aid of the equations just given, we are again faced with the difficulty of having at our choice a large number of unknown velocity constants, without any clue as to the exact values to be assigned to these individual constants. The only limitation imposed by the law of mass action is that k_1'/k_1 must $= K_1$, $k_2'/k_2 = K_2$ *etc.* A full kinetic investigation must therefore wait until the determination of the values of K_1, K_2, K_3 and K_4 has been attempted in the way described in the previous section. In the present section, however, we think it worth while to show that the further development of one particular example of the intermediate theory, namely that which formed the basis of case *A* of the previous section, provides satisfactorily for most of the present known features of the kinetic data. The latter are expressible by the equation

$$\frac{d[\mathrm{XHb}]}{dt} = k'[X][\mathrm{Hb}] - k[\mathrm{XHb}] \qquad \ldots.(4)$$

with the limitation that k' is only constant below $y = 50$ per cent in

the case of combination and k is only constant below $y = 70$ per cent in the case of dissociation. Above these respective ranges, k' tends to rise quite appreciably and k to fall very appreciably (see pp 70—72). For average sheep haemoglobin at *p*H 9·3, 19°C, it is found that $k = 12$ and $k' = 6{\cdot}5$ when O_2 concentrations are measured in mm Hg of partial pressure of dissolved O_2 (so as to be uniform with the units used in the dissociation curve calculations).

Case *A* of the previous section assumes that the first three intermediate reactions are governed by statistical relations, but that the fourth reaction shows large interaction.

Accordingly

$$k_1' : k_2' : k_3' : : 4 : 3 : 2$$
$$k_1 : k_2 : k_3 : : 1 : 2 : 3$$
$$K_1 : K_2 : K_3 : : 1 : \tfrac{3}{8} : \tfrac{1}{6} \text{ and } K_1 = 0{\cdot}32$$

The values of k_4' and k_4 remain to be settled, subject of course to the condition that $k_4'/k_4 = K_4$ (= 8·8 in the present instance). The investigation now proceeds as follows :—

First the question of the combination velocity is tackled. The relationship between y and t (on the basis of equation (3)) is worked out for a series of cases, in all of which $k_1' = 4$, $k_2' = 3$, $k_3' = 2$ but k_4' is given the values of 1·0, (*i.e.* the fourth reaction also ' statistical ') 6·0, 20·0, 36·0, 440 and ∞. For $k_4' = 20$ and above, the values of y are found to be the same to within 0·4 per cent at all times, and the value of k', as calculated for various intervals, ranges from 1·07 (from $y = 0$ to $y = 35$) to 1·42 (from $y = 0$ to $y = 80$). For $k_4' = 1$, the calculated value of k' remains equal to 1·0 over the whole range, and for $1 < k'_4 < 20$, k' shows a smaller rising trend than at $k_4' = 20$. To account for the observed value of $k' = 6{\cdot}5$ from $y = 0$ to $y = 50$ per cent we therefore take $k_1' = 6 \times 4 = 24$, $k_2' = 18$, $k_3' = 12$ and for the moment k_4' must be left undecided, since, as just shown, it can be varied over a very wide range without affecting the calculation of k' appreciably.

Next, the relation between y and t is worked out for the dissociation velocity for a series of cases in all of which $k_4 = 1$ and $k_3 : k_2 : k_1 : : 3 : 2 : 1$, but k_1 is given the values 0·25 (the complete ' statistical ' case), 2·0, 3·0, 5·0, 9·0 and ∞. The values of k are calculated for various intervals over the range of saturation from $y = 100$ per cent to $y = 5$ per cent. For $k_1 \to \infty$, it is found that $k = 1$ over the whole range, *i.e.* the reaction is kinetically unimolecular from start to finish. For $k_1 = 9$, it is found that $k = 0{\cdot}59$ (for $y = 100$ per cent to $y = 90$ per cent) and $= 0{\cdot}93$ (for $y = 90$ per cent to 77 per cent), whilst from $y = 77$ per cent to $y = 0$ per cent, $k = 1$ throughout. For $k_1 = 5$,

k finally reaches the same plateau value of 1·0 but not till y has fallen to 60 per cent. For $k_1 = 3$, the plateau value of $k = 1$ is reached only at $y = 30$ per cent. For $k_1 = 0{\cdot}25$, $k = 0{\cdot}25$ throughout, *i.e.* the reaction is again kinetically unimolecular from start to finish but the rate is exactly a quarter of that with $k_1 \rightarrow \infty$.

To comply with the experimental finding that k reaches a plateau value of 12 at and below about $y = 70$ per cent, it therefore appears that k_4 must be set equal to 12, and k_1 to a value between 9×12, *i.e.* 108, and 5×12, *i.e.* 60.

Since $K_4 = 8{\cdot}8$ and $k_4'/k_4 = K_4$, it follows that $k'_4 = 12 \times 8{\cdot}8 = 106$. The value of k'_4, which was previously indeterminate is thus now fixed.

Since $K_1 = k_1'/k_1$

therefore $k_1 = \dfrac{k_1'}{K_1} = \dfrac{24}{0{\cdot}32} = 75$

similarly $k_2 = \dfrac{k_2'}{K_2} = \dfrac{18}{\frac{3}{8} \text{ of } 0{\cdot}32} = 150$

and $k_3 = \dfrac{k_3'}{K_3} = \dfrac{12}{\frac{1}{6} \text{ of } 0{\cdot}32} = 225$

With $\left.\begin{matrix} k_1' = 24 \\ k_1 = 75 \end{matrix}\right\}$ $\left.\begin{matrix} k_2' = 18 \\ k_2 = 150 \end{matrix}\right\}$ $\left.\begin{matrix} k_3' = 12 \\ k_3 = 225 \end{matrix}\right\}$ $\left.\begin{matrix} k_4' = 106 \\ k_4 = 12 \end{matrix}\right\}$ it is thus possible to account quantitatively (a) for the equilibrium between O_2 and sheep haemoglobin at alkaline *p*H (b) for the kinetics of combination and dissociation over the range where the equation $d[\text{XHb}]/dt = k'[X][\text{Hb}] - k[\text{XHb}]$ holds good, and semi-quantitatively for (c) the divergence of the data from the latter equation when [XHb] is high. It is interesting to see that, on the present view, the large interaction in the last intermediate reaction is due, in almost equal measure, to the respective degrees to which k_4' and k_4 are affected, for the former is increased to 106/6, *i.e.* 18 times the statistical value, and the latter is decreased to 12/300, *i.e.* 1/25 of the statistical value.

The work just described constitutes, I believe, the first thoroughgoing attempt to treat both the kinetics and equilibria of haemoglobin on the basis of the intermediate compound hypothesis, albeit on the assumption of one particular form of the latter. Although successful so far, final judgment as to the status of the work must be reserved until its application has been tested in regard to other types of data than those for which it has so far been used. Several previous theories of the haemoglobin equilibria have, as has been pointed out above, worked quite well until they were applied in directions for which they were not originally designed : and it must be admitted that the basal

assumption used here, namely that the first three intermediate reactions occur 'statistically' and only the final one shows large 'interaction', is hard to reconcile with the present x-ray crystallographic data on the orientation of the haem groups in crystalline horse oxyhaemoglobin. There are, however, two points to be remembered in arguments based on the crystallographic results. *1* It is possible that the configuration of the molecule may change reversibly when it passes from the dissolved to the crystalline form. *2* No x-ray crystallographic data yet exist on the structure of the reduced haemoglobin or of the orientation of the haem groups therein. All that is known is that the structure is drastically different[13, 14] from that of oxyhaemoglobin, methaemoglobin, *etc*, and it is possible that the same may apply to the orientation of the haem groups. It would therefore seem for the present premature to apply arguments based on x-ray data to equilibria in haemoglobin solution in which oxyhaemoglobin, reduced haemoglobin and compounds of intermediate composition are all present.

Although it may eventually be found that other special forms of the intermediate compound hypothesis are more successful in combined handling of the kinetic, the equilibrium and other data, I hope nevertheless that the treatment given in this paper may prove to be the basis for, or at any rate of aid in providing, the final successful attack, whatever this may be.

Received September 1948

REFERENCES

[1] ROUGHTON, F. J. W. *Physiol. Rev.* 15 (1935) 278
[2] FORBES, W. H. and ROUGHTON, F. J. W. *J. Physiol.* 71 (1931) 229
[3] ROUGHTON, F. J. W. *Bio-chem. J.* 30 (1936) 2117
[4] PAULING, L. C. *Proc. nat. Acad. Sci. Wash.* 21 (1935) 186
[5] FORBES, W. H. *Ph.D. Thesis.* Cambridge University (1931)
[6] FERRY, R. M. and GREEN, A. A. *J. biol. Chem.* 81 (1929) 175
[7] ADAIR, G. S. *J. biol. Chem.* 63 (1925) 529
[8] COURTICE, F. C. and DOUGLAS, C. G. *J. Physiol.* 105 (1947) 345
[9] MILLIKAN, G. A. *Ph.D. Thesis.* Cambridge University (1932)
[10] ROUGHTON, F. J. W. *Proc. roy. Soc.* B 115 (1931) 451
[11] BATEMAN, H. *Proc. Camb. phil. Soc.* 15 (1910) 423
[12] ROUGHTON, F. J. W. In course of publication (1949)
[13] HAUROWITZ, F. *Hoppe-Seyl. Z.* 254 (1938) 266
[14] PERUTZ, M. F. Personal communication (1948)

Some Physico-Chemical Evidence Regarding the Structure of Haemoglobin

JEFFRIES WYMAN, JR.

From evidence provided by studies on the effects of pH and temperature on the oxygen equilibrium, it is argued that the four oxygen combining groups of vertebrate haemoglobin, one for each haem, are all identical. Each haem is linked with two acid groups, one of which is strengthened, the other weakened, when oxygenation occurs. Thermodynamic studies indicate that the heat of dissociation of each of these groups is 6,000–6,500 cals, which suggests that they are both imino groups of histidine. The value of $n = 2{\cdot}5 - 3{\cdot}0$ required to describe the oxygen equilibrium in Hill's empirical equation requires the existence of a stabilizing interaction between some, at least, of the four oxygen combining centres. From experiments on the oxygen equilibrium of haemoglobin split by dissolving it in urea solutions, it appears that the four haems occur, not at the corners of a square as originally suggested by Pauling, but in pairs, with strong interaction between members of the same pair and weaker interaction between members of different pairs. The latter, and possibly also the former, interaction seems to involve primarily the un-oxygenated haems. This picture accords with deductions from a recent x-ray study on crystalline vertebrate haemoglobin.

THERE is perhaps no better instance of biological adaptation at a molecular level than the way in which vertebrate haemoglobin reacts with oxygen. The physiological significance of the familiar curve which describes the equilibrium of these two substances has been pointed out repeatedly: its sigmoid character is such as to increase the working range of this molecular mechanism of oxygen transport and at the same time the dependence of the position of the curve on *p*H, reflecting a linkage between the oxygen and acid-base functions of the haemoglobin molecule, adds further to the efficiency of the respiratory exchanges. To provide a physico-chemical explanation of these phenomena has long been a major concern of physiologists and biochemists, but the problem still lacks a complete solution. Closely related problems also occur in several other reactions of haemoglobin, such as oxidation and combination with carbon monoxide. To provide a solution of any of them would mean a substantial step in the understanding of the haemoglobin molecule. In this paper we

shall be concerned mainly with the oxygen equilibrium of horse haemoglobin in its relation to the constitution of the molecule, but we shall also present some considerations involving oxidation.*

In approaching this problem we may initially take it for granted that the power of oxygen to combine with haemoglobin is due to the presence of protohaem in the molecule, for in solutions of haemoglobin as well as of all other haem proteins capable of uniting with oxygen, *e.g.* myoglobins and erythrocruorins, the oxygen capacity, in moles, is equal to the number of haems present, and when the haem is split from the protein the power to take up oxygen is lost. In vertebrate haemoglobin there are four haems per molecule, and therefore four oxygen combining centres. As far as the haem is concerned, these must all be regarded as identical, but this need not be true as regards the local configuration in the globin to which the haem is attached. It cannot be assumed, therefore, without further evidence that the four oxygen combining centres are all alike. Apart from this, the question arises whether or not the four centres are far enough apart in the molecule to be independent of one another, or whether they interact so that the reaction of one affects that of another, and if so, how. Also there is the question whether all the haemoglobin molecules are alike or whether there may be more than one kind, though if so the different kinds would have to be of essentially the same molecular weight ($\sim$ 68,000), in view of the fact that all the experiments show that solutions of native vertebrate haemoglobin are monodisperse. These are all considerations which are significant in the problem of interpreting the oxygen-haemoglobin equilibrium.

Before we proceed to haemoglobin itself, it is instructive to consider the case of myoglobin, which is a closely related but simpler molecule, having a molecular weight of only about 16,000, or approximately one quarter that of haemoglobin, and containing only a single haem. It is satisfactory to find that for myoglobin the oxygen equilibrium, which, unlike that of haemoglobin, is essentially independent of *p*H, is just what would be predicted for the reaction

$$Mb + O_2 = MbO_2 \qquad \ldots.(1)$$

That is, a plot of Y, the fractional saturation of the protein with oxygen, against p, the activity or partial pressure of oxygen, gives a rectangluar hyperbola, in agreement with the mass law equation

$$Y = \frac{Kp}{1 + Kp} \qquad \ldots.(2)$$

* A more complete general account of these and related matters is contained in a recent review by the author, *Advances in Protein Chemistry*, Vol. IV, New York, 1948, Academic Press. References and detailed arguments and derivations are given there.

in which K is the equilibrium constant. This is important in connection with suggestions that the combination of haemoglobin with oxygen may represent an adsorption phenomenon rather than a true chemical reaction. Also it shows that the molecules of myoglobin, if not in all respects alike, are at least alike in their behaviour towards oxygen. We may feel confident, therefore, in regarding the combination of haemoglobin with oxygen as a chemical reaction, and, at least as a working hypothesis, we shall assume that the haemoglobin molecules are all the same in their reactions with oxygen.

Having profited by this view of the behaviour of myoglobin, let us now turn to the question of the identity of the four oxygen combining centres in haemoglobin. There are two sets of observations bearing on this matter, one relating to the heat of oxygenation, and the other to the effect of *p*H on the oxygen equilibrium curves. We shall consider first the *p*H effect (commonly known as the Bohr effect). This effect, extending from *p*H $\sim$ 4 to *p*H $\sim$ 9, is shown in *Figure* 1. In this figure the value of p corresponding to half saturation of haemoglobin with oxygen, which we shall call $p_{\frac{1}{2}}$ and which is by definition the reciprocal of the oxygen affinity, is shown as a function of *p*H. It will be seen that the effect falls into two parts, $p_{\frac{1}{2}}$ having a maximum at *p*H 6·1. Above 6·1 $p_{\frac{1}{2}}$ decreases, below 6·1 it increases, with increasing *p*H. That there is any *p*H effect at all means of course that there are proton dissociating groups in the haemoglobin molecule sufficiently close to the oxygen combining groups to be affected by the uptake of oxygen. The double character of the effect means that there must be one or more such groups which are strengthened and one or more such groups which are weakened by the reaction. Whenever therefore oxygen is introduced into haemoglobin at constant *p*H, there must be a loss or gain of protons, depending on *p*H. This is a reciprocal effect which is well known and has been directly measured by differential titrations of haemoglobin and oxyhaemoglobin. The two effects are in fact counterparts of one another and there is a fundamental relation governing them which may be deduced by quite general reasoning and stated as follows :

$$\left(\frac{\partial X}{\partial Y}\right)_{p\mathrm{H}} = -\left(\frac{\partial \log p}{\partial p\mathrm{H}}\right)_{Y} \qquad \ldots\ldots(3)$$

In this equation X denotes the total negative charge on the haemoglobin molecule resulting from the loss of protons. Now in all cases which have been studied it is found that the equilibrium curves in which Y is plotted against log p are unaffected in shape by changes of *p*H, being subject simply to a displacement along the log p axis. This is a fundamental observation and means that the right-hand member of

equation (3) depends only on pH and not on Y (or y) so that we may rewrite the equation as

$$\left(\frac{\partial X}{\partial Y}\right)_{p\mathrm{H}} = -\frac{d \log p_{\frac{1}{2}}}{dp\mathrm{H}} \qquad \ldots.(3.1)$$

By integrating this at constant pH we see that at *any* pH the number of protons displaced as a result of oxygenation is proportional to the degree of oxygenation.* That is, each successive molecule of oxygen introduced into the haemoglobin molecule causes the same amount of dissociation. This in turn means that each oxygen combining group must be linked with an identical set of acid groups, identical at least as regards their dissociation constants. Clearly in view of the double character of the Bohr effect there must be at least two such groups associated with each oxygen combining centre, one of which is rendered stronger, the other weaker, as a result of oxygenation of the centre.

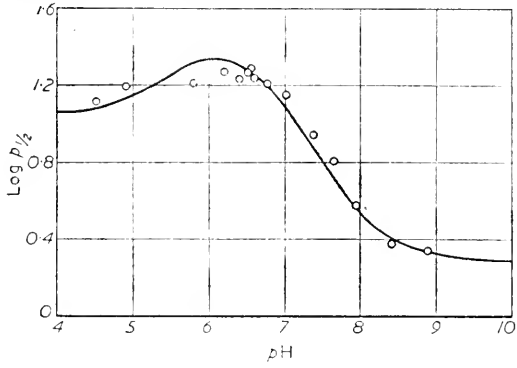


Figure 1. *Values of* $\log p_{\frac{1}{2}}$ *in relation to* p*H for horse haemoglobin at* 25°*C. Smooth curve is calculated from constants for oxygen linked acid groups given in Table I.*

This interpretation may be checked in two ways. In the first place, by direct differential acid base titration we obtain the difference of charge between haemoglobin and oxyhaemoglobin. The data can be satisfactorily fitted by the assumption of just two groups per haem, the same for each haem, with the following choice of constants.

Table I

Apparent Dissociation Constants of Oxygen Linked Acid Groups in Horse Haemoglobin at 25°C and Ionic Strength 0·16

	Haemoglobin	*Oxyhaemoglobin*
pK_1	7·93	6·68
pK_2	5·25	5·75

* $\int_0^Y \left(\frac{\partial X}{\partial Y}\right)_{p\mathrm{H}} dY = -\frac{d \log p_{\frac{1}{2}}}{dp\mathrm{H}} \int_0^Y dY = -\frac{d \log p_{\frac{1}{2}}}{dp\mathrm{H}}\, Y$

In the second place, from these values of the two assumed constants, it is possible, by means of equation (3.1) in integral form, to predict relative values of $p_{\frac{1}{2}}$ as a function of *p*H. When this is done the agreement with the directly measured values of $p_{\frac{1}{2}}$ is satisfactory. The degree of success of these two checks will be seen from *Figures* 1 and 2.*

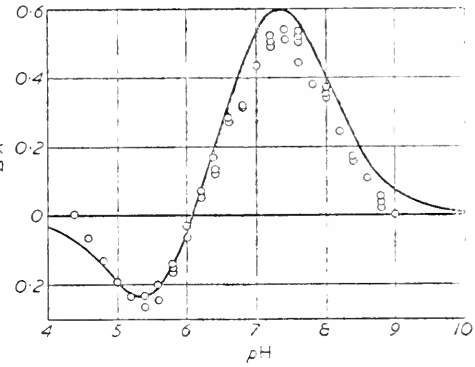


Figure 2. Difference of charge between haemoglobin and oxyhaemoglobin. Smooth curve is calculated from constants given in Table I.

We now turn to the effect of temperature on the oxygen equilibrium. This is exactly like the effect of *p*H : altering the temperature simply shifts the curve of *Y vs* log *p* without changing its shape. From this, by a type of reasoning analogous to, though somewhat more complicated than, that given above and involving the concept of the thermodynamic heat as defined by the van 't Hoff equation, we arrive at the conclusion that the heat absorbed is the same for each successive molecule of oxygen introduced into the haemoglobin molecule, and is given, on a mole basis, by

$$\Delta H = -2{\cdot}303\, RT^2 \left(\frac{\partial \log p_{\frac{1}{2}}}{\partial T} \right)_{pH} \qquad \ldots.(4)$$

Since this heat includes the heat of dissociation of the protons split off as a result of oxygenation, it indicates that this component of the heat is the same for each oxygen combining centre. This in turn indicates once more that the same pair of acid groups is linked with each haem. It is possible to go beyond this. By making differential titrations at different temperatures, which in effect give us the variation of $p_{\frac{1}{2}}$ with *p*H at each of the temperatures and make it possible to determine ΔH as a function of *p*H from its value at any one *p*H, we can obtain the actual value of the heat of dissociation of the oxygen linked acid

* In a sense these two checks are not independent, for it follows from equation (3) that if the hypothesis accounts for the titration data it must account also for the oxygen affinity data. What we have done really is to apply this hypothesis separately to two quite different sets of experimental results.

groups, as distinct from the overall heat of oxygenation. The equation involved is

$$\left(\frac{\partial \Delta H}{\partial \Delta X}\right)_T = -2{\cdot}303\, RT^2 \left(\frac{\partial p\mathrm{H}}{\partial T}\right)_{\Delta X} \qquad \ldots.(5)$$

where ΔX denotes the number of protons dissociated as a result of introducing one molecule of oxygen into the haemoglobin molecule at constant *p*H.

The left-hand member of this equation gives the change in the heat of oxygenation with the change in the number of protons dissociated as a result of oxygenation, and may therefore be interpreted as the heat of dissociation of those protons per equivalent. Now the experiments show that the curves of ΔX *vs* *p*H are simply displaced along the *p*H axis, without change of shape, by changing the temperature. This means that the right-hand member is constant. Its value, the same over the whole *p*H range, is obtained from the size of the displacement as 6,500 calories, though this is subject to considerable uncertainty due to the fact that it is based on second order effects. It has been interpreted however, in connection with other evidence, to mean that both the acid groups associated with each haem are imino groups of histidine, 6,500 being a characteristic value for the heat of ionization of that group. The more essential thing however in the present connection is not the identification of the two oxygen linked groups, but the evidence that they are the same for each haem. The two lines of evidence leading to this conclusion, that based on the *p*H effect and that on the temperature effect, are, taken together, fairly convincing.*

A historically important step in the theory of the oxygen equilibrium of haemoglobin was taken by A. V. HILL when he pointed out that the sigmoid curve of *Y vsp* could be fitted, approximately at least, by the equation

$$Y = \frac{Kp^n}{1 + Kp^n} \qquad \ldots.(6)$$

* While the reasoning involved in this argument is rigorous, it must not be forgotten that the data to which it has been applied are subject to experimental error, and the possibility of slight changes of shape of the oxygen equilibrium curve (*Y vs. log p*) with *p*H and temperature, reflecting real differences between the acid groups linked with different haems, cannot be categorically ruled out. This point was emphasized by Professor Roughton in discussion at the conference. Considerable differences in the heat of dissociation between different haem linked acid groups *need* involve only a slight temperature dependence of the shape of the equilibrium curve. All we can say is that there has been so far no indication of any change of shape of the curve with either *p*H or temperature, and that if there is any such change it lies within the limits of error of the extensive equilibrium measurements.

if n were given a value in the neighbourhood of 2·6. This is the equation for the reaction

$$Hb + nO_2 = Hb(O_2)_n$$

Such an equation implies the simultaneous combination of one molecule of haemoglobin with n molecules of oxygen. With $n = 2{\cdot}6$ however the equation was devoid of physical meaning. In answer to this objection it was therefore suggested that haemoglobin existed as a mixture of polymers, in each of which the reaction occurred with the simultaneous acceptance of the full quota of oxygen molecules. The observed value of n therefore represented kind of average degree of polymerization of the monomer containing one haem. These views were put forward before the accurate determinations of molecular weight by osmotic and ultra-centrifugal methods had demonstrated that haemoglobin solutions were monodisperse and that the molecules all contained four haems. With the advent of that discovery the Hill hypothesis was dropped, and equation (6) remains simply as a useful empirical equation giving an approximate description of the facts. Actually it does not describe the best data very accurately ; one trouble being that it yields a symmetrical plot of Y *vs* log p, whereas the most accurate results show that the curve is not exactly symmetrical. Nevertheless there is an important element of truth in the Hill hypothesis, in so far as it implies the existence of an interaction between the haems, though it is wrong to assume the interaction energy to be infinite as would be required to account for the simultaneous acceptance of two or more molecules or oxygen by a single molecule of haemoglobin.

It should be pointed out here that any reaction occurring in stages by a molecule containing more than one reacting group will be describable with more or less approximation by an equation having the form of (6) with a suitable choice of n, though the approximation will be increasingly poor as n drops below unity. This is so if the reacting groups are unlike and independent, or if, whether alike or unlike, they are subject to destabilizing interactions so that the reaction of one group leads to a decrease in the reactivity of one or more of the others, as in polybasic acids generally. Only if some at least of the groups show a stabilizing interaction will n be greater than unity, and a value of n greater than unity is a sure sign that there is a stabilizing interaction between some at least of the groups. It is in this case that the equation is generally a useful approximation. The equation is of course without *direct* physical significance, but it is a simple matter to relate the value of n, given by the slope of the curve of Y *vs* p or log p at its midpoint ($Y = \frac{1}{2}$) to the various overall constants

for the reaction, and in turn to the interaction constants in accordance with various hypothetical models.

Another landmark in the interpretation of the oxygen equilibrium curve is due to Pauling, and is based on the extensive data of Ferry and Green on horse haemoglobin. In his analysis Pauling accepted the hypothesis discussed above, though without the evidence presented there, that the four oxygen combining centres in haemoglobin were all alike. He then assumed that they were subject to interactions as if located at the corners of a square, with the same interaction between all adjacent centres but with no interaction between centres situated at the ends of a diagonal. This model leads to a formulation of the oxygen equilibrium involving only one constant controlling the *shape* of the curve. This is the interaction constant for adjacent centres. The other constant, representing the inherent affinity of a haem for oxygen, simply affects the *position* of the curve Y *vs* log p along the log p axis. On the basis of this model, by taking the interaction constant as 12 (corresponding to an interaction energy of about 1,500 cals) Pauling found that it was possible to fit the data of Ferry and Green much more exactly than with the Hill equation, in fact, as it appeared, almost if not quite within the experimental error. Since the shape of the curve was independent of *p*H, it was of course necessary to assume that the interaction constant, unlike the other constant, was unaffected by the dissociation of the oxygen linked acid groups.

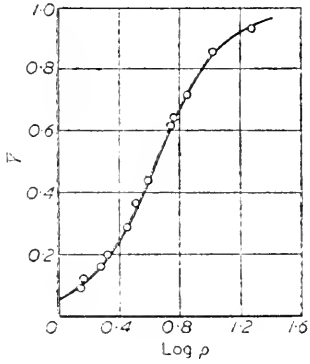


Figure 3. *Data of Wyman and Ingalls on oxygen equilibrium of horse haemoglobin in strong urea solutions. The full curve is calculated for a molecule containing two identical oxygen combining centres having an interaction constant of* 400 *corresponding to an interaction energy of* 3,550 *cals.*

It is perhaps disappointing that this remarkably successful hypothesis of Pauling can hardly be retained, at least as it stands. Yet the very objections to it provide an increased insight into the structure of the haemoglobin molecule and suggest a modification of our views which appears to meet the facts, and which accords astonishingly well with conclusions based on quite different considerations. Let us consider these objections. In the first place the Pauling model implies an exactly

symmetrical curve for Y *vs* log p, as does any theory in which the groups are all identically related to one another. Actually the most accurate data, for example those of Forbes and Roughton on sheep haemoglobin, show that the curve is in fact asymmetrical. In the second place, it does not accord with recently published experiments on the oxygen equilibrium of horse haemoglobin dissolved in strong urea solutions, where the molecules are known to be split in two. In these experiments the curve of Y *vs* log p was found to be exactly symmetrical and corresponded to a value of $n = 1{\cdot}8$–$1{\cdot}9$ in equation (6) (see *Figure* 3). From these results we may conclude that when the haemoglobin molecule is split it divides into identical halves and that there is a strong stabilizing interaction between the two haems present in each half. To be sure any molecule containing only two groups would give a symmetrical curve of Y *vs* log p, but if there were two kinds of such molecule the overall curve would not be symmetrical except in the case of certain compensating effects which are inconsistent with the assumption of the identity of the four oxygen combining centres. Nor would the curve be symmetrical if there were two kinds of molecule, one containing one group, the other three. It is a simple matter to relate the observed value of n to the interaction constant for the case of a model consisting of two identical reacting groups. For $n = 1{\cdot}9$, the required interaction constant is approximately 400, corresponding to an interaction energy of 3,550 cals, though when n is so close to its upper limit 2, corresponding to an infinite interaction energy, n is very insensituve to the value of the interaction energy. We conclude then from these experiments on haemoglobin dissolved in urea solutions that unless there is a profound rearrangement accompanying splitting of the molecule the four haems in the native haemoglobin molecule occur in pairs. Members of the same pair are closely spaced and show a strong stabilizing interaction energy of the order of 3,000–4,000 calories. If we adopt this interpretation, then in order to account for the oxygen equilibrium of the unsplit molecule, for which $n \cong 2{\cdot}8$, we must postulate an additional but smaller stabilizing interaction between haems belonging to different pairs. This much seems clear. It is tempting, however, and perhaps not wholly useless, to go beyond, and try the effect of replacing the Pauling model by a rectangle. If we do, then we find that by retaining the value 400 as the interaction constant for the closely spaced haems (those belonging to the same pair) and assuming an interaction constant of 4 (corresponding to about 800 calories) for haems belonging to different pairs (there being no interaction along diagonals of the rectangle) we arrive at a formulation of the equilibrium which gives a curve almost indistinguishable from Pauling's.

Such a picture of the intact horse haemoglobin molecule, though somewhat arbitrary, accords remarkably well with conclusions reached by Perutz on the basis of x-ray studies of haemoglobin in the crystalline state, which are discussed in another chapter of this volume. According to Perutz the molecule is a right circular cylinder 34 Å high and 57 Å in diameter, having a twofold axis of symmetry perpendicular to the cylinder axis and consisting of two structurally and chemically identical halves. The two haems occur in pairs on the surface of the molecule with their planes parallel to one another and to the axis of symmetry and the cylinder axis. However the rectangular model suffers in one respect from the same difficulty as the original Pauling model : it leads to a symmetrical curve of Y *vs* log p, as indeed does any model in which the haems are all equivalent to one another and the interaction energies are additive. To account for the observed asymmetry we might postulate either that the free energies of interaction in the molecule were not simply additive or that the two identical pairs of haems occupied some arrangement other than a rectangle, which, while satisfying the symmetry conditions, did not make the individual haems all equivalent. For example this would be so if the four haems were situated at the corners of a parallelogram whose plane was perpendicular to the axis of symmetry.

Very similar conclusions as to the existence of the haems in pairs of strongly interacting members are suggested by studies of oxidation-reduction phenomena, to which we can only refer hastily. The theory involved is in all essentials identical with that for the oxygen reaction, the n of the oxidation equation being identical in interpretation with the n in equation (6) and the oxidation voltage E corresponding to log p. In the intact molecule the curve of fractional oxidation *vs* E is asymmetrical with n, as given by the slope of the curve at its mid point, close to 2*. In strong urea solutions the curve becomes exactly symmetrical with n very nearly equal to 2 though apparently slightly less. Thus here again we have evidence that the haems occur in pairs, with very strong interactions between members of the same pair. In the case of this equilibrium however it appears that there is no significant net interaction between members of different pairs.

The oxidation behaviour of haemoglobin is to be regarded as a more general instance of the stepwise oxidation illustrated by the two step oxidation of organic molecules such as hydroquinone, which has been studied so extensively by Michaelis. In these molecules, where the intermediate form is known to be a free radical, the interaction

* This statement is based on experiments by Taylor and Hastings, *J. biol. Chem.* 131 (1942) 649. More recent experiments by R. Havermann, *Biochem. Zs.* 314 (1943) 118 indicate that the curve is symmetrical with $n = 1{\cdot}12$.

constant is of the same order of magnitude as what we have deduced for the paired haems in haemoglobin. (Michaelis's semiquinone formation constant is simply four times the reciprocal of our interaction constant, the factor four arising from symmetry considerations.) In close analogy with haemoglobin, the two oxidizable groups of the molecules investigated by Michaelis are indistinguishable and there are differences of acid base properties between reductant, semiquinone, and oxidant so that here also we encounter oxidation linked acid groups. In contrast to haemoglobin, however, there is only one such group for each oxidizable group. Owing to the compactness of the molecule, there is direct interaction between these acid groups with the result that the overall interaction constant of the oxidizable groups is dependent on *p*H. This is in contrast to what is found in haemoglobin, where the shape of the curve of Y *vs* log p or E is independent of *p*H.

One final question now presents itself. This is whether the stabilizing interactions operative in the oxygen equilibrium stem from the haems in their uncombined state or from the haems when united with oxygen. According to one hypothesis the first oxygen would enter the molecule with more difficulty than the next (and so on) because of a pre-existing stabilization of the uncombined haems. According to the other hypothesis, the second oxygen would enter more readily than the first because of a kind of attraction exerted by the presence of the first. Since both interpretations lead to identical formulations of the equilibrium it might seem that it would be impossible to decide between them. Nevertheless there is a basis for doing so, at least for interactions between haems of different pairs, in the data obtained on haemoglobin dissolved in urea solutions. These data show that there is a decrease in $p_{\frac{1}{2}}$ by a factor of about 2·7 when the molecule splits in two. Reflection will show that this is qualitatively just what would be expected on the hypothesis that the stabilization involves *unoxygenated* haems, for when the molecule is split, this stabilization disappears and an obstacle to oxygenation is removed. On the other hand it is the opposite of what would be expected if stabilization involved oxygenated haems. Quantitatively, the magnitude of the effect is not too far from what would be expected on the basis of our rectangular model, for it is possible to show that $p_{\frac{1}{2}}$ should be diminished by a factor equal to the square root of the interaction constant involved, *i.e.* $\sqrt{4} = 2$. All conditions (*e.g.* high dilution) which have the effect of diminishing the value of n seem to lead to an increase in the oxygen affinity (decrease of $p_{\frac{1}{2}}$). It is further suggestive that the value of $p_{\frac{1}{2}}$ for horse myoglobin (with one haem) at 25°C and *p*H7 is about 1/20 that for horse haemoglobin. On the basis of the constants

400 and 4 for our rectangular model we should predict that completely separating the haems of horse haemoglobin would lead to a decrease of $p_{\frac{1}{2}}$ by a factor of 40. However, we are hardly justified in thinking of myoglobin as a quadrant of a haemoglobin simply because the molecular weight is one quarter, and too much cannot be argued from this effect.

The conclusions of this paper, based mainly on considerations relating to the oxygen equilibrium of horse haemoglobin, but strengthened also by data on other haemoglobins and by oxidation-reduction phenomena, some of which have been mentioned and some not, may be stated as follows. The four haems are all bound to structurally identical local configurations of the globin, and each interacts with two acid groups, one of which, the one active in the physiological range, is rendered stronger, the other of which, the one active at more acid reactions, is rendered weaker when an oxygen molecule combines. These groups are probably imino groups of histidine. The four haems occur in pairs, with strong stabilizing interactions between members of the same pair and weaker interactions between members of different pairs. In the oxygen equilibrium the interaction between members of different pairs, amounting to about 800 cals, involves primarily the unoxygenated haems and there is some indication that the same may be true of the much stronger interaction ($\sim$3,500 cals) between members of the same pair.

III

ANALYSIS AND AMINO ACID COMPOSITION

The Amino Acid Composition of the Haemoglobins of the Blood and Muscle of the Horse

G. R. TRISTRAM

The respiratory proteins of blood (haemoglobin) and muscle (myoglobin) have parallel functions, and it is attractive to conjecture that myoglobin, molecular weight 17,000, might be a precursor of haemoglobin, molecular weight 68,000. Early centrifugal studies led T. Svedberg[1] to advance this suggestion;[2] a similarity is also suggested by x-ray analysis of single crystals. It is clear, however, that only by the accurate estimation of the relative amino acid compositions of the two proteins might the validity of such a hypothesis be confirmed. Recent work in the Department of Biochemistry, Cambridge, in which the amino acids were estimated by a variety of modern methods, previously tested by the analysis of ad hoc *mixtures of amino acids, has suggested that the two proteins are completely dissimilar in amino acid composition. The present analyses of myoglobin show significant differences from those previously reported, particularly with regard to the sulphur containing amino acids in the protein.*

SOURCE OF PROTEINS AND METHODS OF ANALYSIS

Haemoglobin— The amino acid data reported in *Table I* were collected from the literature, care being taken to select values which had been obtained by the analysis of well characterized samples of this protein.

1 The values for the basic amino acids are taken from H. T. MACPHERSON[2] and were obtained by electrodialysis, arginine and histidine being estimated ultimately by colorimetry and lysine by means of a specific decarboxylase (*cf* Gale[7]).

2 The dicarboxylic acids, leucine, glycine and tyrosine were obtained by G. L. FOSTER[8] using isotope dilution.

3 The hydroxyamino acids were obtained by M. W. REES[3] using his modification of the Nicolet-Shinn procedure. This worker also estimated amide-N.

4 Proline, alanine and phenylalanine were obtained by partition chromatography of the acetylamino acids (Tristram, unpublished experiments).

5 Various estimations of the sulphur acids have been reported and there are wide divergencies in the values obtained by different workers.

Table I

Amino acid composition of haemoglobin and myoglobin

1 Wt of amino acid in 100 gm protein.

2 Number of residues per molecule.

2a Number of residues in myoglobin assuming M.W. to be 68,000 (i.e. 4 × 17,000).

	Myoglobin (T.N. 16·9%)			*Haemoglobin* (T.N. 16·8%)	
	1	*2*	*2a*	*1*	*2*
Arginine	2·2	2	8	3·65	14
Histidine	8·5	9	36	8·71	36
Lysine	15·50	18	72	8·51	38
Cystine/2	0	—	—	0·45	2·5
Cysteine				0·56	3
Tyrosine	2·4	2	8	3·03	11
Tryptophan	2·34	2	8	1·70	5
Alanine	7·95	15	60	7·40	54
Isoleucine	16·80	22	88	0	0
Leucine				15·4	75
Valine	4·09	6	24	9·10	50
Proline	3·34	5	20	3·9	22
Glycine	5·85	13	52	5·60	48
Phenylalanine	5·09	5	20	7·9	33
Methionine	1·71	2	8	1·0	4·5
Glutamic acid	16·48	19	76	8·15	36
Aspartic acid	8·20	10	40	10·60	51
Amide–N	0·66	8	(32)	0·93	(36)
Serine	3·46	6	24	5·68	35
Threonine	4·56	7	28	4·36	24
Total	108·49	143	572	106·43	542
Nitrogen recovered (Excluding Haem–N)	96·02			95·3	

Those reported in *Table I* for cystine and methionine were obtained by R. KUHN *et al*[9] using an iodometric procedure. The cysteine value was obtained[10] by titration of the intact protein. There are other values for these acids, *e.g.* methionine 1·5 per cent (Lyman *et al*[11] by means of microbial assay) and 0·95 per cent (Rees, in unpublished experiments using differential oxidation) and cystine-cysteine 0·72 per cent

(differential oxidation—Rees in unpublished experiments). The values reported in *Table I* may therefore require modification, but even if the highest value for cystine-cysteine (1·0 per cent) be taken and it is assumed that only cystine is present, then a maximum of 3 disulphide groups may exist in the haemoglobin molecule. It is known however that haemoglobin does contain SH groups (*cf* Greenstein[10], Mirsky and Anson[12]) so that S–S cannot exceed two groups. Since the work of R. R. PORTER and F. SANGER (p. 121) suggests the presence of six constituent chains in the haemoglobin molecule, it would appear that either the distribution of the sulphur-containing amino acids requires re-investigation, or that the intramolecular chains are cross-linked by some system as yet unknown but which is covalent.

Table II
Distribution of Groups in Haemoglobin and Myoglobin

	Myoglobin		*Haemoglobin*	
Total No. of residues	146		580	
Mean residue weight	112		112·5	
Percentage distribution of groups				
1 Polar groups				
Free anionic	14·4	34·2	9·5	25·7
Cationic	19·8		16·2	
Amide	5·5		6·7	
Phenolic	1·4		2·0	
Aliphatic hydroxyl	8·9		10·8	
	50·0		46·8	
2 Non polar groups				
Hydrocarbon side-chain	39·0		43·0	
Glycine	9·0		10·1	
	48·0		53·1	

Myoglobin— This protein was analysed in the above laboratory by H. T. MACPHERSON[2], M. W. REES[3], the author[4] and G. WILTSHIRE. The last named estimated the dicarboxylic acids by a modification of the ion-exchange method of R. CONSDEN, A. H. GORDON and A. J. P. MARTIN[13]. The myoglobin analyzed was identical with that used by J. C. KENDREW (p. 148) for crystallographic studies. It was prepared by Rees from horse heart muscle, being finally crystallized from phosphate buffers.

Before the two proteins are compared the distribution of the sulphur acids in myoglobin must be discussed in some detail. J. ROCHE *et al*[5] and A. ROSSI-FANELLI[6] (see p. 115) both reported the presence of cysteine equivalent to one molecule of SH in a protein molecule of 17,000. Rees (unpublished experiments) using an elaboration of the method of sulphur distribution[14] could find no evidence for the presence of cystine-cysteine and this was confirmed by the absence of any nitroprusside test, even after treating the protein with cyanide. It is probable that the protein used by the earlier workers was contaminated by a cysteine-containing impurity since the cysteine content was, on occasions, considerably lower than the minimum required for one molecule[6].

DISCUSSION OF ANALYTICAL DATA

The amount of any one amino-acid has little significance in the interpretation of structure and biogenetic relationships[15].

Similarly, no significance can at present be attached to the ratio of one amino acid to another unless there is definite evidence that the amino acids or other groups concerned are functionally interrelated. Thus Roche[5] and Rossi-Fanelli (p. 115) have compared the histidine/iron ratios of various haemoglobins and also the sulphur/arginine ratios. Thus while the former may be significant because of possible connections between histidine and haem, the latter ratio is without meaning because sulphur is not present in the form of a single substance but, in the pigments of the various species, may be contained in varying mixtures of cystine, cysteine and methionine.

K. BAILEY[15] has suggested that it is probably more valuable to compare the distribution of the various polar and non-polar groups (*cf Table II*).

Comparison of the proteins— The amino acid data in *Table I* demonstrate the basic nature of the two proteins and show that it is produced by a significantly different mixture of amino acids. Thus in myoglobin 62 per cent of the basic groups are due to lysine and 31 per cent to histidine whereas lysine and histidine each contribute 40 per cent of the basic groups of haemoglobin. The total ionic groups of the proteins are also significantly different. Haemoglobin contains 27 per cent of ionic groups (cationic and free anionic) and is similar to other serum proteins (*cf* Tristram[16]). Myoglobin contains 34 per cent of such groups and in this respect bears a distinct resemblance to the muscle proteins myosin (34·1 per cent) and tropomyosin (45 per cent) (see Bailey[15]).

Myoglobin as a precursor of haemoglobin— The simple relationship between the molecular weights of myoglobin (17,000) and haemoglobin

(68,000) and certain similarities in crystal structure (*cf* Kendrew (p. 148)) made it attractive to suggest that the simpler protein might be a precursor of the more complex molecule. Whilst their overall amino acid compositions are dissimilar, the possibility that myoglobin may be the precursor of a portion of the haemoglobin cannot be excluded. That this is not so seems certain by virtue of the findings of Porter and Sanger (p. 121) that whereas haemoglobin contains six intramolecular chains each with a valine residue as the terminal amino group, myoglobin has but a single chain with a glycine residue in the corresponding position.

The proteins haemoglobin and myoglobin must therefore be regarded as strictly independent molecules, with parallel functions in blood and muscle respectively, and as far as amino-acid composition is concerned similar only in their content of haem and in the overall basic character of the protein moieties.

My thanks are due to Dr H. T. Macpherson, Mr M. W. Rees and Mr G. Wiltshire for permission to make use of unpublished analyses and to Professor A. C. Chibnall for advice and valuable criticism.

Received September 1948

REFERENCES

[1] SVEDBERG, T. *The Ultracentrifuge* (1936) Clarendon Press, Oxford
[2] MACPHERSON, H. T. *Bio-chem. J.* 40 (1946) 470
[3] REES, M. W. *Bio-chem. J.* 40 (1946) 632
[4] TRISTRAM, G. R. *Bio-chem. J.* 40 (1946) 721
[5] ROCHE, J., DERRIEN, Y. and VIEIL, H. *Bull. Soc. chim. Biol. Paris* 24 (1942) 1016
[6] ROSSI-FANELLI, A. *Arch. Sci. biol. Napoli* 26 (1940) 244
[7] GALE, E. F. *Bio-chem. J.* 39 (1945) 46
[8] FOSTER, G. L. *J. biol. Chem.* 159 (1945) 431
[9] KUHN, R., BIRKOFER, L. and QUACKENBUSH, F. W. *Ber. dtsch. chem. Ges.* 72 (1939) 409
[10] GREENSTEIN, J. *J. biol. Chem.* 128 (1939) 233
[11] LYMAN, C. M., MOSELEY, O., BUTLER, B., WOOD, S. and HALE, F. *J. biol. Chem.* 166 (1946) 161
[12] MIRSKY, A. E. and ANSON, M. L. *J. gen. Physiol.* 19 (1936) 439
[13] CONSDEN, R., GORDON, A. H. and MARTIN, A. J. P. *Bio-chem. J.* 42 (1948) 443
[14] BLUMENTHAL, D. and CLARKE, H. T. *J. biol. Chem.* 110 (1935) 345
[15] BAILEY, K. *Bio-chem. J.* 43 (1948) 271
[16] TRISTRAM, G. R. *Advances in Protein Chemistry* V (1949) Academic Press, New York

The Chemical Composition of Human Myoglobin

A. ROSSI-FANELLI

These researches have demonstrated the possibility of obtaining human myoglobin from skeletal muscles by a relatively simple procedure ; they have also shown that human myoglobin crystals differ in form from those of haemoglobin, and some crystallographic data for human myoglobin are given. Human myoglobin and haemoglobin differ in the chemical nature of their globins. The nitrogen distribution (by the Van Slyke method) is different in the two pigments. The amino-acid content is also different, myoglobin being poorer in arginine and richer in lysine and tryptophan.

THE haemoglobin of muscle (myoglobin) is similar to that of blood, but differs from it in certain properties, such as position of the spectral absorption bands, affinity for oxygen and carbon monoxide, and the Bohr effect. Moreover both pigments have highly specific physiological functions. Haemoglobin and myoglobin both contain the same prosthetic group (protoferrohaem), and it was consequently presumed that the difference between the two pigments lay in the difference between their globins. In 1940 we gave the first experimental evidence for this hypothesis[1-5], showing that the protein components of haemoglobin and myoglobin (obtained in crystalline form from horse myocardium) are chemically different substances. The most striking differences were observed in the arginine, lysine, and tyrosine content, but the differences in proline, hydroxyproline, and tryptophan were also noteworthy. J. ROCHE[6] contributed a good deal to this work. Studies on the chemical nature of myoglobin, however, included only those derived from the more common laboratory animals (horse, ox, dog, pig) ; human myoglobin was neglected because attempts to prepare it in crystalline form had been unsuccessful. Nevertheless during recent years human myoglobin has been crystallized by D. L. DRABKIN[7], by H. THEORELL[8], and by the present author[9], using a variety of different procedures for the isolation of the protein. Our method, already used successfully for the crystallization of horse and bovine myoglobins, is based on the different solubilities of haemoglobin and myoglobin in ammonium sulphate solutions, a fact already observed by V. E. MORGAN[10], E. COHN[11], J. ROCHE[6], and others. This method does not involve any previous perfusion of the muscle and may be applied to any type of skeletal muscle.

It is first necessary to determine the solubility curve of haemoglobin in ammonium sulphate solution, since haemoglobin is always present in skeletal muscle. These solubility curves of haemoglobin were measured either by determining the concentration of pigment photometrically, or by measuring the nitrogen content according to the procedure proposed by Roche and Marquet[12]. The solubility of human haemoglobin in ammonium sulphate is a function of the *p*H and, to a minor extent, of the haemoglobin concentration itself. But in concentrations approximately equal to those found in muscle extracts haemoglobin is wholly precipitated in ammonium sulphate solutions of 75 per cent saturation at a *p*H of 6·8 to 7·0. Under these conditions myoglobin remains in solution. It was, however, not easy to separate the two pigments quantitatively under these conditions, and we found it advisable to increase the concentration of ammonium sulphate to the point where a certain amount of myoglobin is precipitated as well.

The technique used for the crystallization of human myoglobin was as follows. The muscle, taken as soon as possible after death or surgical removal, was freed from fat and subcutaneous and connective tissue and passed twice through a latapie (mincer). 1·2 volumes of water were added and the *p*H adjusted to 6·8–7·0 with N.NaOH, using a glass electrode. The mixture was kept for 12 or 14 hours in the ice box, being shaken intermittently. It was filtered through linen cloth, the mass of muscle tissue being carefully squeezed, and the liquid extract then being filtered through paper and the *p*H again controlled. Solid ammonium sulphate was added gradually up to 78 per cent saturation, complete solution being ensured after each addition. After storing for 1–2 hours at 0°C the solution was filtered, and the filtrate distributed in Petri dishes of about 25 cm diameter, the depth of liquid not being greater than 1 or 1·5 cm. The dishes were stored at 0°C in a dry room. Crystallization began after 36 to 48 hours or sometimes longer. In the first instance yellow-red, strongly birefringent spheres were formed (*Figure* 1) ; these later developed ray-like prominences and finally gave rise to typical crystals (*Figures* 2 to 4).

Crystallization was allowed to continue for a further 12 to 24 hours at 0°C in order to increase the crystal size. The crystals were then separated by centrifugation at moderate speeds, and washed repeatedly with saturated ammonium sulphate solution at *p*H 7·0 and at low temperature. The iron content was checked by a method described previously[2] and if necessary crystallization was repeated, after dissolving the crystals in the minimum amount of water.

This procedure should be repeated until a constant iron content of 0·34 per cent is reached. The best results are obtained if all operations are carried out at 0°C in a refrigerated room. Owing to the great

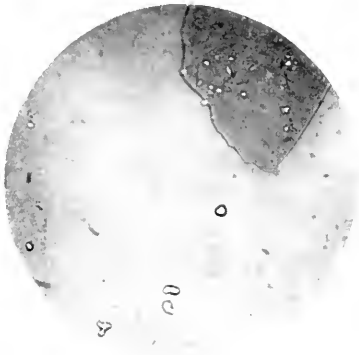


Figure 1. *Human myoglobin. Beginning of crystallization*

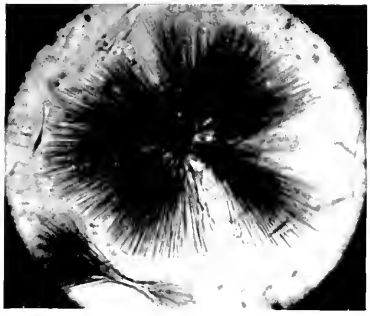


Figure 2. *Human myoglobin. Crystals of myoglobin prepared from normal human skeletal muscles (traumatic accident)*

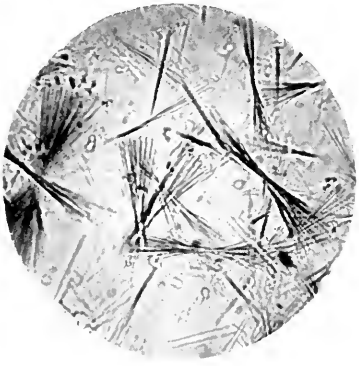


Figure 3. *Human myoglobin. Crystals of human myoglobin prepared from limb amputated for Leo-Buerger's disease*

Figure 4. *Human myoglobin. Crystals of human myoglobin prepared from limb amputated for sarcoma*

affinity of myoglobin for oxygen the above procedure leads to the formation of crystals of metmyoglobin.

In order to obtain the best crystals it is necessary to control the temperature and to protect all solutions from dust and from any possible colloidal impurities. We are, however, convinced that the most important factor in the production of good crystals is the state of the protein itself. Similar observations have been made with methaemoglobin by J. BOYES-WATSON, E. DAVIDSON, and M. F. PERUTZ[13].

By these procedures crystalline samples of myoglobin from several human skeletal muscles have been obtained (*Figures* 2, 3, and 4). It should be noted that the preparation of *Figure* 2 was obtained from the muscles of a healthy man, removed after a traumatic accident. The preparation of *Figure* 3 was made from the muscles of a man suffering from Leo-Buerger's disease, only those muscles which appeared to be in good condition being taken ; in cases such as this myoglobin crystallizes with difficulty, and many of our attempts were unsuccessful —probably the anoxic state, the deficiency of pigment, and even the denaturation of the protein, are responsible for this state of affairs. The preparation of *Figure* 4 was obtained from the limb muscles in a case of sarcoma, only muscles far removed from the site of the tumour being taken ; crystallization was good although there was considerable contamination from the lymphatic ganglia.

The micrographs show that the myoglobin crystals obtained from the various preparations have very much the same form. The crystals of metmyoglobin are definitely birefringent and consist of very thin needles which unite in more or less parallel bundles or in spheroidal aggregates of radiating fibres. The birefringence is negative, α being parallel to the long axis of the needle. There is straight extinction and marked pleochroism, α' (brown-reddish) being parallel to the needle axis and γ' (pale yellow) being perpendicular to it. The refractive index is greater than 1·514 when compared with balsam in xylene solution.

In order to investigate the chemical constitution of myoglobin, crystals from normal muscles were dissolved in water, the protein was carefully denatured by heating, and ammonium sulphate was completely removed. Fat was removed with alcohol and ether, and the protein was dried at 100°C and finally kept in a desiccator until its weight was constant. The following analyses were then carried out : nitrogen distribution by a micro-modification of the Van Slyke method[2], sulphur and iron content, and finally the content of various amino-acid residues by micromethods previously described[2, 14]. These require only 300 mg of protein in all. For comparative purposes analyses were also made of microcrystalline samples of human haemoglobin prepared by the methods of Drabkin[15].

Table I

Distribution of Nitrogen in Human Haemoglobin and Myoglobin
(Values are quoted as gm amino-acid N *per 100 gm protein* N)

	Haemoglobin	*Myoglobin*
Amide N	5·86	6·57
Humin N	3·93	4·66
Arginine N	7·86	3·98
Histidine N	13·37	13·85
Lysine N	9·34	13·10
Cysteine N	0·50	0·47
Non-NH_2 N (*imino-acids* + 1/2 N *of tryptophan*)	4·13	5·06
Filtrate-NH_2 N (*monoamino-acids*)	54·52	51·75
Total N *recovered*	99·55	99·44

Table I gives the nitrogen distribution of the two pigments, and reveals differences in the distribution of the various amino-acid

Table II

Percentage concentration and molecular ratios of certain components of human haemoglobin and myoglobin

(Values are quoted as weight of amino-acid per 100 gm of dry protein)

Pigment	*Iron*	*Histidine*	*Lysine*	*Arginine*	*Cysteine*	*Tryptophan*	*Sulphur*		*Nitrogen*
							Total	*Non cysteine*	
				Percentage compositions					
Haemoglobin	0·34	8·1	7·98	4·0	0·71	2·0	0·65	0·47	16·7
Myoglobin	0·34	8·2	11·00	1·98	0·65	3·4	0·57	0·40	16·4
				Molecular ratios					
Haemoglobin	4	35	37	16	4	7			
Myoglobin	1	9	13	2	1	3			

fractions. The most evident difference concerns the basic nitrogen, minor differences also being found in the non-amino nitrogen of the filtrate (imino-acids + 1/2 tryptophan nitrogen) and in the nitrogen of the monoamino and dicarboxylic acids.

Table II gives the data for percentages of iron, sulphur, histidine, lysine, arginine, cysteine and tryptophan. These data show that human haemoglobin and myoglobin have the same iron content and probably the same prosthetic group (protoferrohaem). The proteins differ in amino-acid content, myoglobin being poorer in arginine and richer in lysine and tryptophan : only small differences in sulphur and cysteine content were noted. In consequence the molecular ratios are different (see second part of *Table II*). Human myoglobin has one residue of cysteine only, in good agreement with a minimum molecular weight of 17,000 (*cf* the constancy of cysteine content of myoglobin of different species, claimed by Roche[6]).

The differences in chemical composition are accompanied by differences in the physico-chemical behaviour of the two pigments. For example, the positions of the spectral absorption bands and the resistances to alkaline denaturation are different[16, 17, 18]. On the latter property was based a spectrophotometric method for the simultaneous determination of the two pigments[19].

Table III

Arginine/Sulphur and Histidine/Iron Ratios of Haemoglobins and Myoglobins of different Species

Nature of pigment	*Arginine/sulphur*	*Histidine/iron*
Haemoglobin of		
*Ox**	1·20	0·82
Dog	1·18	0·86
Horse	1·35	0·90
Man	1·13	0·85
Myoglobin of		
Ox	—	1·22
Dog	—	1·11
Horse	0·63	0·93
Man	0·64	0·86

* The data for ox and dog myoglobin and haemoglobin are calculated from figures given by Roche[6].

Finally we compared the arginine/sulphur and histidine/iron ratios of human haemoglobin and myoglobin with those of the corresponding pigments from other species. The results are summarized in *Table III.* It will be noted that the arginine/sulphur ratio, which Roche considers to be very important for the classification of proteins[20], has a constant value for haemoglobins of different species, and a different constant value for myoglobins of different species ; myoglobin has an arginine/sulphur ratio half that of haemoglobin—another index of the different nature of the two pigments. On the other hand the histidine/iron ratio, while constant for haemoglobins of different species, is not constant for the different myoglobins. This fact is of particular interest in view of the fact that this amino-acid is believed to have a considerable importance in binding the iron of the prosthetic group to the globin.

Received August 1948

REFERENCES

[1] ROSSI-FANELLI, A. and TRAVIA, L. *Boll. Soc. ital. Biol. sper.* 15 (1940) 484
[2] — *Arch. Sci. biol. Napoli* 26 (1940) 244
[3] — and TRAVIA, L. *Boll. Soc. ital. Biol. sper.* 16 (1941) 768
[4] — and VESCIA A. *Boll. Soc. ital. Biol. sper.* 17 (1942) 291
[5] — and ARAGONA, C. *Boll. Soc. ital. Biol. sper.* 17 (1942) 206
[6] ROCHE, J., DERRIEN, Y. and VIEIL, H. *Bull. Soc. Chim. biol., Paris* 24 (1942) 1016
[7] DRABKIN, D. L. *Amer. J. med. Sci.* 209 (1945) 269
[8] THEORELL, H. *Arch. Biochem.* 12 (1947) 119
[9] ROSSI-FANELLI, A. and PERRI, G. C. *Boll. Soc. ital. Siol. sper.* 23 (1947) 113
[10] MORGAN, V. E. *J. biol. Chem.* 112 (1936) 557
[11] COHN, E. J. *Physiol. Rev.* 5 (1925) 359
[12] ROCHE, J. and MARQUET, F. *Bull. Soc. Chim. biol., Paris* 17 (1935) 1630
[13] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. F. *Proc. roy. Soc. A.* 191 (1947) 83
[14] ROSSI-FANELLI, A. and VESCIA, A. *Boll. Soc. ital. Biol. sper.* 17 (1942) 129
[15] DRABKIN, D. L. *J. biol. Chem.* 164 (1946) 703
[16] ROSSI-FANELLI, A. and GIULOTTO, L. *Boll. Soc. ital. Biol. sper.* 23 (1947) 253
[17] — *Boll. Soc. ital. Biol. sper.* 23 (1947) 852
[18] — *Boll. Soc. ital. Biol. sper.* 24 (1948) 410
[19] — and GIULOTTO, L. *Boll. Soc. ital. Biol. sper.* 24 (1948) 506
[20] ROCHE, J. *Essai sur la biochemie générale et comparée des pigments respiratoires* Masson et Cie, Paris

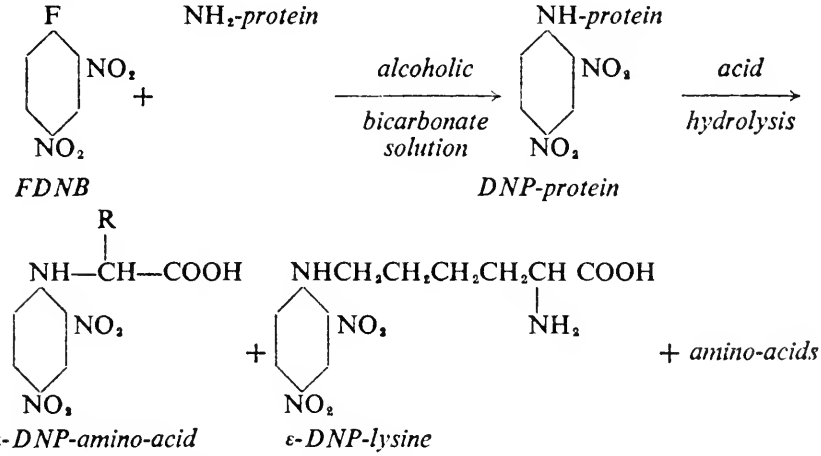
The Free Amino Groups of Haemoglobins

R. R. PORTER and F. SANGER

A comparative study of certain features of the structures of a number of mammalian haemoglobins has been carried out.[1] *Information as to the structure was obtained by identifying and estimating the amino-acids which carry a free amino group.*[2] *It was hoped that such an investigation of the general structure of haemoglobins from closely related and more distant species might suggest a chemical basis for the immunological specificity of these proteins. Their physical and chemical properties differ according to the species of origin but only their serological behaviour shows a systematic variation which follows their genealogical relationships. A graduated variation in structure was in fact revealed and in horse haemoglobin a preliminary study of the terminal peptides of the polypeptide chains has given further information about the molecular pattern.*

THE DNP METHOD

THE principle of the method used for the identification and estimation of free amino groups in proteins may be illustrated by the following formulae :



The reagent used is 1 : 2 : 4-fluorodinitrobenzene (FDNB), which reacts quantitatively with the free amino groups of the proteins at room temperature. On hydrolysis of the DNP-protein by strong acid the yellow DNP-amino-acids, which are relatively stable, may be separated from the other amino-acids and can be fractionated successfully using partition chromatography on wet silica gel.[3] In this manner they can be identified and estimated colorimetrically.

Table I summarises the results that were obtained using the DNP technique.

Table I. The Numbers of Free Amino Groups of Haemoglobins

Haemoglobin	*Assumed mol wt*	*Terminal residues*	*Free lysine ϵ-amino groups*
Horse	66,000	6 *valine*	41
Donkey	66,000	6 *valine*	41
Human adult	66,000	5 *valine*	43
Cow	66,000	2 *valine*, 2 *methionine*	47
Sheep	66,000	2 *valine*, 2 *methionine*	47
Goat	66,000	2 *valine*, 2 *methionine*	48
Human foetal	66,000	2·6 *valine*	47
Horse myoglobin	17,000	1 *glycine*	20

It can be seen from these results that the haemoglobins from closely related animals, such as the horse and donkey or the cow, sheep and goat have the same number of open polypeptide chains per molecule and that these chains have the same terminal amino-acids, although the two groups are distinct from each other and from the other haemoglobins examined. A detailed investigation of the serological relationships of these proteins has been carried out[4, 5, 6] and it was found that the haemoglobins from the closely related animals, which we have now shown to have a similar structure, cross-reacted strongly with each other but not with other haemoglobins. Also L. HETKOEN[7] found in the dog that myoglobin was serologically distinct from haemoglobin and R. R. DARROW, S. NOWAKOVSKY and M. H. AUSTIN[8] showed that human adult and foetal haemoglobin did not cross-react. Thus the parallel between the immunological behaviour of the haemoglobins and the general structure as revealed by this technique is striking. It may be, however, that though both these properties are dependent on the genealogical origin of the proteins, they are not interdependent. A much more detailed study would be necessary to establish the precise features of the protein structure which control immunological specificity.

Preliminary investigations of serum globulins[9] showed that there were similar structural variations from species to species and it would be interesting to know if this could be a basic property of all proteins. Insulin has indeed been found to be an exception in that cattle, sheep

and pig insulin have the same terminal amino-acids[9] but it is in many ways a unique protein; one of its exceptional properties is non-antigenicity.

FURTHER DETAILS OF THE STRUCTURE OF HORSE HAEMOGLOBIN

If we assume that these haemoglobin molecules are made up of several open polypeptide chains, we presuppose that these chains are bound in some manner to form the whole molecule. The method of binding may in some instances be of a labile character, as it has been shown that mild changes of conditions will reduce the molecular weight of horse haemoglobin to half. However stronger cross-linkages between the groups of three chains probably exist.

Comparison of the numbers of ε-amino groups of lysine which reacted with FDNB, with the analytical figures for the total number of lysine residues per molecule shows good agreement. This is in contrast to results found for certain other proteins (PORTER[10]) and shows that in the haemoglobins no interchain linkages occur through the lysine side chains.

The only known covalent linkage is the —S—S— bridge of cystine. The content of this amino-acid in horse haemoglobin is low, not more than three residues being present (see G. R. TRISTRAM, this volume, p. 109), and some of these are known to be in the reduced state, as —SH groups become reactive on denaturation. It is clear therefore that should the six polypeptide chains of horse haemoglobin be covalently bound into two groups of three, there must exist another type of covalent cross-linkage besides the —S—S— bridges of the cystine residues.

A further important point which requires to be established in elucidating the structure of horse haemoglobin, is whether the six chains, which all have the same terminal amino-acid, are in fact identical. Information has been obtained on this by an investigation of the peptides produced by partial hydrolysis of DNP-globin. Partial hydrolysis was carried out by dissolving the DNP-globin in 10 N. HCl and keeping the solution at 37°C for 4 hours. The terminal DNP-peptides could then be fractionated on silica gel columns using suitable solvents. It was possible to crystallize three DNP-valyl peptides purified in this manner, and their structure was found to be as follows :

1 DNP-valyl-leucine
2 DNP-valyl-glutamyl-leucine
3 The amide of *2*.

It is clear that at least two distinct types of chains must exist in the molecule, as two different penultimate residues, leucine and glutamine,

have been found. It may well be that other different terminal peptides occur and that the polypeptide chains vary significantly in the sequence of amino-acids, making the final solution of the detailed structure of this protein an even more formidable problem.

REFERENCES

[1] PORTER, R. R. and SANGER, F. *Bio-chem. J.* 42 (1948) 287
[2] SANGER, F. *Bio-chem. J.* 39 (1945) 507
[3] GORDON, A. H., MARTIN, A. J. P. and SYNGE, R. L. M. *Bio-chem. J.* 37 (1943) 79
[4] HETKOEN, L. and SCHULHOF, K. *J. infect. Dis.* 33 (1923) 224
[5] — and BOOR, A. K. *J. infect. Dis.* 49 (1931) 29
[6] HEIDELBERGER, M. and LANDSTEINER, K. *J. exp. Med.* 38 (1923) 561
[7] HETKOEN, L. *J. infect. Dis.* 42 (1928) 31
[8] DARROW, R. R., NOWAKOVSKY, S. and AUSTIN, M. H. *Arch. Path. Lab. Med.* 30 (1940) 873
[9] PORTER, R. R. *Ph.D. Thesis.* Cambridge University (1948)
[10] — *Biochimica et Biophysica Acta* 2 (1948) 105

The Simultaneous Spectrophotometric Determination of Haemoglobin and Myoglobin in Extracts of Human Muscle

CHRISTIAN DE DUVE*

A differential spectrophotometric method is described, by which haemoglobin and myoglobin can be simultaneously determined in extracts of human muscle. The method is based on the spectral differences between the CO*-compounds of the two pigments. Readings are taken at* 575·7 mμ, *where they have identical extinction-coefficients, at* 568 *and* 583·8 mμ, *where* CO–Mgb *has the same extinction-coefficients,* CO–Hb *widely different ones. Subsequent calculations are facilitated by the choice of these particular wave-lengths. Perfect reproducibility of the wave-lengths is an essential condition of accuracy and is ensured by preliminary checking by means of a coloured glass filter.*

The extract used for the determination must be crystal-clear, strongly buffered, and truly representative of the pigment-content of the muscle. A method is briefly described by which these requirements can be met as completely as possible. Appreciable amounts of unidentified haemin have been found, however, in the tissue residues. By all criteria, they appear to belong to an insoluble component.

THE method which is briefly described in this paper was worked out at the suggestion of Professor H. THEORELL in order to furnish clinical research workers with a suitable analytical tool, which would make it possible to investigate the myoglobin-content of human heart- and skeletal-muscle under various conditions. The present paper constitutes a preliminary report; detailed publication of the method described has recently been made in *Acta Chemica Scandinavica*.†

The main difficulty in the determination of myoglobin is brought about by haemoglobin, which is invariably present in muscle extracts and cannot be removed quantitatively without some loss of myoglobin as well. It was therefore necessary to work out a differential method, taking advantage of the slight spectral differences which exist between

* Present address: Department of Physiological Chemistry, University of Louvain, Belgium.

† 2 (1948) 264.

the two pigments. Their carbon-monoxide compounds were found particularly suitable for this purpose. The Beckmann spectrophotometer was used for the determinations.

Standard absorption curves were carefully determined for solutions of pure CO-haemoglobin and CO-myoglobin, the latter being purified and crystallized according to a method previously reported[1]. The pigments were dissolved in M/15 Na_2HPO_4 and pure CO was bubbled through the solution after addition of an excess of $Na_2S_2O_4$. The solution was then poured into a 1 cm cuvette, care being taken to fill the cuvette completely and to cover it immediately. Readings were taken at a number of wave-lengths on pure solutions and on mixtures. The applicability of Beer's law was checked by varying the dilution widely.

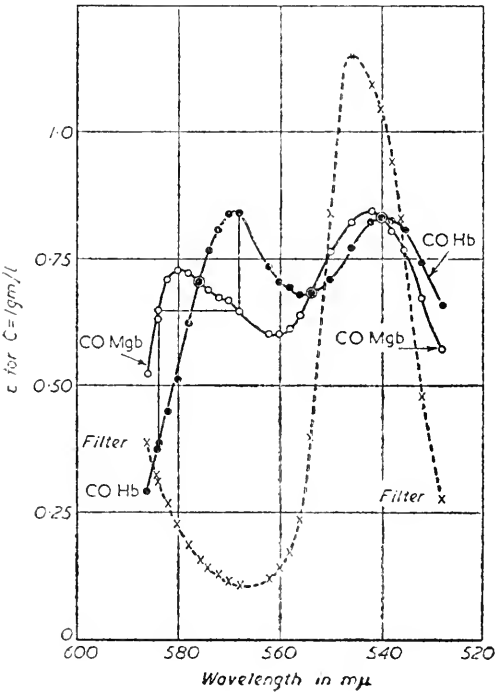


Figure 1. *Standard extinction curves of* CO–Hb (●—●), CO–Mgb (O—O) *and light-filter* (x—x)

ε = *extinction coefficient for* $C = 1$ *gm/l of dry pigment and* $d = 1$ *cm*

Figure 1 shows part of the spectral curves of pure CO–Hb and CO–Mgb in the range of the alpha and beta bands. For differential measurements it was found very convenient to take readings at 575·7 mμ (isobestic point) and at 568 and 583·8 mμ, where CO–Mgb has identical, CO–Hb widely different extinction-coefficients. The following simple set of equations is then valid :

$$\left.\begin{aligned} C_{tot} &= \frac{A_{(575\cdot7)}}{\varepsilon_{(575\cdot7)}} \\ C_{Hb} &= \frac{A_{(568)} - A_{(583\cdot8)}}{\varepsilon_{Hb(568)} - \varepsilon_{Hb(583\cdot8)}} \end{aligned}\right\} \quad C_{Mgb} = C_{tot} - C_{Hb}$$

where C = concentration of dry pigment in gm/l, $A_{(x)}$ = density read at x mμ, $\varepsilon_{(x)}$ = extinction coefficient at x mμ.

By replacing the extinction-coefficients by their value, one obtains:

$$\left.\begin{aligned} C_{tot} &= 1\cdot416 \times A_{(575\cdot7)} \\ C_{Hb} &= 2\cdot2123 \times (A_{(568)} - A_{(583\cdot8)}) \end{aligned}\right\} \quad C_{Mgb} = C_{tot} - C_{Hb}$$

The total pigment concentration (C_{tot}) was also determined independently by the reduced pyridine-haemochromogen method, thus providing a check of the value obtained at the isobestic point.

If 1 ml of the solution to be analyzed is mixed with 3 ml of a solution containing: pyridine 100 ml, N NaOH 30 ml, aq dist *ad* 300 ml, and if the reading is taken at a wave-length of 557·5 mμ immediately after the addition of an excess of $Na_2S_2O_4$, a value of C_{tot} directly comparable with the one above is obtained from the equation:

$$C_{tot} = \frac{A_{(557\cdot5)}}{0\cdot494}$$

Results obtained in this way can only be accurate if wave-lengths can be reproduced within a fraction of a mμ and this was found not to be the case with the apparatus used. In order to ensure perfect reproducibility of the wave-lengths, the spectrum of a suitably chosen coloured glass filter (Seitz VG 20 1 mm) was determined against air, simultaneously with the two standard spectra, and subsequently used as reference spectrum. Standard readings were made in such a way that each set of three points, corresponding respectively to CO–Hb, CO–Mgb and the filter, were determined without moving the monochromator of the apparatus. It was then possible to reproduce subsequently any of the wave-lengths used for the standard readings by moving the monochromator to a position where the required extinction-value could be read for the filter.

The use of this simple device led to a very satisfactory degree of accuracy, the error being less than 1 per cent in most of the trial tests on mixtures of the purified pigments. Good reproducibility was also obtained on muscle extracts, but the preparation of extracts both truly representative of the myoglobin content of the tissue and suitable for spectrophotometric determination turned out to be a problem by itself. After numerous trials, the following method was found most satisfactory:

Approximately 500 mgm of tissue are cut into small pieces, frozen,

and ground with dry ice in a mortar until a pinkish homogeneous powder is obtained. This powder is then transferred into a Pyrex glass homogenizer, further ground until thawing is completed and then thoroughly homogenized with 3 ml of a dilute acetate buffer, N/100, *p*H 4·5. An aliquot of the homogenate is taken for dry weight determination and the remainder weighed and extracted three times with 2 to 3 ml acetate buffer. The three extracts are pooled and their total volume read accurately. After mixing, the pooled extract is examined for clarity and eventually recentrifuged if found not to be crystal-clear. Six ml of the clear supernatant are then pipetted out and alkalinized by the addition of 25 mgm solid Na_2HPO_4, 12 H_2O per ml of fluid, with a resulting increase in volume of 1·2 per cent. One ml of the alkaline extract is used for pyridine-haemochromogen determination and the remaining 5 for conversion into the CO-compounds and spectrophotometric analysis. It is important to mention that clear extracts can only be prepared by using a slightly acid extracting fluid. If distilled water or alkaline buffers are used, the extracts are turbid and cannot be clarified by centrifuging. Alkalinizing of the extracts before they have been centrifuged to clarity similarly creates unremovable turbidity.

Final results are expressed in per cent of dry weight and can be calculated by means of the following formula :

$$\% \text{ Mgb} = C_{Mgb} \frac{1{\cdot}012 \times V \times 100}{d.w.}$$

in which V is the volume of the extract and C_{Mgb} its Mgb concentration, *d.w.* the dry weight of the amount of tissue analyzed, which is obtained by multiplying the weight of homogenate extracted by the ratio : dry weight/wet weight of the homogenate.

Results obtained so far in duplicate and serial determinations on different pieces of the same muscle suggest that the range of error of the method rarely exceeds 10 per cent.

Appreciable amounts of unextracted haemin have been found, however, in the tissue residues and considerable work has already been devoted to this aspect of the problem. No definite conclusion can be formulated as yet but the evidence available so far does not support the simple explanation that the insoluble haemin residue belongs to unextracted myoglobin. Further results will be communicated by Dr G. Biörck, who has undertaken a systematic study of the myoglobin content of human muscle.

It is a pleasure to acknowledge the invaluable help and advice of Professor H. Theorell. *Received August 1948*

REFERENCE

[1] Theorell, H. and de Duve, Chr. *Arch. Biochem.* 12 (1947) 113

IV

X-RAY CRYSTALLOGRAPHY

An X-Ray Investigation of Haemoglobin and Haemocyanin in Aqueous Solution

D. G. DERVICHIAN, G. FOURNET and A. GUINIER

The scattering of x-rays at low angles has been used to obtain information on the dimensions of molecules of haemoglobin and haemocyanin in aqueous solutions. A gyration radius of 23 Å *is found for haemoglobin, which fits with the flat cylindrical model of the molecule* (57 Å *diameter and* 34 Å *height*). *Haemocyanin shows a spacing of* 220 Å inside *the particle. The same proteins were examined in presence of urea but showed no marked sign of dissociation.*

THE method of x-ray scattering at low angles can supply information on the size of particles in solution. In a previous work we investigated the organization of the haemoglobin molecules in the red cell[1]. It was shown that there existed a certain type of regularity in the mutual arrangement of the haemoglobin molecules. In the present article we are concerned with solutions of horse haemoglobin of concentrations much weaker than in the red cell. These concentrations varied from 1·2 to 12 per cent. In these conditions organization is absent. But, owing to the relative independence of the molecules it is possible to deduce information on the size of the dissolved haemoglobin molecules from the shape of the scattering intensity curve.

Solutions of haemocyanin (from *Helix*) were also investigated for concentrations ranging between 0·7 and 3 per cent. They show an internal spacing of the particle in solution in agreement with the value already found by O. KRATKY[2].

Finally we tried to detect the action of urea on the size of the particles of haemoglobin and haemocyanin.

Technical details of the method and the theory of the calculation have been given elsewhere by one of us[3]. It should be recalled here that the gyration radius of the scattering particle is calculated from the curve of the logarithm of intensity of scattered x-rays against the square of the angle. This gyration radius ρ has the same definition as is given in the calculation of moments of inertia, *i.e.* ρ represents the radius of a particle having the same moment of inertia as the particle studied but in which the mass of the particle is entirely localized at the distance ρ. Thus, for example, for a homogeneous sphere, ρ is equal to 0·78 times the actual radius. By adopting a certain definite shape, *e.g.* cylindrical, spherical, cubic, *etc*, and assuming that the particle is

homogeneous, it is possible to calculate the gyration radius of the adopted model and to test if it coincides with the gyration radius deduced from the X-ray scattering measurements.

HAEMOGLOBIN

The curve of *Figure* 1 represents the variation of the scattered intensity with the angle of scattering for haemoglobin. It was deduced from the microphotometric recording of the X-ray photograph. The calculated gyration radius is equal to 23 Å $\pm$ 1. This same value is found either with recrystallized horse haemoglobin dissolved in saline or with the crude contents of the red cell after lysis and dilution with saline. The same value was also found whatever the concentration, this varying between 1·2 and 12 per cent.

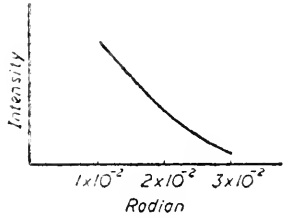


Figure 1. *Variation of the scattered intensity with the angle of scattering. Haemoglobin*

A gyration radius of 23 Å may be attributed to a variety of forms of particles. Thus, if a spherical form were assumed for the haemoglobin molecule in solution, this value of the gyration radius would correspond to a sphere of 59 Å diameter. The interest of this result is that if we assume that the haemoglobin molecule in solution has the flat cylindrical form proposed by one of us[4] as the general shape of protein molecules in solution and if we take for this form the dimensions found by M. F. PERUTZ *et al*[5] for the molecule in the crystal of haemoglobin, *i.e.* 57 Å diameter and 34 Å height, the gyration radius calculated is found equal to 23 Å, coinciding with the value deduced from the scattering intensity variation curve.

From the type of variation of the scattering intensity it is possible to conclude that haemoglobin solutions are monodisperse, *i.e.* containing mainly particles of the same size. In fact, the study of other protein solutions with the same method shows that solutions of serum globulins for example behave as mixtures of particles having different sizes. On the contrary, solutions of serum albumin do behave as if they were monodisperse.

HAEMOCYANIN

While the intensity curve slopes down regularly with haemoglobin, with haemocyanin there appears a well marked hump as is seen on

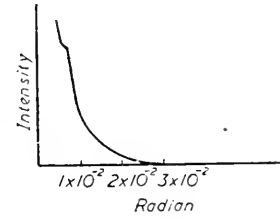


Figure 2. *Variation of the scattered intensity with the angle of scattering. Haemocyanin*

Figure 2. The angle at which this hump appears does not vary with the dilution of the haemocyanin solution. The conclusion is therefore that the singularity is due to an internal structure of the particles.

If Bragg's law is assumed, the value of the angle at which the hump occurs corresponds to a period of 220 Å. This, as said above, appears to be an internal spacing of the particles in solution. But, in contrast to the case of haemoglobin, the particular form of the experimental curve does not permit the use of the above mentioned simple theory to obtain information about the size of the entire particle of haemocyanin.

O. KRATKY[2] found with haemocyanin a similar scattering curve showing a hump. The deduced spacing was 260 Å, but apparently this author has not examined solutions at different dilutions. Kratky thinks that it is plausible to conclude that the primary particles are really spherical and that the value of 260 Å corresponds to the diameter of these particles. This dimension should be compatible with the molecular weight of $8{\cdot}9 \cdot 10^6$ found by ultra-centrifugation. According to Kratky these spheres would be capable of forming threadlike aggregates responsible both for flow birefringence and for the hump in the x-ray scattering diagram.

According to the electron micrographs obtained by A. POLSON and R. W. G. WYCKOFF[6] with haemocyanin preparations (although the haemocyanin was from *Busycon canaliculatum*), each haemocyanin particle appears to consist of rod-like sub-units stacked together. The dimensions of these sub-units, which can be appreciated from the picture of Polson and Wyckoff, are of the same order of magnitude as the 220 Å found in the present work. Nevertheless, a model formed of four ellipsoids stacked together does not fit with the complete curve of scattering intensity.

ACTION OF UREA

By the same method of x-ray scattering, solutions of haemoglobin or haemocyanin containing different concentrations of urea were studied. Urea was added up to a concentration of 25 per cent and left in contact with the protein from a few hours to 24 hours. With haemoglobin, the

scattering curve appears practically the same as the curve obtained with the untreated protein and gives again a gyration radius of 23 Å (see *Figure* 3). With haemocyanin, the presence of urea does not modify the general shape of the scattering curve, but the hump is slightly displaced as if the internal spacing was reduced to 170 Å (by assuming again the validity of Bragg's law).

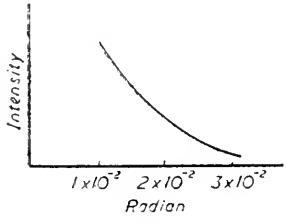


Figure 3. *Variation of the scattered intensity with the angle of scattering. Haemoglobin with* 25 *per cent urea*

The negative result with haemoglobin is rather surprising since it has been shown, particularly by J. STEINHARDT[7], that already at urea concentrations of the same order of magnitude the haemoglobin molecules are dissociated giving smaller sedimentation constants. It would be interesting to reconcile these contradictory results obtained on the one hand by measurements of sedimentation velocities and on the other hand by our x-ray scattering analysis. The solutions studied by Steinhardt contained only 0·43 to 0·65 per cent haemoglobin while those we have submitted to the action of 25 per cent urea contained from 3 to 12 per cent haemoglobin. The quantity of urea relative to the quantity of protein may perhaps have an importance.

Received September 1948

REFERENCES

[1] DERVICHIAN, D. G., FOURNET, G. and GUINIER, A. *C. R. Acad. Sci., Paris* 224 (1947) 1848
[2] KRATKY, O. *J. Polymer Sc.* 3 (1948) 195
[3] GUINIER, A. *Ann. Phys., Paris* 12 (1939) 161
[4] DERVICHIAN, D. G. *J. Chim. phys.* 38 (1941) 59
(*) *J. chem. Phys.* 11 (1943) 236
[5] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. F. *Proc. roy. Soc. A* 191 (1947) 83
[6] POLSON, A. and WYCKOFF, R. W. G. *Nature, Lond.* 160 (1947) 153
[7] STEINHARDT, J. *J. biol. Chem.* 123 (1938) 543

(*) For reasons indicated in the Editor's footnote, the translation of this article in J. chem. Phys. had not been submitted to the author. Consequently the meaning of some sentences has been obscured or modified. Other sentences express the reverse of the idea found in the original text, *e.g.* title of Table I, where ' elongated ellipsoids ' should read ' oblate ellipsoids'.

Recent Developments in the X-Ray Study of Haemoglobin

M. F. PERUTZ

The paper reviews the x-ray analysis of horse methaemoglobin, especially the results of the recently completed three-dimensional Patterson synthesis. A brief description of the principal features of that synthesis is followed by an account of its interpretation. An arrangement of the polypeptide chains compatible with all the chemical and x-ray data is suggested and the possible positions of the haem groups are discussed. The paper concludes with a brief account of comparative x-ray studies of different haemoglobins, discussing possible correlations between structural data and physico-chemical properties.

HORSE METHAEMOGLOBIN

Introduction— Most x-ray crystallographers would agree that the work involved in the analysis of a crystal structure increases as an exponential function of the structure's complexity. Due largely to the absence of any direct method for obtaining the atomic positions from the observed intensities of the diffracted rays, a detailed analysis of an organic compound of comparatively moderate size, such as sucrose or cholesterol, takes two or more man-years to complete. On the face of it, therefore, an attempt to analyze the crystal structure of haemoglobin, or of any crystalline protein for that matter, looks about as promising as a journey to the moon. Indeed, had it not been for an improbably large share of good luck, which confuted all the more sober assessments of my chances, I should not know more about the structure of haemoglobin now than when I started. Fortunately, the first crystalline protein which came my way was methaemoglobin of horse which has since proved to have the simplest crystal structure of any protein of comparable molecular weight, with features so favourable that they make a crystallographer's heart leap with joy.

The crystals are easily grown in the right habit for x-ray analysis and can be kept in salt solutions for long periods. The molecules occupy known positions in the unit cell, each of them being placed on a diad axis of symmetry which requires each molecule to consist of two identical halves. As all the diad axes in the crystal point the same way, the molecules must do the same. I soon found that the crystals consist of layers of haemoglobin molecules alternating with layers of liquid ; it was this factor, combined with certain other useful

properties, which enabled me to calculate the electron density distribution in projection along a line through the molecule. Finally, the structure happens to be one of those exceptional types mentioned in the article with J. C. KENDREW (p. 149), where the vector structure bears a strong resemblance to the real one. It was the parallelism of the polypeptide chains in each haemoglobin molecule, and of all the molecules in the crystal, which facilitated the interpretation of the three-dimensional Patterson synthesis. This synthesis forms the main theme of the present article, but before passing on to it I shall review briefly some of the more important properties of the haemoglobin crystals, and describe their molecular structure as we saw it before the results of the Patterson synthesis became available. Both this work and the results of the Patterson synthesis have been published in full elsewhere (J. BOYES-WATSON, E. DAVIDSON and M. F. PERUTZ[1]; M. F. PERUTZ[2]; henceforth referred to as *I* and *II*). For readers who are unfamiliar with the methods of X-ray crystallography and especially with the meaning of Patterson syntheses, a brief outline of X-ray analysis as applied to the study of crystalline macromolecules is provided in this volume (Kendrew and Perutz, this vol. p. 161).

Over 50 per cent of the volume of wet haemoglobin crystals consist of liquid of crystallization. More than a third of this is water 'bound' to the protein molecules and therefore not available as solvent to electrolytes, but the remainder is free and acts as a medium through which a variety of ions can diffuse without damaging the structure of the crystals[3]. This sponge-like character is a unique and peculiar property of protein crystals, and can be used with great advantage in their X-ray analysis. For instance, by allowing heavy ions to diffuse into the liquid of crystallization, the X-ray scattering power of the liquid can be enhanced relative to that of the protein; in effect, the liquid regions in the crystal can be 'stained' as far as their X-ray scattering power is concerned, with resulting changes in the relative intensities of certain diffracted rays. These changes can give important information about the distribution of liquid in the unit cell, and hence about the shape of the molecules.

Protein crystals suspended in solutions of different electrolytes or in atmospheres of different humidity can be made to swell or shrink. A great deal was learnt by comparing the diffraction patterns of the same crystal at different states of swelling and shrinkage. It was found, for instance, that the haemoglobin molecules themselves were rigid and impenetrable to liquid, and that it was merely their relative arrangement and the distances between them which alter during swelling and shrinkage. It was also through shrinkage experiments that the layer structure of the crystals was first discovered.

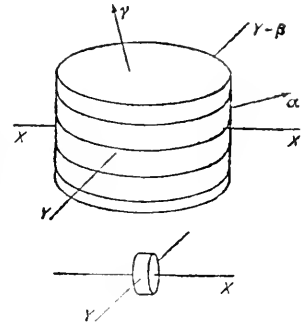


Figure 1. *Diagrammatic model of haemoglobin molecule, showing its orientation with respect to the crystal axes.* Y *is the diad axis. The small disk underneath represents a haem group drawn on the same scale and in the correct orientation with respect to the crystal axes. The four lines on the cylinder surface represent four layers of scattering matter.* (*Reproduced from* I.)

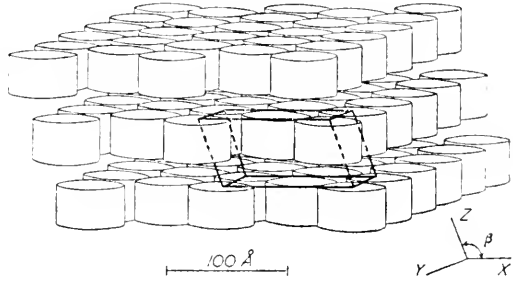


Figure 2. *Packing of haemoglobin molecules in the crystal structure, showing layers of close-packed molecules separated by liquid. One unit cell is shown in the foreground on the right.* (*Reproduced from* I.)

The information derived in those studies is summarized in *Figures* 1 and 2 which show the shape of the haemoglobin molecules and their arrangement in the lattice of the normal wet crystals. The haemoglobin molecule is pictured in an idealized form as a cylinder of 57 Å diameter and 34 Å height ; the four horizontal lines indicate four layers of scattering matter whose presence was inferred from a one-dimensional Fourier projection on to the cylinder axis. The small disk underneath represents a haem group whose orientation relative to the crystal axes can be found from the optical dichroism of the crystals (Perutz[5] and *I*). Some indirect evidence regarding the positions of the haems on the globin molecule will be discussed below. In the crystal the cylindrical haemoglobin molecules are packed into layers which alternate with layers of liquid. Within each layer every cylinder is surrounded by

six nearest neighbours and the interstices between the cylinders are filled with liquid.

Of necessity only a very small part of the total diffraction pattern from haemoglobin was used for the studies just described (*i.e.* the reflexions from sets of planes parallel to the principal crystal axes) while the remainder had to be disregarded for lack of any method of interpretation. It was obviously desirable to prepare a three-dimensional Patterson synthesis which was intrinsically much more likely to lend itself to reasoned interpretation than the two-dimensional projections which had been employed in the earlier work, yet for a small research team this seemed a vast and very risky enterprise. Had haemoglobin not been one of the favourable structure types mentioned before—and we had little evidence to suggest that it was—the meaning of the vector structure might have been impossible to decipher. If I decided nevertheless to take the chance, it was partly because the stakes seemed to make it worth while, and partly because youthful enthusiasm made me underestimate the years of soul-destroying routine work which lay ahead.

The Three-dimensional Patterson Synthesis— The physical principles underlying this method are described by Kendrew and Perutz (p. 161). In practice, the intensities of about 20,000 diffracted rays had to be recorded, indexed, measured, corrected and tabulated. In the work of indexing the reflexions and measuring their intensities, the longest stage of all, I was helped by Miss Edna Davidson and Miss Joy Boyes-Watson. After allowance had been made for overlapping and symmetry I was left with about 7,000 relative intensities which were to be the coefficients of the terms of the Fourier summation. Each intensity formed the amplitude of a three-dimensional wave giving a sinusoidal variation of vector density through the unit cell. In theory, 28,000 such waves, each of different amplitude, wave-length and orientation, would have had to be summed for each of 54,000 points in the unit cell. Actually this labour can be much reduced by making use of the symmetry properties of cosine functions, but even so a calculation of this magnitude is beyond the patience even of a crystallographer. It was done for me by the Scientific Computing Service using punched card calculating machines, and did not take more than one person's working time for about four months.

The results of the three-dimensional synthesis were plotted in the form of contour maps, each of which gives the distribution of vector density in a section through the unit cell. The sections are parallel to the plane containing the X and Z axes (see *Figure* 2) and extend over the whole of c and one quarter of a. Altogether 31 sections were plotted, at intervals of just over 1 Å, starting from the origin

and extending halfway along b. Thus the contour maps cover $\frac{1}{8}$ of the unit cell—the remaining $\frac{7}{8}$ are related to them by symmetry.

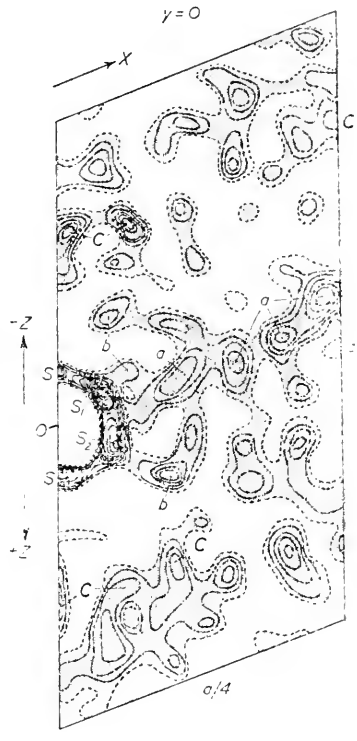


Figure 3. *Section of vector structure through the origin. Scale in this and the following diagrams* 1 Å = 7 *mm.* (*Reproduced from* II).

Figure 3 shows the section through the origin. The peak at the origin itself has been omitted ; the average level of vector density in the unit cell was chosen as a kind of sea level and only the contours of the islands were drawn, those of the hollows below sea level being omitted. All areas above sea level are shaded. The most striking features of *Figure* 3 are first a ring of high vector density surrounding the origin at a distance of about 5 Å (marked S) which contains a very high concentration of vector density, and secondly a rod-like concentration of high vector density which is centred at $z = \mathrm{O}$ and runs parallel to X. This rod contains four maxima (marked a), spaced at intervals of 5 Å along the length of the rod.

A survey of the other sections showed that the ring at 5 Å was part of a roughly spherical shell of high vector density around the origin, but that the density within that shell was greatest in the plane of *Figure* 3. This survey also revealed more rod-like structures running

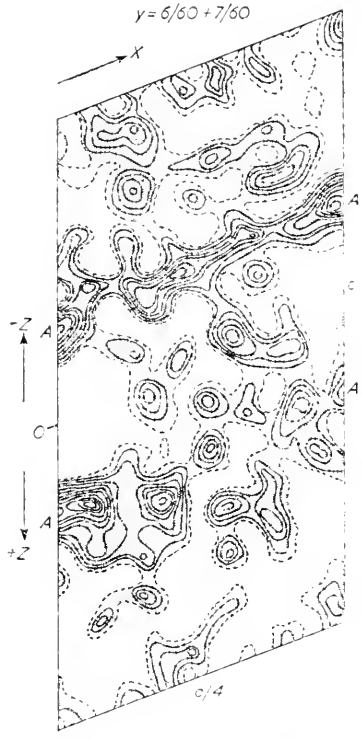


Figure 4. *Contour map obtained by adding densities of sections* 6·3 *and* 7·4 Å *above the origin.*

Figure 5. *Contour map obtained by adding densities of sections* 9·5, 10·5 *and* 11·6 Å *above the origin.*
(*Reproduced from* II.)

parallel to the *X*-direction which are shown in *Figures* 4 and 5. These contour maps were drawn by superimposing two (or three) sections and adding the densities at each point. In this way features which extend over more than one section can be made to show up very clearly. In *Figure* 4 two rods of high vector density (marked *A–A*) are visible ; these are really part of one continuous rod-like structure which runs through most of the unit cell—a fact which can be visualized if the map is imagined to be repeated by symmetry, involving rotation through 180° around the points $x = z = 0$ and $x = \frac{1}{4}$, $z = 0$. Finally *Figure* 5 shows a structure, rod-like though rather tortuous, winding its way along the *X*-direction at the centre of the diagram (marked *B–B*). Both the '*A*' and the '*B*' rods are spaced 10–11 Å from the central one. As they are all parallel to the *X*-direction

they show up most strikingly on a planar projection of the vector density along the *X*-axis (*Figure* 6), *i.e.* a projection on to a plane which is normal to the length of the rods. Here the rods appear as six peaks surrounding the origin at a distance of 10–11 Å, in the exact positions of the rods in the three-dimensional synthesis.

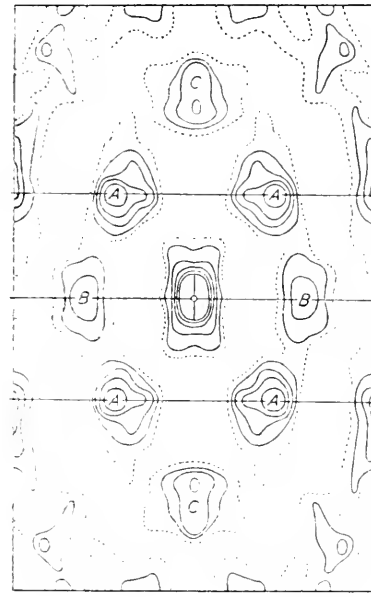


Figure 6. *Patterson projection normal to x-axis. The origin is marked by a cross at the centre.* (*Reproduced from* II.)

Figure 7 gives a perspective view of this rod structure in an idealised form. It shows the central rod sliced open along half its length,

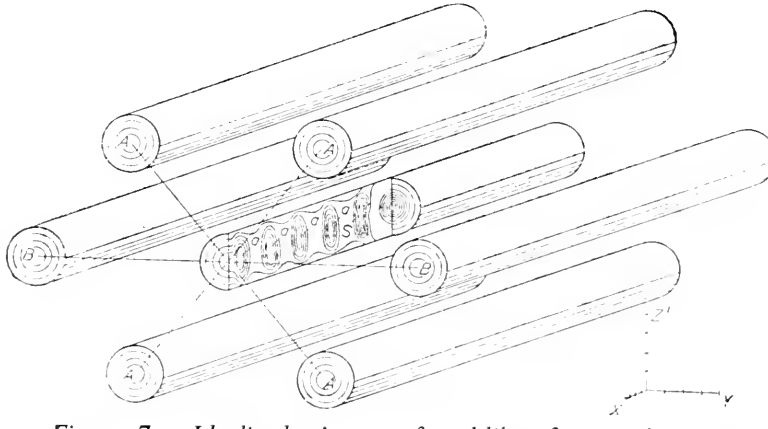


Figure 7. *Idealized picture of rod-like feature in vector structure. The scale marks on the axial cross give Ångstrom units.* (*Reproduced from* II.)

revealing the arc of high vector density at 5 Å from the origin (*S*) and the maxima along the length of the rods. The main rod is surrounded by six others at a distance of 10·5 Å, arranged at the corners of a regular hexagon, which can be identified with the rods '*A*' and '*B*' in the sections.

It will be shown elsewhere in this volume (see p. 176) that this is the type of vector structure to be expected from a set of parallel chain molecules. According to the relationship between vector structure and molecular structure outlined in *Figure* 10 of that paper (p. 177), the arrangement of the rods suggests that the haemoglobin molecule contains chains parallel to *X* with a molecular pattern repeating at 5 Å intervals along the chain direction and a distance of 10–11 Å between neighbouring chains. This conclusion emerges from the vector structure alone and is quite independent of other evidence, comprising both chemical and x-ray data, which can be used to carry the interpretation further. The detailed argument was given in *II* and need not be repeated here. I showed there that the molecular chains whose presence is indicated by the vector structure can be none other than the polypeptide chains themselves. The repeat of the molecular pattern at intervals of 5 Å along the chain direction implies that the polypeptide chains are folded, since in a fully extended chain the amino-acid residues repeat at intervals of 3·4 Å and not of 5 Å; hence the 5 Å vector would appear to represent the distance between atoms which are spaced two or more amino-acid residues apart. According to *Figure* 1 the chains would have to run parallel to the base of the cylindrical haemoglobin molecule. These chains would probably be arranged in the form of four layers, to conform with the layered arrangement of the vector peaks in *Figure* 6, and the layered structure of the haemoglobin molecule previously deduced from one-dimensional Fourier projections (see *I*; also this volume, *Figure* 1 p. 137).

Arguing purely from considerations of packing there should be 20 such chains in the haemoglobin molecule. R. R. PORTER and F. SANGER (p. 121), on the other hand, have shown the horse haemoglobin molecule to contain six terminal α-amino groups. Hence the 20 chains cannot be independent, but must be combined into six bigger chains folded backwards and forwards in long zig-zags. Alternatively there might be six open chains together with a number of closed rings.

The foregoing conclusions are summarized in *Figure* 8 which is an idealized drawing showing the *type* of chain configuration and packing which would be compatible with the Patterson synthesis. (*a*) shows a polypeptide chain with a pattern repeating at intervals of 5 Å in the

chain direction (as nothing is known about the geometry of this pattern, its existence is merely indicated by a series of lines normal to the chain length), and a long-range zig-zag leading to distances of 10·5 Å between neighbouring portions of the same chain folded back on itself. In (*b*) an arrangement of the chains has been chosen in which the two prominent interchain vectors '*A*' and '*B*' are the most frequent. The details in the two pictures are of course purely imaginative, but the general layout which they indicate follows from the vector structure.

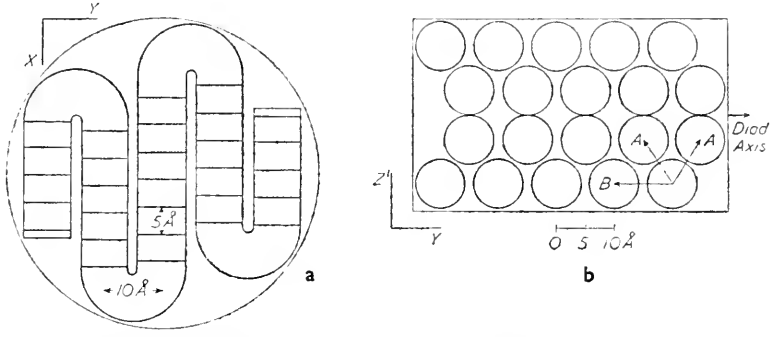


Figure 8. *Idealized picture of type of haemoglobin structure compatible with Patterson synthesis.* (a) *Shows a basal section through the cylindrical molecule with one polypeptide chain folded in a plane.* (b) *Represents a vertical section through the cylinder normal to* X *with the chains seen end on.* (*Reproduced from* II.)

Discussion: *The Positions of the Haems*— In the following paper Kendrew discusses the close resemblance between the basic structural features of haemoglobin and myoglobin. X-ray studies of other crystalline proteins indicate that the layered arrangement of the polypeptide chains and their packing at distances of 10–11 Å may be frequently occurring features of globular protein structure. There also appears to be a close connection between the polypeptide chain structure in haemoglobin and that in the group of fibrous proteins giving the well-known α-keratin pattern. This pattern generally consists of only two reflexions, one at 5·1 Å in the direction of the fibre axis and another at 9·7 Å at right angles to it (see for instance plate 16 in W. T. ASTBURY[4]). It seems that the 5·1 Å reflexion in α-keratin corresponds to the 5 Å vector peak along the chain direction found in haemoglobin. If we assume the chains in α-keratin to be arranged in hexagonal close packing, the average distance between neighbouring chains would be 9·7/sin 60° = 11·2 Å, which is the same within the limits of definition of the vector peaks as the interchain distance of 10·5 Å found in the present analysis. This leads us

to the conclusion that the fundamental stereo-chemical configuration of the polypeptide chains in haemoglobin and myoglobin, and in the large group of proteins of the α-keratin type, are the same.

The position of the haem groups is one of the most vital structural problems in the X-ray analysis of haemoglobin, vital not only for the understanding of the reaction mechanism between haemoglobin and oxygen, but also because it affects the much wider problem of chemical interaction between prosthetic groups across the framework of protein molecules. The scattering contribution of the haems relative to that of the globin is very small, and at the present stage they cannot be located by direct X-ray analysis. On the other hand, we now have a variety of crystallographic and other data which serve at least to narrow down their possible positions. I have described in earlier papers (Perutz[5] and *I*) how the orientation of the haem groups can be deduced from the pleochroic absorption of haemoglobin crystals. These observations showed that the four haem groups in the haemoglobin molecule must be approximately parallel to each other, with their flat sides normal to the *X* axis. In the light of the evidence just described this would mean that their flat sides would also be approximately normal to the length of the polypeptide chains.

There is also certain evidence in favour of the haem groups lying on the surface of the haemoglobin molecules[3]. It was mentioned before that the haemoglobin molecules are rigid and impenetrable to liquid. On the other hand, reactions between the haem groups and diffusing ions take place inside the haemoglobin crystals, just as though the haemoglobin molecules were in solution, and without causing any change in unit cell dimensions. For instance, crystals of acid methaemoglobin suspended in ammonium sulphate solutions can be transformed into alkaline methaemoglobin by adding diammonium phosphate to the suspension medium ; on addition of sodium azide, crystals of acid methaemoglobin are transformed into the addition compound azide methaemoglobin. Such reactions can be performed in a drop of liquid on a slide and watched with a microspectroscope. Now if it is true that the haemoglobin molecules are impenetrable to liquid and if reaction between the haem group and the liquid of crystallization can take place nevertheless, the haem groups must obviously be located on the surface of the globin portion of the molecule.

As regards their positions on the surface of the globin molecule the following considerations are relevant. (*1*) A diad axis of symmetry passes through the centre of the molecule, which means that a rotation by 180° about that axis must bring one half of the molecule into congruence with the other. (*2*) J. WYMAN, Jr. (see p. 95) shows that when the haemoglobin molecules split into halves in urea solution,

one pair of haems is associated with each half. The two pairs, therefore, must be related by the diad axis and they must lie on opposite sides of the plane of splitting. (*3*) The haem groups can be pictured as disks of 15 Å diameter and 3·7 Å thickness. These must be so placed that they fit into the unit cell of the dried as well as the wet crystals. For instance, if the haem group stuck out above and below the top and bottom surfaces of the globin cylinder, there would not be room for them in the dried unit cell ; hence they are more likely to be attached to the sides of the cylinder. (*4*) An azide group can be attached to each iron atom without causing a change in unit cell dimensions. Since an azide group is over 4 Å long, this means that the haem groups cannot be concerned in the bonding of neighbouring haemoglobin molecules in the crystal lattice.

This is as far as the data go at the moment. We may hope that circumstantial evidence will continue to accumulate and that by fitting this together bit by bit we may eventually be able to define the positions of the haem groups within close limits.

COMPARATIVE STUDIES

Derivatives of Horse Haemoglobin— Having spent so much time on an intensive x-ray analysis of methaemoglobin of horse I was naturally curious to see how this structure compared with those of other haemoglobin derivatives of the same species, especially whether the physiologically more important derivatives oxy-, carboxy- and reduced haemoglobin have the same molecular structure as methaemoglobin. I soon found that the situation was complicated by the polymorphism of many of these substances. It seems that met-, oxy- and carboxy-haemoglobin can be crystallized in either of two forms. One is the monoclinic one described in the foregoing pages and the other an orthorhombic one, with a structure far less favourable for analysis (mentioned in *I*, *Table I*, p. 93). Crystalline oxy- or carboxyhaemoglobin can be changed into methaemoglobin without change in crystal structure and therefore any structural conclusions derived from an analysis of the latter appiy with equal force to the former. In addition to the two forms just mentioned, carboxyhaemoglobin has recently been crystallized in a second monoclinic form, entirely different from the one described here and containing a more complex arrangement of the molecules in the unit cell.

As F. HAUROWITZ discovered by optical studies[6], the crystal structure of reduced haemoglobin is different. Crystals of methaemoglobin cannot be transformed into reduced haemoglobin without being broken up, and the isotropic, pseudo-hexagonal plates of reduced

haemoglobin are transformed into bundles of birefringent needles in the presence of oxygen. X-ray analysis shows the crystals to be rhombohedral with six haemoglobin molecules arranged in a row along an axis of triad symmetry. The dichroic absorption of the crystals suggests that the flat sides of the haem groups are normal to the triad axis, while a vector projection on a plane normal to that axis gives an indication, but no more than an indication, that the polypeptide chains are parallel to that axis. On this basis the triad in reduced haemoglobin would correspond to the *X* axis in methaemoglobin, as far as the orientation of the haemoglobin molecules is concerned. It is very difficult, however, to fit a molecule into the unit cell of dried reduced haemoglobin which also fits into the unit cell of dried methaemoglobin. This, and the totally different crystal form of reduced haemoglobin indicates that a molecular change more profound than the mere oxygenation and de-oxygenation of the haem groups accompanies the transformation between the two compounds. This had already been suggested by Haurowitz[6], but so far no other evidence has appeared which would provide any clue as to the nature of the change. Neither Haurowitz nor Gutfreund (private communication) could detect any difference in viscosity, nor could the latter find any difference between the sedimentation constants of carboxyhaemoglobin and reduced haemoglobin. There are, of course, great differences between (*a*) the solubilities of these two derivatives, and (*b*) the stabilities of the two types of derivatives on storage (Roughton, private communication). It remains to be seen whether these are due to a change in structure on oxygenation.

Haemoglobins of Other Species— A comparative study of foetal and and adult sheep haemoglobin, undertaken at Sir Joseph Barcroft's suggestion, has recently been published[7] and has provided strong additional evidence for the non-identity of the two proteins. Our study was more concerned with a general survey of the types of crystal structure which occur in the foetal-adult haemoglobin system of sheep than with a detailed analysis of any one of them. We did, however, find indications of a difference in molecular symmetry between the two proteins: in the adult haemoglobin the molecular weight of the asymmetric unit* was 68,000, while in the foetal one it was 34,000. This unit of 34,000 in turn seemed to consist of two at least closely similar sub-units of molecular weight 17,000. These differences in molecular symmetry can be correlated with different splitting properties of the two proteins in solution. Gutfreund (p. 195) who measured the sedimentation constant of the two proteins found that adult sheep haemoglobin does not dissociate, whereas the foetal haemoglobin—

* For definition of this term see page 162.

which has the same molecular weight as the adult one in concentrated solutions—tends to split into quarter molecules in dilute solutions.

Thanks to the courtesy of Dr. O'Brien and Mrs. Jope who are supplying us with a variety of beautiful crystals, we have recently embarked on a study of the human haemoglobin system. All the crystals examined so far had complex structures with several molecules in different orientations in the unit cell. The form studied in most detail was carboxyhaemoglobin which forms beautiful orthorhombic octahedra, but owing to the complexity of the intermolecular arrangement the X-ray analysis yielded disappointingly meagre results[8]. In the human haemoglobin system oxy-, carboxy- and methaemoglobin crystals again seemed to form isomorphous crystals, while reduced haemoglobin crystallized in two modifications which were both different from the orthorhombic form of the other three derivatives. (See this vol., p. 269.) Foetal human carboxyhaemoglobin forms truncated rhombohedra which may be either mono- or triclinic and are different from any crystals observed in the adult haemoglobin system.

CONCLUSION

To the outside observer the effort that has to be spent on the X-ray analysis of a crystalline protein may seem out of proportion to the gain. Yet this method, though slow and laborious, still offers the only way by which the molecular structure of the crystalline proteins can be studied, and to date it has given us at any rate a glimpse of the general layout of the polypeptide chains in two protein molecules, some reasonably accurate data regarding their shape, and a number of useful hints about the possible positions of the haems on the globin. The haemoglobins are among the easiest proteins to crystallize. They offer a wealth of crystalline compounds and derivatives, thus opening a variety of different ways of approach to the problem of protein structure. Some of these ways have proved dead ends, but many are still leading on.

Received September 1948

REFERENCES

[1] Boyes-Watson, J., Davidson, E. and Perutz, M. F. *Proc. roy. Soc. A* 191 (1947) 83
[2] Perutz, M. F. *Proc. roy. Soc. A* 195 (1949) 474
[3] — *Trans. Faraday Soc.* 42*B* (1946) 187
[4] Astbury, W. T. *Proc. roy. Soc. B* 134 (1947) 303
[5] Perutz, M. F. *Nature, Lond.* 143 (1939) 731
[6] Haurowitz, F. *Hoppe-Seyl. Z.* 254 (1938) 266
[7] Kendrew, J. C. and Perutz, M. F. *Proc. roy. Soc. A* 194 (1948) 375
[8] Perutz, M. F. and Weisz, O. *Nature, Lond.* 160 (1947) 786

The Crystal Structure of Horse Myoglobin

J. C. KENDREW

The preliminary results of an x-ray study of horse myoglobin are described. They suggest the hypothesis that the myoglobin molecule is similar in shape and dimensions to one of the four layers in the molecule of horse haemoglobin. That is to say, it consists of a disk about 9 Å *thick and with other dimensions not greater than* 57 Å *; the most probable values are* 51·5 *by* 37 Å. *In the crystal there are two of these disks in each unit cell, parallel to one another and separated by a layer of liquid of crystallization* 6·6 Å *thick. The molecule consists of a single layer of parallel polypeptide chains about* 9·5 Å *apart, having a repeat distance of about* 5 Å *along the chains; this arrangement is similar to that found in haemoglobin and apparently to that in α-keratin. The haem group, which is about* 15 Å *across, lies perpendicular to the plane of the disk and approximately perpendicular to the polypeptide chains. The arrangement is illustrated diagrammatically in Figure* 4.

THE preceding paper describes the results of a prolonged and intensive study of horse haemoglobin. The present one gives an account of a much more superficial examination of horse metmyoglobin* ; more detailed study has so far been prevented by technical difficulties, which are mentioned below and which will take time to overcome. Fortunately the structure of the myoglobin crystals is simpler than that of haemoglobin crystals, and with the aid of the interpretations already put upon the haemoglobin data it is possible to make a number of interesting deductions even from the preliminary results. For further details of the X-ray crystallographic principles used in this research, the reader is referred to the succeeding paper (p. 161).

I have felt for some time that myoglobin would be a subject of particular interest for X-ray study. There are several reasons for this view. First, its molecular weight is only about 17,000[1, 2] which is a very low value by protein standards, so that its structure may be expected to be correspondingly simple. Second, there is a close relationship, in function and in properties, between myoglobin and haemoglobin, and we may anticipate a similarly close relationship between the structures of the two proteins. Third, myoglobin is being closely studied from the chemical point of view by other workers (see the contributions by G. R. TRISTRAM, p. 109, and by R. R. PORTER and F. SANGER, p. 121).

* In the interests of brevity, the term ' myoglobin ' will be used throughout this paper to denote horse metmyoglobin, and ' haemoglobin ' to denote horse methaemoglobin.

Unfortunately crystals of myoglobin, though relatively easy to prepare by salting out the aqueous solution of the protein with ammonium sulphate, are normally obtained in a habit which is most unsatisfactory from the point of view of the x-ray crystallographer, whose ideal is a single crystal with no dimension less than about 0·25 mm. The myoglobin crystals are amply long in one dimension, but extremely small in the other two—in other words they form thin needles and, as ordinarily prepared, lie just beyond the useful range of present-day single crystal x-ray technique.

The fortunate circumstance which made x-ray work possible was the discovery by M. W. REES[3] of the Department of Biochemistry at Cambridge that, if myoglobin is crystallized from very concentrated phosphate buffer solutions instead of from ammonium sulphate, it assumes a rather different habit, the needles becoming flattened to long lath-like plates (see *Figure* 1). Phosphate concentrations between 3 and 4 molar, and concentrated protein solutions, are required; mixtures of NaH_2PO_4, $2H_2O$ and K_2HPO_4 were used since these are the most soluble acid and basic phosphates available, the proportions being adjusted to give a *p*H of about 6·4. Details of an almost exactly similar crystallization of whale myoglobin (but at slightly higher *p*H) are given by J. KEILIN and K. SCHMID[4]. The crystals so prepared are often several mm. long and about one mm. across, but even the largest of them is still very thin, so that x-ray exposures of up to 200 hours were necessary to obtain usable diffraction pictures. After irradiation of this duration protein crystals appear to suffer some sort of internal disintegration so that, although outwardly unaltered, their diffraction patterns become weaker and weaker. This limits the amount of information which can be obtained from small crystals, and in these myoglobin crystals this limit has just about been reached. This is the reason why it is unlikely that the results about to be described can be greatly added to until even larger crystals can be prepared.

CRYSTAL SYMMETRY AND CELL DIMENSIONS

Myoglobin crystals are monoclinic, that is to say the unit cell dimensions are all unequal, while the angles between the *a* and *b* axes, and between the *c* and *b* axes, are 90° each, and the angle β between *a* and *c* has a value different from 90°. The actual dimensions are: $a = 57{\cdot}3$ Å, $b = 30{\cdot}8$ Å, $c = 57{\cdot}0$ Å, $\beta = 112°$, and a perspective drawing of the cell is given in *Figure* 2*a*. For comparison a drawing of the crystal in the same orientation is given in *Figure* 2*b*.

The space group is $P2_1$. This implies that a screw diad axis runs through the cell parallel to *b*. The meaning of this is that any piece

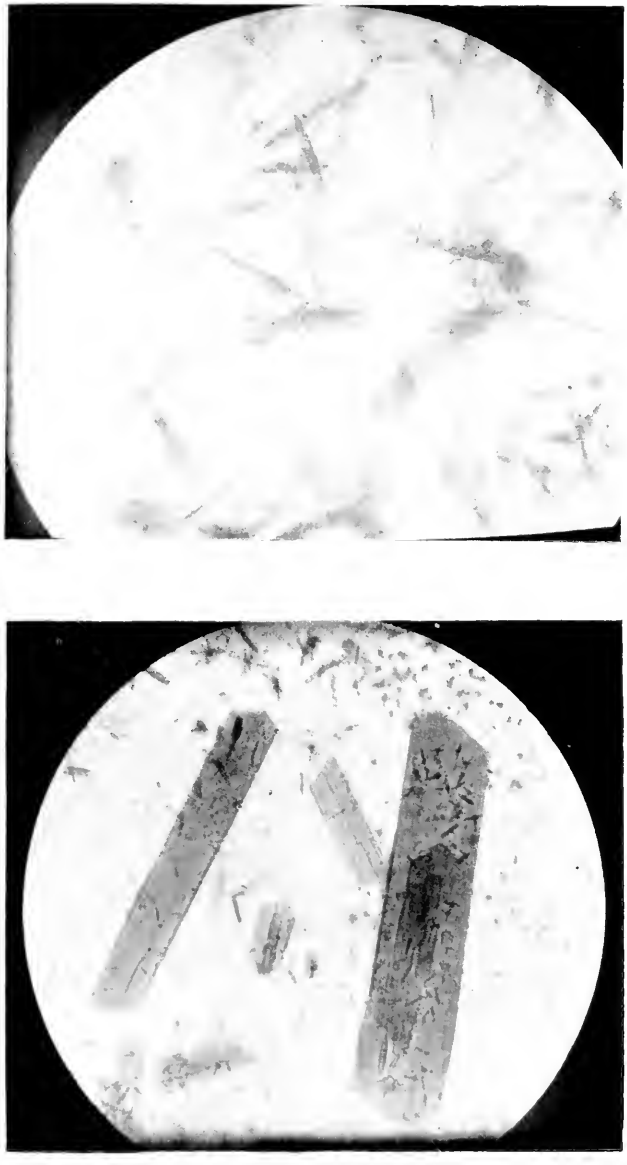
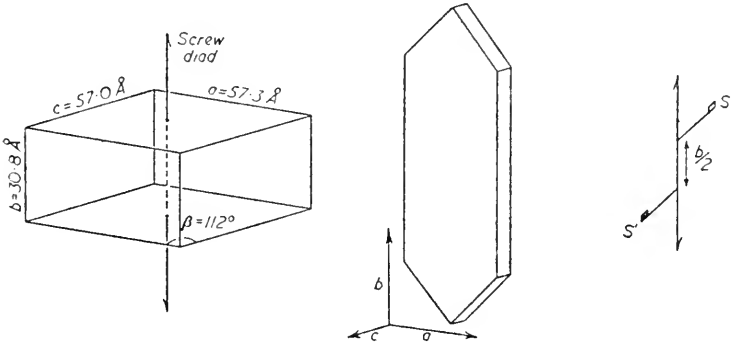


Figure 1. *Crystals of horse metmyoglobin.*
(By courtesy of M. W. Rees.)

of scattering matter S (see *Figure* 2*c*), if rotated through 180° about the axis, and then translated through a distance $b/2$ parallel to it, must come into coincidence with an *identical* piece of scattering matter S′.



a *b* *c*

Figure 2. *Unit cell and morphology of horse myoglobin crystals.*

The above data refer to the crystal as grown in approximately 3M phosphate buffer at *p*H 6·4, and they will be referred to as lattice A. Crystals of myoglobin, however, like other protein crystals, alter their dimensions when placed in different solutions or when dried, and although the poor quality of the crystals has so far prevented a complete exploration of the possible lattices, two others have been encountered. One, known as lattice B, is found when the crystals are placed in *saturated* phosphate buffer ; the other, known as the dry lattice, is obtained by taking the crystals out into air. The dimensions of all three lattices are given in *Table I.*

Table I

Cell Dimensions of Horse Metmyoglobin

Lattice	*Conditions*	*a*	*b*	*c*	*β*	*Cell vol.*
A	3M *phosphate*	57·3 Å	30·8 Å	57·0 Å	112°	93,400 Å^3
B	*Sat. phosphate*	57·3	30·8	43·1	98°	75,500
Dry	*Air-dried*	51·5	28·0	37·0	98°	53,300

It will be noted that the dimensions do not all shrink at equal rates ; *a* and *b* are unchanged between A and B, while *β* remains unaltered during the transition from B to Dry.

Assuming a normal density of about 1·27 for the dry crystals (this could not be directly measured because salt-free crystals were not

available, but the value is known to vary little from protein to protein), we may calculate the weight of the dry unit cell as 40,800 in molecular weight units. This is 2·4 times the molecular weight of myoglobin (16,850), so it may be deduced that each unit cell contains two protein molecules, the surplus weight being accounted for by residual water which always persists in protein crystals dried at room temperature. The two molecules must be related to one another by the screw diad axis of symmetry mentioned above.

PROPOSED MOLECULAR MODEL FOR MYOGLOBIN

In order to shorten the discussion it will be convenient to anticipate an account of the Patterson projections of myoglobin by describing the molecular model which appears to agree most closely with all the available data ; as each projection is described in turn its correspondence to the model will be indicated.

M. F. PERUTZ has shown (see p. 137) that the haemoglobin molecule has a four-layer structure, each layer consisting of a circular disk 8·8 Å thick and 57 Å in diameter. It is known that myoglobin has about one quarter the molecular weight of haemoglobin, and that it contains one haem group instead of four. The postulate for myoglobin is that its molecule consists of a single layer of parallel polypeptide chains, with dimensions approximately the same as one of the four layers of haemoglobin.

The first observation which suggested this hypothesis is the close correspondence between the *a* and *c* dimensions of the myoglobin unit cell and the diameter of the haemoglobin molecule : *Figure* 3*a* shows the *ac* face of the cell and a haemoglobin disk superimposed. This correspondence is suggestive since, although there is no absolute compulsion for a molecule to crystallize with a unit cell whose dimensions closely resemble those of the molecule, there is often in practice a near agreement if the molecule has what may loosely be called a reasonable shape.

Since the unit cell contains two molecules it is necessary to fit in two disks and to arrange them so that they are related by the screw diad. This has been done in *Figure* 3*b*, which is shown in projection on to the *ab* face in *Figure* 3*c* ; the arrows indicate the orientations of the molecules. It will be seen that the two disks fit very conveniently into the cell, with a layer of about 6·6 Å of liquid of crystallization between them. It should be mentioned that the data would equally fit a ' staggered ' arrangement of the kind shown in *Figure* 3*d*. Furthermore the disks need not necessarily be strictly coplanar, though the Patterson projections indicate that they must be approximately so.

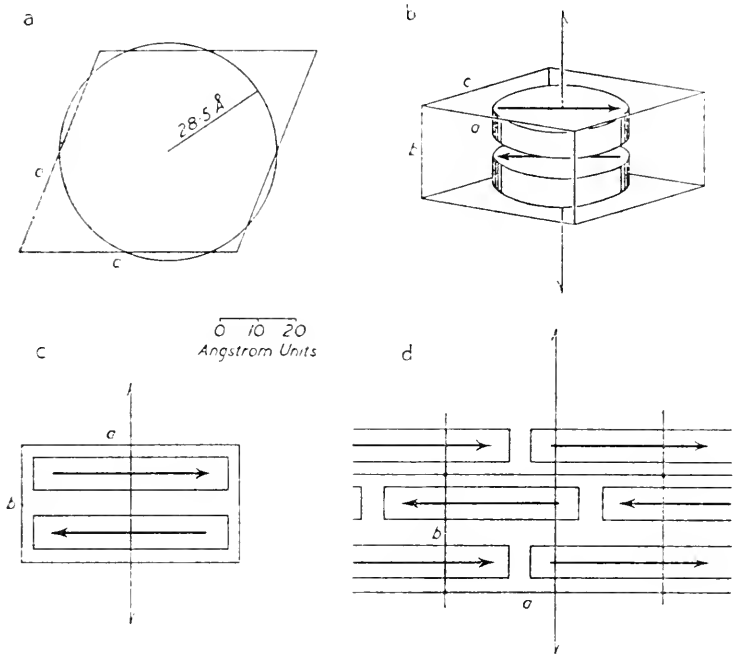
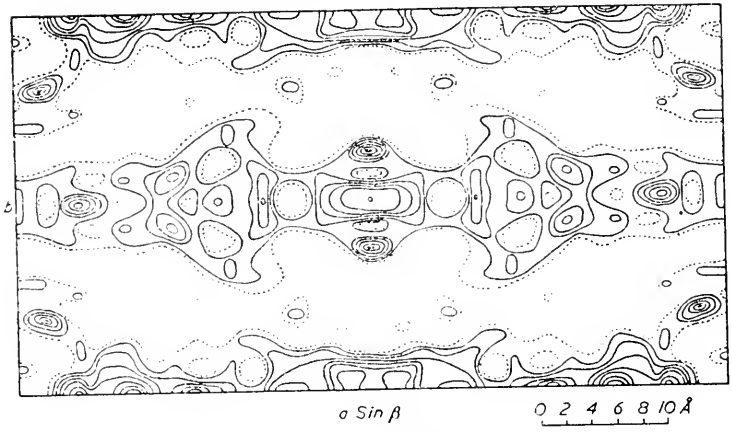


Figure 3. *Suggested mode of packing of myoglobin molecules in the unit cell.*

THE PATTERSON PROJECTIONS AND THEIR INTERPRETATION

I now proceed to give an account of various Patterson projections of the wet myoglobin unit cell, and to indicate how they support, and add further detail to, the molecular model just described. So far it has not been practicable to collect the data which would be required for a three-dimensional Patterson synthesis on the lines of that described by Perutz for haemoglobin (p. 138) ; fortunately the structure of myoglobin is so much more simple that two-dimensional projections contain many features which can be interpreted.

The three most important projections are illustrated in *Figure* 4, showing the main peaks of vector density. Each projection shows the whole of the unit cell, and in each the origin is drawn at the centre of the cell. In a monoclinic unit cell it is convenient to project, not on to the *ab* and *cb* faces of the cell, but on to planes through *b* and respectively perpendicular to *c* and *a* ; these projections have dimensions $b \times a \,.\, \sin\beta$ and $b \times c \,.\, \sin\beta$ and may be referred to as the *c* and *a* projections. The third projection, perpendicular to *b*, is on to the *ac* face of the cell and has dimensions *a* and *c*.



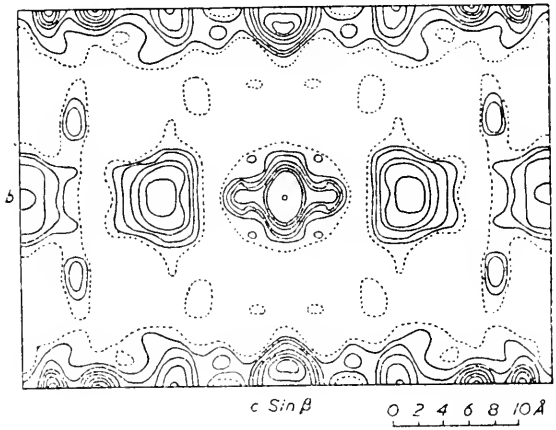
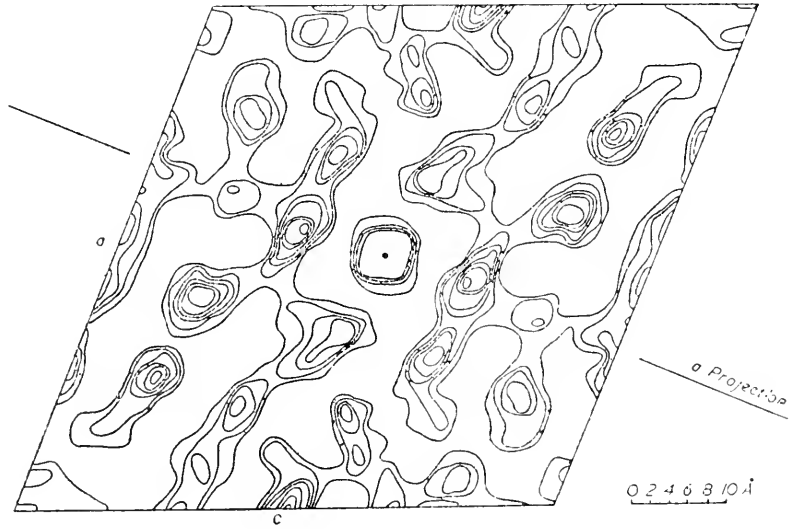


Figure 4.
Patterson projections of horse myoglobin.

Top—c projection. Lattice B.

Centre—a projection. Lattice B.

Bottom—b projection. Lattice A.



It will be noted that one of the projections is derived from lattice A, and the other two from lattice B. These have been chosen because they illustrate most clearly the striking features which will be mentioned below. Comparison between Patterson projections from slightly different lattices of the same crystal will not affect our deductions about the shape of the molecule since studies of other protein crystals have shown that during shrinkage the *intra*molecular distances, and hence those peaks in the Patterson diagrams which correspond to *intra*molecular vectors, do not alter ; it is only the *inter*molecular distances which shrink. In the present instance this has been confirmed for the *b* projection in the transition A—B and for the *a* projection in the transition B—Dry though not all the relevant projections are illustrated here.

(*a*) *The* c *projection* (*Figure* 4*a*)—The most prominent feature of this projection is the very powerful ridge of vector density through the origin and perpendicular to *b*. Parallel to it are two further ridges at the top and bottom of the cell, spaced from it by half the *b* dimension, *i.e.* 15·4 Å. These extremely striking features must be due to parallel concentrations of projected electron density, also 15·4 Å apart (see p. 178). Thus the projection fits in well with the scheme of *Figure* 3*b*, since the two disks in that scheme would project into two parallel bars of projected electron density separated by *b*/2 (this separation is obligatory owing to the operation of the screw diad axis). We may also note the prominent peaks at 4 Å from the origin in a direction parallel to *b* ; these must be part of the intramolecular pattern, though their interpretation is not at present clear.

(*b*) *The* a *projection* (*Figure* 4*b*)— This projection is surprisingly similar to the *c* projection just discussed, exhibiting the same general structure, perhaps even more strikingly than before. This is exactly what the model would lead us to expect, since the two disks would project into two parallel bars of projected electron density on this plane as well—or, indeed, on *any* plane parallel to *b*. There is, however, a new phenomenon—each of the ridges of vector density is partly segmented into prominent peaks about 10 Å apart. To understand these it is first necessary to discuss the third projection.

(*c*) *The* b *projection* (*Figure* 4*c*)— Here we have an entirely different type of diagram, the most prominent feature of which is an irregular but distinct ridge of vector density through the origin at an angle of about 20° to the *a* axis. Parallel to this, and about 9·5 Å from it, are two further ridges ; beyond these again and spaced from them by a similar distance can be seen traces of a further pair. The interpretation placed on these ridges is that they are the vector equivalent of the parallel polypeptide chains, seen in plan. The distance between

the ridges is also the distance between polypeptide chains, *viz*, 9·5 Å.

A further and better resolved *b* projection of lattice B (not illustrated here) shows that along each ridge is a series of vector peaks spaced about 5 Å apart. This must correspond to a repeat distance of 5 Å along the chain.

We are now in a position to interpret the peaks of the *a* projection, mentioned in (*b*) above. This projection is made on to a plane which intersects the *ac* plane along the line marked '*a* projection' in *Figure* 4*c*, and it will be seen that the polypeptide chains are almost end-on as viewed in this projection. The peaks of the *a* projection are, in fact, the interchain vectors of the end-on projection of the chains. Since the two molecules in the cell are related by a screw diad, one is turned through 180° relative to the other, so that the chains in the two molecules are parallel and do not interfere with one another in the *b* projection.

THE ORIENTATION OF THE HAEM GROUP

The strong absorption in the visible region of the spectrum of haem-containing pigments is attributed to a selective absorption by the planar conjugated ring system of the haem group itself, and such absorption is suffered only by that component of the electric vector of the incident radiation which lies in the plane of the ring system. Where the crystal displays powerful pleochroism—that is to say, where the absorption of transmitted plane-polarized light is a function of the direction of polarization relative to the crystal axes—one may deduce that all the haem groups throughout the crystal are more or less parallel, and proceed to relate the plane in which they lie to the crystal axes.

Myoglobin crystals are strongly pleochroic, and it emerges that the haem groups lie in a plane containing the *b* axis and approximately bisecting β. Reference to *Figure* 4*c* shows that they must therefore lie roughly perpendicular to the direction of the polypeptide chains, one forming part of each molecule : a similar arrangement exists in haemoglobin. The operation of the screw diad ensures the parallelism of the two haem groups in each unit cell.

CONCLUSIONS : THE STRUCTURE OF HORSE MYOGLOBIN AND ITS RELATION TO HORSE HAEMOGLOBIN

I conclude by showing how all these observations may be integrated to give a picture of the myoglobin molecule. This picure may perhaps not be regarded as more than plausible at the present stage ; however it can be correlated reasonably well with all the x-ray data so far obtained, and its close relation to the haemoglobin structure deduced by Perutz may be considered a further point in its favour.

Myoglobin, then, appears to consist of an approximately circular disk whose diameter (deduced from the dimensions of the wet unit cell) is not more than about 57 Å, and whose thickness is about 9 Å. Each unit cell contains two of these disks arranged parallel to one another and perpendicular to the *b* axis, with their centre lines 15·4 Å apart in the wet condition. The molecules are separated by about 6·6 Å of liquid of crystallization. (There is no direct evidence from the myoglobin crystal about the thickness of the molecule—the figure given is by analogy with a single layer of haemoglobin—though the value of *b* in the dry cell sets an upper limit of 14 Å.)

Each molecule consists of a single layer of parallel polypeptide chains, about 9·5 Å apart and having repeats of about 5 Å along the chains. As in haemoglobin, we may note a close analogy to the structure of α-keratin, in which the chains are separated by a distance of 9·3 Å and which gives a ' backbone spacing ' of 5·1 Å.

The haem group lies perpendicular to the plane of the disk and also approximately perpendicular to the polypeptide chains. Since a haem group is about 15 Å across and we have postulated a disk thickness of only 9 Å, the haem must stick out on one or both sides of the disk.

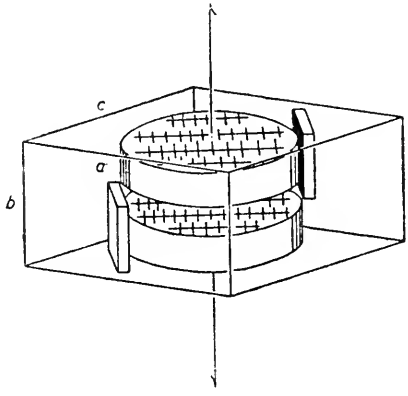


Figure 5. Diagrammatic sketch of a possible arrangement of myoglobin molecules in the unit cell.

Figure 5 is an attempt to illustrate this scheme diagrammatically ; it is in fact identical with *Figure* 3*b* with the addition of polypeptide chains and haem groups. In drawing such a scheme, however, it is difficult to avoid being more concrete than the evidence warrants, and the following provisos must be made :

1 The actual shape of the disk is only very approximate.

2 The two shaded rectangles show only the *orientations* of the haem groups and not their actual positions, though the positions illustrated may perhaps be regarded as more plausible than any

others, if we accept a close similarity between the myoglobin molecule and a single layer of haemoglobin, since in the latter there is evidence that the haem groups are on the outer surface of the molecule.

3 The detailed structure of the polypeptide chains is unknown, as is the relation between them (but see below).

4 The two molecules in the cell may be 'staggered' as shown in *Figure* 3*d*, instead of being directly over one another—in fact other evidence (not mentioned here) makes a staggered arrangement in the wet crystal rather more likely. Also the two molecules may not be strictly parallel, though they must be nearly so.

Throughout I have stressed that there seems to be a close relationship between the myoglobin molecule and a single layer in horse haemoglobin. This relationship includes the overall dimensions of the disk, and also the arrangement of haem groups and polypeptide chains—and in the latter respect there is also a close similarity to α-keratin. Furthermore, the *b* projection of myoglobin when superimposed on the zero-level *b* Patterson section of haemoglobin (so that rod corresponds with rod) shows some surprising coincidences of peaks which, while they cannot as yet be interpreted, can hardly be accidental.

Finally I wish to indicate how the crystallographic data may be correlated with two other and quite distinct lines of evidence.

First, R. R. PORTER and F. SANGER have shown by the end-group method that the horse myoglobin molecule contains only a single free α-amino group ([5] ; also see this volume, p. 122). If we provisionally exclude the possibility of closed polypeptide rings, this means that the molecule consists of a single polypeptide chain. To accommodate it within our model, the chain must be folded in a zigzag somewhat as in *Figure* 6. We may go so far as to calculate the dimensions of such a model, assuming that the myoglobin molecule contains in all about 146 amino-acid residues (see p. 111), and using the α-keratin model with 3 amino-acid residues per unit backbone repeat of 5·1 Å and chains 9·3 Å apart. This gives a total chain length of 248 Å. In the dry crystal it may be supposed that adjoining molecules are in fairly close contact with one another so that the *a* and *c* dimensions should approximate to those of the molecule ; the values found are 51·5 and 37·0 Å (see *Table I*). The *c* dimension is roughly perpendicular to the chains, and would exactly accommodate four of them ($4 \times 9{\cdot}3$ Å $= 37{\cdot}2$ Å). *Figure* 6 shows an arrangement of the chain in 4 lengths (assuming all the loops of equal length, a scheme which gives the most compact molecule) ; it is easy to calculate that a molecule of 146 amino-acids arranged in this way would have an overall length of about 60 Å. (Similar arrangements containing closed loops of poly-

peptide chain would have about the same dimensions.) This is 8·5 Å longer than *a* so the agreement, though reasonable, is not exact, but there are not at present enough data to enable us to discriminate between various arrangements which would account for this discrepancy. At least it seems reasonable to suppose that the molecule consists in its main part of four parallel lengths of polypeptide chain.

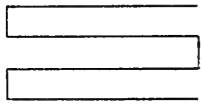


Figure 6. Diagram of a single polypeptide chain folded into four parallel segments.

Second, there should be an agreement between the molecular dimensions postulated above and the asymmetry ratio deduced from properties in solution such as sedimentation rate, viscosity, and dielectric constant. Clearly our model demands a high degree of asymmetry, with an axial ratio of the order of 4:1, though this would be reduced somewhat if we may assume that in solution the molecule is covered by a layer of tightly-bound water, and particularly if this water is adsorbed preferentially on the flat faces of the disk (as would happen if the structure follows Astbury's model for α-keratin, so that the polar, hydrophilic, side chains stick up and down, perpendicular to the plane of the disk). Thus the model requires a degree of asymmetry greater than that postulated for most other globular proteins. Unfortunately the properties in solution referred to above do not give an unequivocal answer. The question has been discussed by J. WYMAN[6, 7] who suggests, on the basis of all the available data, that if the equivalent ellipsoid is an oblate one (as seems likely), its asymmetry ratio is 3·6:1. The sedimentation constant alone would suggest a considerably lower figure but, especially in the absence of data about the hydration of myoglobin, the question must be regarded as still unsettled in spite of the very satisfactory agreement between the compromise figure given by Wyman and the X-ray data. We cannot even exclude the possibility of a reversible change of configuration during the transition from crystal to solution. The X-ray crystallographer would fervently hope that this does not turn out to be the truth, since it would mean that his studies of protein crystals are of less direct interest to the biologist, who is mainly concerned with the state of the protein molecule in solution.

Received September 1948

REFERENCES

[1] SVEDBERG, T. *Proc. roy. Soc. A* 170 (1939) 40
[2] ROCHE, J. and VIEIL, H. *C.R. Acad. Sci. Paris* 210 (1940) 314
[3] REES, M. W. *Personal communication* (1947)
[4] KEILIN, J. and SCHMID, K. *Nature, Lond.* 162 (1948) 496
[5] PORTER, R. R. and SANGER, F. *Bio-chem. J.* 42 (1948) 287
[6] WYMAN, J. and INGALLS, E. N. *J. biol. Chem.* 147 (1943) 297
[7] — *Advances in Protein Chemistry* 4 (1948) 436 Academic Press Inc., New York

The Application of X-Ray Crystallography to the Study of Biological Macromolecules

J. C. KENDREW and M. F. PERUTZ

A brief account is given of methods of analyzing crystal structures by means of x-rays, with particular reference to their application to crystals of large molecules of biological origin. Fourier and Vector (Patterson) syntheses are described, and it is shown that where the molecule is large there is sometimes a general resemblance between some features of the vector structure and the molecular structure from which it is derived.

THIS paper serves as an introduction to the two which precede it, and is intended as a brief conspectus of the applications of X-ray crystallographic techniques, particularly to the study of large molecules (but excluding fibrous structures), for the benefit of workers in other fields who may be unfamiliar with these methods. It will not be possible in the short space available to provide mathematical proof or even convincing evidence for many of the statements made. We are trying to give the reader a glimpse of the physical principles underlying the different methods of analysis ; for more detailed information some of the references given at the end of this paper should be consulted.

THE STRUCTURE OF CRYSTALS

A perfect crystal consists of a three-dimensional array of molecules arranged regularly in repeating units of pattern. In two dimensions a piece of patterned wallpaper is analogous ; if corresponding points in the pattern (known as ' lattice points ') are joined by two sets of parallel lines the wallpaper is divided into ' unit cells ' of identical size, shape, and contents. These unit cells are the smallest and therefore the fundamental units of pattern, and in the three-dimensional array they can be drawn in exactly the same way, and are defined by three sets of parallel lines known as the crystal axes (denoted by *a*, *b* and *c*). An example of a two-dimensional pattern is shown in *Figure* 1.

An array of a large number of unit cells is known as the space lattice, and the shape of the unit cell determines the crystal system to which the crystal belongs ; seven such systems are possible, ranging from triclinic (with three unequal axes and no right angles), through monoclinic (three unequal axes, two angles of 90°, one angle not 90°), orthorhombic (three unequal axes at right angles), hexagonal (two equal axes at 120°, the third at right angles to the first two and

of different length), rhombohedral (three equal axes, angles all equal but not 90°), tetragonal (two axes equal and different from the third, all angles 90°) to cubic (three equal axes at right angles).

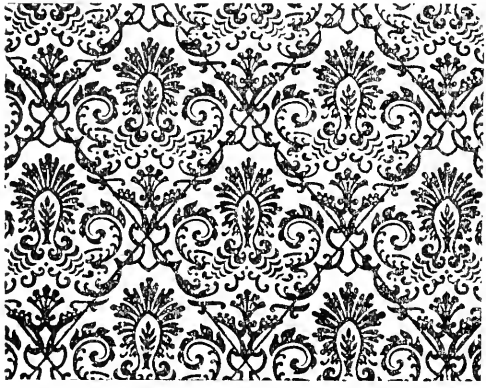


Figure 1. *Patterned wallpaper.* '*Unit cells*' *are indicated by lines.*

Each unit cell may contain one, two, or more molecules, oriented at various angles to one another. The contents of the cell nearly always exhibit certain symmetry properties. For example, it may be possible to draw through the cell a plane such that one half of the cell is the mirror image of the other half (mirror plane) ; or a line such that any piece of matter in the cell if rotated through 180° about the line comes into exact coincidence with an identical piece of matter (diad axis of rotation) ; or an axis such that rotation through 90° produces coincidence (tetrad axis). Other symmetry properties may be exhibited, and mathematical analysis has shown that in three dimensions the possible combinations of symmetry elements yield in all 230 distinct *space groups.*

Although the unit cell is strictly the smallest repeating unit of pattern, the existence of symmetry elements implies that within the cell there are generally still smaller units, identical but in different orientations. (In *Figure* 1 the unit cell of the wallpaper contains two identical units, related by a mirror plane.) The smallest such unit has the property that, if the symmetry elements be allowed to operate upon it (if, for example, it is reflected in any mirror planes, and both it and the reflexions are rotated round axes of rotation, *etc*), the whole contents of the cell are produced. This unit, which may consist of a single complete molecule, or of part of one molecule and part of another, or of part of one molecule only (where the molecule is built up of identical sub-units), is known as the *asymmetric unit.* Its size can at once be determined if the unit cell dimensions and space group (*i.e.* symmetry properties) are known.

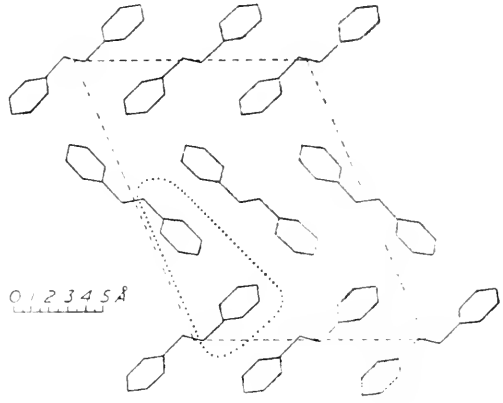


Figure 2. *Structure of stilbene projected on plane normal to b axis. The asymmetric unit is marked by dotted lines. (Reproduced from Bunn*[6].)

An example is shown in *Figure* 2, which is the *b* projection of the monoclinic cell of stilbene (in other words a projection on to the plane perpendicular to the *b* axis). The cell contains four molecules (some fall partly in adjoining cells, but the total per cell is 4), and the asymmetric unit is shown surrounded by a dotted line—here it consists of half of one molecule and half of another. In projection the asymmetric units are related by a diad axis passing through the centre of the projected cell and normal to the plane of the paper.

THE REFLEXION OF X-RAYS FROM CRYSTALS

The distances between the atoms in a crystal are of the same order as the wave-length of x-rays (wave-length of Kα line in x-radiation from copper = 1·54 Ångstrom units. 1 Å = 10^{-8} cm), and it can be shown that a set of parallel and equidistant ' net planes ' through the crystal (that is to say, planes drawn so as to contain a large number of lattice points) diffracts a beam of x-rays just as an optical grating diffracts visible light. A very large number of such sets of planes can be drawn through a given lattice, and each of these may give rise to a diffracted x-ray beam (*Figure* 3 is a crystal lattice showing a few types of planes).

We are dealing, however, with a three-dimensional array in the crystal, and unlike the two-dimensional grating which diffracts visible light for all angles of incidence, mutual interference between the crystal planes of a set suppresses the diffracted beams except at certain angles of incidence given by the Bragg Law

$$n\lambda = 2d.\sin\theta$$

where λ is the wave-length of the x-rays, θ is the angle of incidence (which is also equal to the angle of diffraction), d is the spacing between planes, and n is an integer. Thus as a crystal is rotated in a mono-

chromatic x-ray beam, successive diffracted rays will ' flash out ' when the angle of incidence becomes appropriate for each plane in turn ; the various types of x-ray camera are designed to photograph these rays and their geometry is arranged so that each ray can readily be identified with the set of planes which caused it. This kind of diffraction is rather like optical reflexion from a set of parallel glass plates, and it is usually referred to as x-ray ' reflexion ', and the diffracted rays from a set of lattice planes are simply called ' reflexions '.

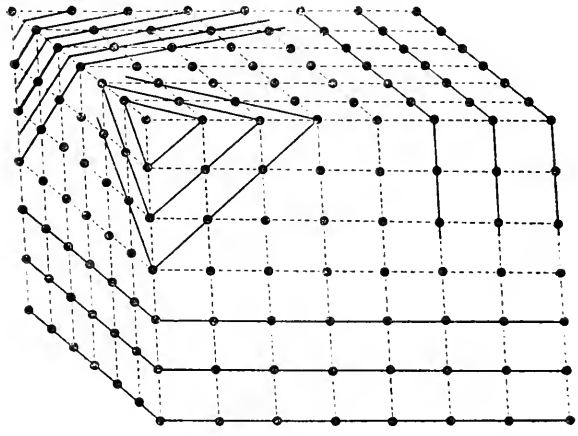


Figure 3. *Space lattice showing lattice planes in different orientations.*
(*Reproduced from Bunn*[6].)

Just as the diffraction angle of the rays from a grating can be used to deduce the periodicity of its rulings, so the diffraction angle of the x-ray reflexions can be used to deduce the distance between the planes in the crystal (the spacing d in the above equation), and hence to find out the dimensions of the unit cell—always the first step in the x-ray analysis of a crystal.

Returning to the optical grating, we note that while the *diffraction angle* is a function of the periodicity of the rulings (the scattering units), their *relative intensities* are governed by the shape of the rulings. This shape will depend on the tool used to engrave the grating. Indeed the diffracted rays will differ not only in intensity (or amplitude) but also in phase, though differences in the latter are of course not apparent to the eye, and both amplitude and phase will be functions of the shape of the rulings.

If a beam of x-rays falls on matter the electrons contained in the matter are responsible for the scattering which takes place, and so the

intensity of scattering at any point in a crystal is a function of the electron density (*i.e.* the number of electrons per unit volume) at that point. The amplitudes and phases of the diffracted beams of x-rays will be a function of the distribution of electron density within each repeat scattering unit, in this case the unit cell.

THE ANALYSIS OF X-RAY DIFFRACTION PATTERNS

In the grating it would be possible, by measuring the amplitudes and phases of all the diffracted rays, to deduce the shape of the lines in the grating. Actually calculation is unnecessary, since by a suitable optical arrangement the rays can be recombined to form an image of the original grating, the process of recombination automatically taking care of both amplitudes and phases ; this, of course, is the basis of Abbe's treatment of the optical microscope, where the object is conceived as replaced by a small ideal grating[1].

Unfortunately there are no such things as x-ray lenses (in other words we do not know how to *refract* x-rays appreciably), so it is not possible to construct an x-ray microscope which would recombine the reflexions of the diffraction pattern to form an enlarged image of the unit cell of the crystal. Furthermore, it is not generally possible to measure the phase of a reflected x-ray beam ; all that can be recorded in the x-ray camera is the intensity of the reflexion, the square root of which is its amplitude. The x-ray diffraction pattern in itself, therefore, does not provide sufficient data for a direct calculation of the electron density in the unit cell which produced it. This is the fundamental difficulty of all x-ray analysis, but it is encountered in its most acute form in the analysis of very large molecules.

Progress can be made in simple cases where it is possible, by making certain assumptions, to deduce or guess the phases of some or all of the reflexions. For example, one simplifying factor may be that the symmetry of the crystal is such that the phase angles between all the diffracted rays and the incident ray must be 0 or π—in other words the diffracted rays are all exactly in or exactly out of phase with the incident ray ; the problem is then reduced to one of finding out merely the *signs* of the amplitudes of each reflexion.

In such cases it may be possible to apply various tricks to find out the signs of some reflexions. For example, if one atom in the unit cell has very much larger atomic number than any of the others, its contribution to each reflexion will also be very large by virtue of its high electron density, and will effectively determine the sign of all but the weakest reflexions ; once the position of the heavy atom is known direct calculation will often give the signs of enough strong reflexions to enable a first approximation to the crystal structure to be computed.

This is the *heavy atom* method ; another is the method of *isomorphous replacement*, where a series of isomorphous crystals differing only in the nature of one atom in the molecule (*e.g.* the alums) is examined. Progressive changes in the amplitudes of corresponding reflexions in successive members of the series may be attributed to the different scattering contribution of the isomorphously-replaced atom, and hence some signs may be deduced.

Given the amplitudes and phase angles of all diffracted rays, the positions of the atoms in the unit cell may be found by means of a mathematical method known as a Fourier summation. The Fourier method is the basis of all x-ray analysis of more complex types of structure today, no matter whether it be that of a metal, a mineral or an organic substance like penicillin or calciferol. One of its many advantages is that it presents molecular structures in a realistic form so that different types of atom and interatomic bonding are immediately obvious to the eye (see *Figure* 6). The development of the Fourier method in 1925 and 1926 by Compton, Havighurst and W. L. Bragg was perhaps the most important advance in crystallography since Bragg's original discovery of the structure of NaCl in 1913. The nature of this method will now be explained.

DETERMINATION OF STRUCTURE : FOURIER SUMMATION

By the methods of Fourier analysis it is possible to represent any periodic function mathematically as the sum of a series of sinusoidally varying quantities whose wave-lengths are 1, $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, . . . $1/n$ of the fundamental wave-length : each term in the series is fully determined by its amplitude and phase. (The amplitude of a term of a Fourier series is commonly referred to as its coefficient—a name which will frequently recur below.) W. H. BRAGG realized as early as 1915 that a crystal structure in which a pattern repeats at regular intervals in three dimensions could be represented *mathematically* by a Fourier series, but at that time atomic theory was not sufficiently advanced for the *physical* relationship between the observed diffracted rays and the terms of the series to be fully understood. It was not discovered until 1925 that the amplitudes of the diffracted rays themselves formed the coefficients of the Fourier terms, that the relative phases of the diffracted rays formed the phase factors, and that the summation of the series produced a periodic function in three dimensions which is the *electron density* distribution in the crystal.

It will be remembered that it is the electrons and not the atomic nuclei which are responsible for the diffraction pattern, so that a crystal structure will appear to the ' x-ray eye ' as a cloud of electrons

of varying density. The cloud will be densest near the centre of an atom and thinnest halfway between two atoms. Given then the amplitudes and phases of all the reflexions in the diffraction pattern, and applying the process of Fourier summation, the electron density can be calculated throughout the unit cell and the positions of all the atoms can thus be determined. The kind of crystal whose structure interests people today produces a very large number of reflexions ; hence the number of data will be large and the work of computation great. However, various devices have been constructed to shorten this labour and at the present time a number of projects are on foot to design electronic computers for this purpose.

Often such a calculation of the electron density in three dimensions may be impossible, because the phase factors cannot be found ; but much useful information can be derived by calculating the electron density projected on to a plane, or even on to a line passing through the unit cell. We should like to dwell in detail on examples of the latter kind because they afford convenient and simple illustrations of the Fourier method.

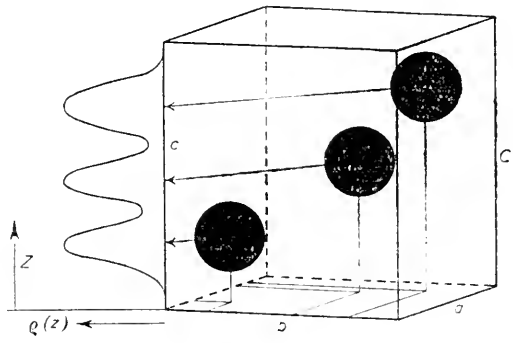


Figure 4. *Hypothetical unit cell containing three atoms. The curve on the left is a linear electron density projection on the* c *axis, obtained by projecting all the electrons in the unit cell onto* c *as indicated.*

A linear Fourier projection can be visualized in the following way. Consider the array of atoms in the hypothetical unit cell of *Figure* 4. If all the electrons in these atoms are projected on to the line marked *c*, along directions which are perpendicular to *c*, we shall obtain a varying distribution of projected electron density along this line. We may now plot this in the form of a curve which will actually show the height of each atom above the base of the unit cell. To calculate the electron density distribution in the unit cell projected on to this line, the intensities of the different order diffracted rays from the set of lattice planes normal to that line would have to be measured. Their amplitudes would form the coefficients of the terms of the series, and provided the phase factors could be found, the series could be summed.

Let us now consider as a real example the linear Fourier projection of horse haemoglobin. An x-ray study of this protein showed that the phase angles for the reflexions from a certain set of planes could be found by experiment ; symmetry conditions were such that these phase angles were restricted to 0 or π. The Fourier series consisted of only seven terms, each of which forms a cosine wave (*Figure* 5) ; the first term has a wave-length equal to the whole length of the unit cell, its amplitude (*i.e.* its coefficient) corresponds to that of the first order diffracted ray and its phase angle is 0, which means that the wave has a maximum at the centre of the unit cell. The second term

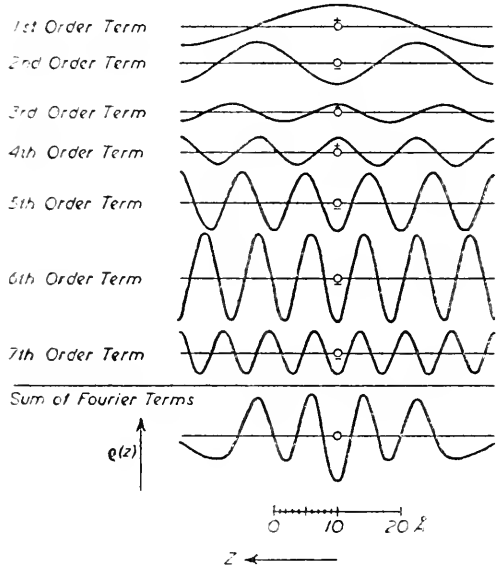


Figure 5. *Graphical representation of Fourier summation. Each sinusoidal wave represents one Fourier component. The linear electron density projection of haemoglobin obtained by summing all the components is shown at the bottom. The origin is marked by a small circle for each curve.*

has a wave-length corresponding to one half of the length of the unit cell, its amplitude corresponds to that of the second order diffracted ray and its phase angle is π, which means that the wave has a minimum at the centre of the unit cell ; and so on. When all the terms are summed they make up the curve shown at the bottom of the figure which gives the distribution of electron density through the unit cell projected on to a line. It will be noted that this distribution shows four peaks of approximately equal height which are spaced just under 9 Å apart. This fact provided valuable information on the structure of the haemoglobin molecule and will be recalled later on when the Patterson equivalent of this curve is discussed.

In order to calculate the electron density distribution in a unit cell projected on to a plane, the intensities of the reflexions from all those

sets of planes which are normal to the plane of projection have to be measured, and their amplitudes form the coefficients of the terms. Each of these terms will now be a two-dimensional wave, corresponding to a wave on a water surface, with a characteristic wave-length and orientation relative to the edges of the projected unit cell. An example of a two-dimensional Fourier projection is shown in *Figure* 6, which also indicates for comparison the layout of the molecule as viewed in projection. The projection is plotted as a contour map of projected electron density : a peak corresponds to the presence of an atom, and the higher the peak the higher the atomic number of the atom. Generally a projection is made on to each of the three faces of the unit cell in turn ; from the three projections the arrangement of the atoms in space in the unit cell can be deduced. Of course such projections are liable to be complicated by the fact that certain atoms in the unit cell may overlap when viewed in projection on to any given plane, but in simple cases it is generally possible, by comparison between the three projections, to see where such overlapping has occurred. Another difficulty is that hydrogen, since it contains but one electron, makes slight contributions to the reflexions and does not as a rule show up in the final result ; it is then necessary to guess the positions of all hydrogen atoms after the main outlines of the structure have been determined.

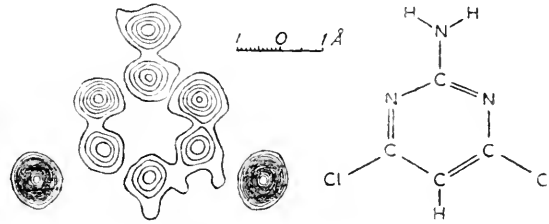


Figure 6. *Planar electron density projection of a pyrimidine compound. (Reproduced from Clews and Cochran*[8]*).*

Because of overlapping of the atoms in planar projection, or because of the extreme weakness or even absence of the higher order diffracted rays, the resolving power of planar projections may be insufficient to find the exact positions of all the atoms. This happened, for instance, in the x-ray analyses of cholesterol iodide and penicillin ; in such cases it is necessary to calculate the electron density in space by means of a three-dimensional Fourier summation. This requires measurement of the intensities of all the diffracted rays from the crystal in question. As a rule the phase angles of these can be found only when the structure is already approximately known. The terms of this Fourier series will be three-dimensional waves, forming a sinusoidal variation of electron density in space. Such waves may be pictured rather like the density variation produced at a given instant by 'monochromatic' sound

waves travelling through a solid body. Again each term has its characteristic wave-length and orientation in the unit cell, while its phase angle (if restricted to 0 or π) will determine whether the wave has its density maximum or minimum at the centre of the unit cell. Summation of such a series involves summation of the density of each of perhaps more than a thousand waves for each of tens of thousands of points in the unit cell. The labour, even if done by machines, is great, but so is the amount of detailed and often new information on molecular structure which can be obtained from such summations.

The results of three-dimensional Fourier summations are normally represented in the form of a series of plane sections through the unit cell, the electron density distribution within each section being drawn as a contour map. Wherever such a section intersects an atom the contour map will show a peak. It has become customary, therefore, to refer to concentrations of density within the three-dimensional electron cloud quite generally as peaks and this expression has been carried over into the jargon associated with the Patterson functions discussed below.

THE X-RAY ANALYSIS OF BIOLOGICAL MACROMOLECULES : PATTERSON SUMMATIONS

Where a macromolecule (such as a protein) forms crystals it is possible to record the diffraction pattern and to deduce the dimensions of the unit cell (usually very large), the crystal symmetry, and hence immediately the size of the asymmetric unit (if this is found to be smaller than the size of the molecule, it can be assumed that the latter consists of identical sub-units whose number and symmetry relationship are given by the symmetry properties of the cell). The next step, however, is the difficult one, for the patterns produced are generally very complex and comprise thousands of reflexions, and none of the methods devised for guessing phases in simple crystals can be applied. For example even the heaviest of heavy atoms would make a negligible contribution to the reflexions from so large a unit cell, so it would exert no control over the phases. Only in a single case, studied by one of us[2] has it so far been possible to make a Fourier projection of a protein molecule (haemoglobin), and then only in one dimension and in simplified form.

We now proceed to discuss a method whereby at any rate some information about the crystal structure can be obtained without knowledge of any of the phases of the reflexions in the diffraction pattern. This method owes its discovery to A. L. PATTERSON[3] who found by mathematical reasoning that a Fourier series, summed with the intensities (instead of the amplitudes) as coefficients of the terms, has

a physical meaning. Since the intensities are proportional to the squares of the amplitudes and the sign of the (amplitude)^2 will always be positive, the unknown signs of the amplitudes do not enter into the series. But even if the phase angles are not restricted to values of 0 or π, it can be shown mathematically that they do not enter into the Fourier series which therefore does not contain any quantities that cannot be measured by direct experiment. The physical meaning of this so-called Patterson synthesis is one of the most difficult conceptions in crystallography. It would hardly seem justified in a survey like the present one to dwell on a method as abstract as Patterson's, were it not for the supreme importance which this method has now assumed in the analysis of macromolecular structures. Nearly all the information which has been derived from x-ray studies of crystalline proteins is based on the results of Patterson syntheses. Moreover, the phase angles which have to be known before an ordinary Fourier summation can be performed, can only be found either if the structure is already approximately known, or if the positions at least of certain heavy atoms are known. In most present-day structure analysis this type of information is sought from Patterson syntheses. The meaning of this synthesis will best be explained by beginning with a number of simplified and sometimes hypothetical cases. For the sake of completeness two mathematical formulae are included in the exposition given below, but the treatment is intended to be intelligible also without these.

Consider once more the electron density distribution of haemoglobin projected on to a line (*Figure* 5). It was shown how this was obtained by summing a series of cosine waves whose coefficients were the amplitudes of the different orders of diffracted rays ; the waves were placed either with a maximum or a minimum at the centre of the unit cell, depending on whether the signs of the amplitudes were positive or negative, *i.e.* the phase angles 0 or π. Suppose we now wish to calculate the corresponding Patterson function. The coefficients of the cosine waves in this case will correspond to the *intensities* of the different order diffracted rays ; as their signs are all positive, all the cosine waves have to be placed with their maxima at the centre of the unit cell (*Figure* 7). We shall choose this as the origin.

The resulting curve has its highest maximum at the origin (obviously, since the maxima of all the constituent waves coincide there) and a number of subsidiary maxima on either side. The first of these (peak 1) is 9 Å away from the origin, corresponding to the distance between any pair of neighbouring peaks in the electron density projection. The next maximum (peak 2) is 18 Å from the origin, corresponding to the distance between next but nearest peaks in the electron density projection, and so on. In fact the distances of the maxima from the

origin in this Patterson projection correspond to the distance between successive maxima of electron density in *Figure* 5.

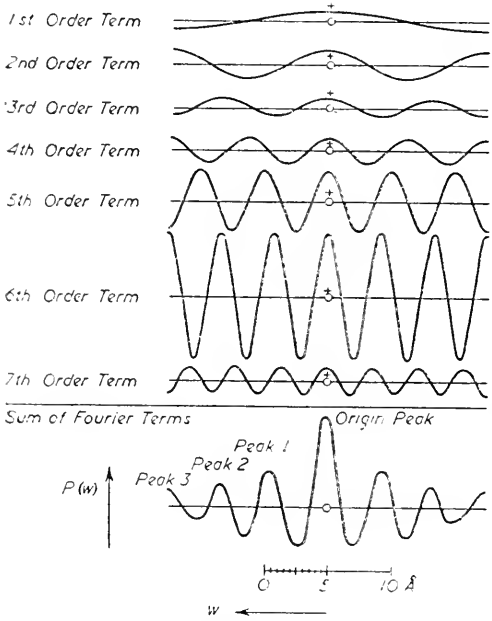


Figure 7. Graphical representation of Fourier summation for Patterson projection. Each sinusoidal wave represents one Fourier component. The bottom curve shows the linear Patterson projection obtained by summing all the components; its vertical scale is half that of the other curves. The origin is marked by a small circle for each curve.

Following Patterson's original paper we can state the meaning of the projection as follows. Consider an electron density distribution ρ along a line z as in *Figure* 5, which may be written as $\rho(z)$; the Patterson equivalent of this function gives the distribution of a quantity which we may call $P(w)$, where the direction of w coincides with z (*Figure* 7). It can be shown mathematically that $P(w)$ is the integral over the length of the unit cell of the products of the electron density at any point z, multiplied by the electron density at another point which is a distance w away from z.

$$P(w) = \frac{1}{c \sin \beta} \int_0^{c \sin \beta} \rho(z)\, \rho(z + w)\, dw$$

In the example we have chosen, the peak (1) arises as the integral of all the products of the electron density at any point z, multiplied by the electron density at any point $(z + 9)$. Since the electron density projection contains four peaks which are spaced approximately 9 Å apart $P(w)$ will contain a maximum at $w = 9$. On the other hand, the curve contains no maximum at $w = 6$, since there is no pair of points 6 Å apart in the electron density distribution where the electron density is high at *both* points. The Patterson projection, then, has maxima

whose distance from the origin corresponds to the distance between two or more peaks in the electron density projection.

The matter becomes more complex if a crystal structure in three dimensions is considered. Just as the results of a three-dimensional Fourier summation with the amplitudes as coefficients of the terms can be expressed as a series of contour maps showing the distribution of *electron density* in sections through the unit cell, the Patterson function when calculated in three dimensions leads to a series of contour maps showing the density distribution within a cloud of ' something ' in the unit cell. Patterson called this ' something ' the weighted density distribution about any point in the crystal structure. What does this mean ? By analogy with the one-dimensional example just given the density of this ' something ' at any point of the unit cell having the coordinates u, v, w, should be equal to the integral of the product of ρ at any point x, y, z in the crystal structure and ρ at any other point having the coordinates $x + u$, $y + v$, $z + w$.

$$P(u, v, w) = \frac{1}{V} \int_o^a \int_o^b \int_o^c [\rho(x, y, z)] [\rho(x+u), (y+v), (z+w)] \, du. \, dv. \, dw$$

Supposing there were two atoms, one at x, y, z and the other at $x + u$, $y + v$, $z + w$. The electron density at both these points will be large and hence their product

$$[\rho(x, y, z)] [\rho(x + u), (y + v), (z + w)]$$

will also be large ; the Patterson function will therefore show a peak at the point u, v, w. The line joining this peak to the origin (the centre of the unit cell, say) will correspond in length and direction to the line joining the two atoms in the crystal structure. Since a quantity having both length and direction is known as a vector, the density of ' something ' in the Patterson synthesis is now generally called *vector density*. In a sense this name is misleading, because the peak in a Patterson synthesis is characterized not only by its position with respect to the origin, defining the length and direction of the vector, but also by the magnitude of the density at the centre of the peak, usually called its height. The height of a vector peak depends on two factors : one is the number of pairs of atoms in the unit cell which are joined by corresponding lines of the same length and direction, and the other the atomic number of the atoms concerned.

Consider now the relationship between the distribution of ' ideal ' atoms in a crystal structure and the distribution of ' ideal ' vectors in the corresponding Patterson synthesis, which follows from the abstract reasoning just given. For this purpose it will be convenient to think of point atoms which scatter X-rays only at their centres and give rise

to point peaks in the Patterson synthesis, and to examine a structure containing only three such atoms in the unit cell (*Figure* 8). If the coordinates of two atoms, for example *A* and *B*, differ by 2, 3, and 4, say, the vector structure will show a peak at the distance $\sqrt{2^2+3^2+4^2}$ from the origin. The vector joining the origin to this peak, drawn in the figure as a wedged line, corresponds in length and direction to the line joining the two atoms in the crystal structure on the left. The same will apply to the lines joining any other pair of atoms—they will all appear in the Patterson synthesis as lines radiating from a common origin. It can be shown mathematically that if there are n atoms in the unit cell of the crystal structure, the unit cell of the Patterson synthesis will contain $n(n-1)$ vector peaks. In a real Patterson synthesis the height of each peak will of course be equal to the product of the electron densities at the centres of the corresponding pair of atoms.

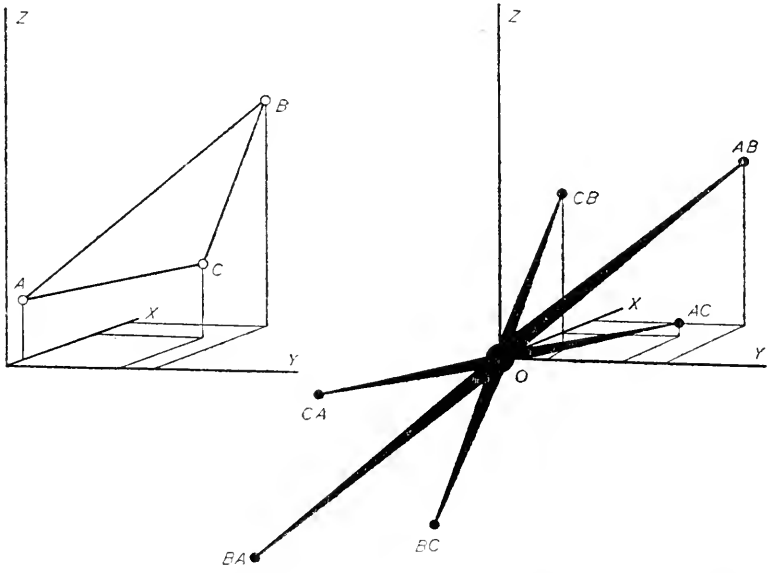


Figure 8. *Left : Hypothetical structure showing three point atoms A, B and C. Right : Corresponding vector structure. The dot AB marks the vector peak corresponding to the line joining the atoms A and B, and so on. O marks the origin.*

Let us now examine an actual experimental example of the relationship between electron density map and Patterson synthesis in a simple organic structure. The substance chosen is penta-erythritol which crystallizes in the tetragonal system. *Figure* 9*a* shows this structure in projection along the tetrad axis, with a quarter molecule at each corner and a complete molecule at the centre of the unit cell (the latter lies

halfway up the unit cell above the plane of projection). This picture is derived from the electron density *projection* of *Figure 9b* where each atom in the unit cell appears as a peak on a contour map ; it will be seen that the peaks corresponding to oxygen atoms are slightly higher than those corresponding to carbon atoms. *Figure 9c* is a *section* of the three-dimensional Patterson synthesis through the origin. Unlike the electron density *projection* of *Figure 9b* which shows *all* the atoms in the unit cell, this *section* shows only those vectors which lie in the basal plane passing through the origin. This means that only those pairs of atoms in *Figure 9b* which are situated at the same level in the unit cell, so that the line joining the pair (and hence its corresponding vector) is parallel to the plane of projection, will give rise to a peak in this Patterson section. In *Figure 9b* three such pairs are marked, one by a full, one by a dashed and one by a dot-dashed line ; the corresponding vectors are seen radiating from the origin in *Figure 9c*, each giving rise to a different type of peak. As all the other peaks in the section are related to these three peaks by symmetry, we have already accounted for all the peaks which occur. In the actual analysis of the structure the reasoning would of course be reversed and the orientation of the penta-erythritol molecules would be found from the position of the peaks on the Patterson section.

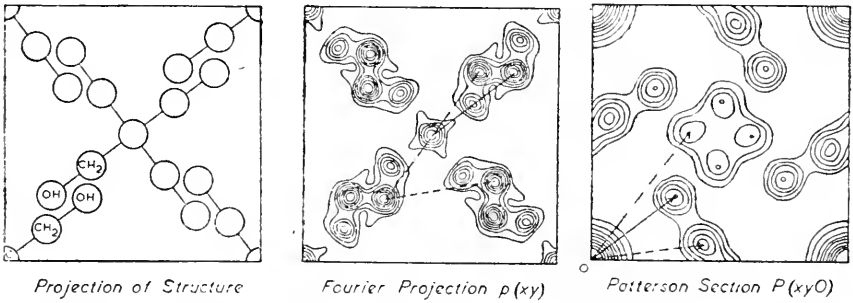


Figure 9. (a) Unit cell of penta-erythritol projected on the face normal to the c *axis. (b) Electron density* projection *from which (a) was derived. Atoms lying at equal levels above the plane of projection are connected by lines. (c)* Section *through the origin of the three-dimensional vector structure.* O *marks the origin. Vectors correspond to lines in (b). (Reproduced from Llewellyn, Cox and Goodwyn*[9].)

A more complex structure would contain a far more intricate system of vector peaks. In fact, since the number of vector peaks in the unit cell increases almost as the square of the number of atoms, the vector equivalent of any but the simplest crystal structure would at first sight appear to be of forbidding complexity. There are, however, special cases where a certain resemblance arises between the real structure of

the unit cell and the vector structure derived from it. Such cases were actually met with in the analyses of haemoglobin and myoglobin described elsewhere in this volume. It will help us to understand the Patterson syntheses of these two substances, if we consider the comparatively simple example of a crystal consisting of parallel straight chain hydrocarbons (*Figure* 10). The picture on the left shows just two such hydrocarbon chains, with lines joining different pairs of atoms drawn in different colours. The equivalent vector structure is drawn on the right. According to definition this should contain a high peak at the origin (shown at the centre of the picture) surrounded by a series of smaller peaks. Each of these peaks should be at the end of a vector from the origin, corresponding to a line joining two atoms in the hydrocarbon chains. The first four peaks around the origin, drawn in black, correspond to the bonds between neighbouring atoms in the hydrocarbon chains which are also drawn in black. The four yellow peaks which are all on a line passing through the origin correspond to the yellow lines joining pairs of next but nearest atoms along the chains. Similarly the four blue peaks originate from the pairs of atoms joined by blue lines. All the peaks which correspond to lines joining atoms within any one chain form a row, with a pattern which repeats at the same intervals along the row as does the pattern of carbon atoms along the molecular chains.

Consider now the lines joining pairs of atoms situated in neighbouring chains. A few of these lines are drawn in the diagram in red ; they are numbered to make the correlation with their vector equivalent more obvious. The red vectors are drawn radiating from the origin towards the right. In order not to confuse the diagram only a very few of the inter-chain vectors are drawn, but the ones that appear should enable the reader to visualize the way in which the other red peaks arose. It is seen that these inter-chain vectors produce another row of vector peaks which is parallel to the first row and spaced at a distance equal to that between a pair of hydrocarbons in the crystal structure. There is one such row on each side of the origin and, if the unit cell of the crystal structure contained not just two but several hydrocarbon chains side by side, the central row of vector peaks through the origin would be flanked on either side by several vector rows. Suppose in space each hydrocarbon chain were surrounded by six neighbouring ones, then the origin in the vector structure would be surrounded by six rows of vector peaks. The vector structure in this case would therefore reveal the direction of the chains with respect to the crystal axes, the repeat distance of the atomic pattern along the chains, the distance between neighbouring chains and their relative arrangement. This information would be sufficient to allow the crystal

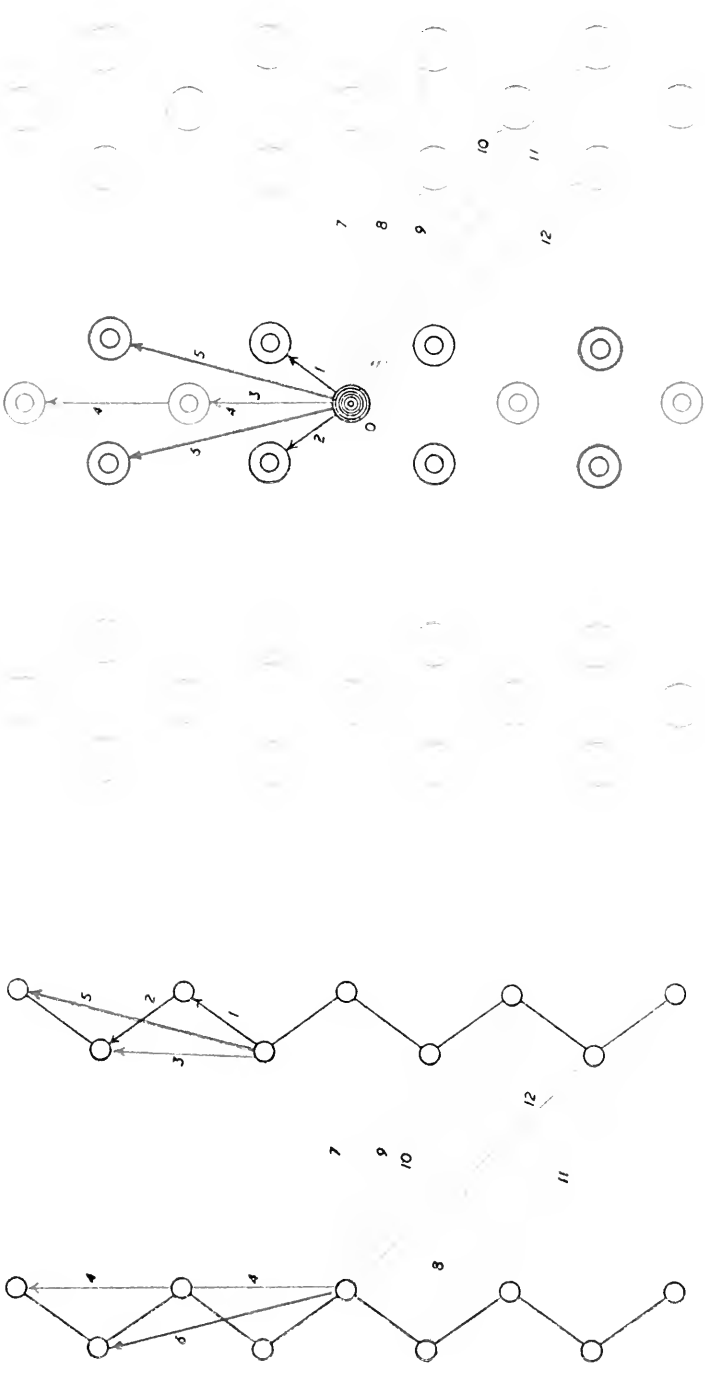


Figure 10. *Left : Hypothetical structure consisting of two parallel hydrocarbon chains. Right : Corresponding vector structure.* O *marks origin.*

structure to be solved. The haemoglobin molecule has a structure of this kind, though more complex than the example just given.

Similarly a layer structure in the crystal will give rise to a vector structure showing a layer of high vector density passing through the origin, and other layers at equal distances on each side of the origin. From the orientation of the vector layers and the distances between them, the corresponding data for the molecular layers can then be deduced. This was the type of structure met with in myoglobin.

Of course it is possible (just as in the case of electron density) to calculate vector densities either in three dimensions, or in projection in two dimensions on to a plane, or in projection in one dimension on to a line. It is to be noted that the labour that has to be spent to obtain these different forms of vector maps is related in an inverse proportion to the ease with which they can be interpreted. For a vector projection on to a line only the reflexions from one set of lattice planes are required ; the computation can be done in half an hour, but rarely provides much useful information. A two-dimensional projection needs the reflexions from all the sets of lattice planes which are parallel to one crystal axis and may take a few days to compute. Such projections may be extremely useful in favourable cases (*e.g.* myoglobin), but often cannot be interpreted because peaks at different levels above the plane of projection overlap. In the case of macromolecules complete calculations of vector density in three dimensions have only been made possible by the introduction of punched card calculating machines. Since such three-dimensional syntheses make use of all the reflexions in the entire diffraction pattern, they provide the maximum of *direct* information which that pattern can give. This was the reason why such syntheses were computed for horse haemoglobin and also for insulin (Crowfoot, Private Communication).

In x-ray studies of crystalline proteins the relative phases of the reflexions are generally unknown, and it is only the Patterson synthesis which allows the wealth of data contained in the diffraction pattern to be translated into a form which is at least potentially capable of interpretation in terms of molecular structure. It often happens, of course, that the unit cell contains several protein molecules in different orientation, which makes it impossible to decipher the vector structure. Fortunately, in the two analyses described in this volume this was not so ; both vector structures revealed relatively simple systems of parallel rods or layers which could be interpreted in terms of polypeptide chains.

A more detailed treatment of the principles and practice of x-ray crystallography may be found in various monographs : those by R. W. JAMES[4], W. L. BRAGG[5] and C. W. BUNN[6] are especially recommended (in increasing order of completeness).

Both Bragg and Bunn and especially J. M. ROBERTSON[7] deal in some detail with the Fourier method of obtaining electron densities. About the Patterson method little can be found as yet in text-books. Bunn's book contains a short section on it, but the treatment presented here is based on Patterson's original paper, and on examples of actual structure determinations.

Received September 1948

REFERENCES

[1] PORTER, A. B. *Phil. Mag.* 11 (1906) 154
[2] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. F. *Proc. Roy. Soc. A* 191 (1947) 83
[3] PATTERSON, A. L. *Z. Kristallogr.* 90 (1935) 517
[4] JAMES, R. W. *X-Ray Crystallography* London (1930)
[5] BRAGG, W. L. *The Crystalline State* London (1933)
[6] BUNN, C. W. *Chemical Crystallography* Oxford (1945)
[7] ROBERTSON, J. M. *X-Ray Analysis and Application of Fourier Series Methods to Molecular Structures; Reports on Progress in Physics*, p. 332 London (1937)
[8] CLEWS, C. J. B. and COCHRAN, W. *Acta Cryst.* 1 (1948) 4
[9] LLEWELLYN, F. G., COX, E. G. and GOODWYN, T. H. *J. Chem. Soc.* (1937) 883

V

PHYSICO-CHEMICAL PROPERTIES

The Effects of Salts on the Activity and Solubility of Haemoglobin

G. S. ADAIR

Mixtures of haemoglobin and phosphate buffers have special properties that make it possible to evaluate the solubility and the activity of the protein at the same pH value and salt content.

Functions termed excess coefficients can be computed from measurements of the distribution of phosphates across collodion membranes, using highly soluble species of haemoglobin and super saturated solutions of horse haemoglobin.

Methods and formulae for calculating the activity of haemoglobin from measurements of excess coefficients are described.

It is shown that salts cause relatively large changes in the activity of haemoglobin in solution, and that there must be relatively large changes in the activity of the protein in the solid phase.

THE composition of crystals of haemoglobin is influenced by the medium in which they are suspended, and it has been suggested that changes in the composition of the crystals are important for the interpretation of measurements of solubility of proteins in terms of activity coefficients.[1] E. J. COHN and J. T. EDSALL[2] on the other hand have suggested that the activity of the protein component in the crystal is approximately constant. They have shown that the effects of salts on the solubilities of amino acids and of proteins are similar, and it is known that the activity is constant in all saturated solutions of a pure substance. They also refer to electrometric measurements made with metallic or amalgam electrodes[3] in support of the hypothesis that the activity of the protein component is a constant.

Direct electrometric methods are not available for the buffer mixtures usually employed for studying the solubilities of proteins. Phosphate buffers have been used for extensive series of measurements of the solubility of haemoglobin. In the present communication an independent method is developed for calculating the activity of haemoglobin in phosphate buffers from measurements of membrane equilibrium.

The addition of a protein to a dilute solution of an inorganic salt usually tends to diminish the activity of the salt. If only one salt be present, at a constant molality, variations in the activity of the salt can be correlated with variations in the activity of the protein by a formula due to N. BJERRUM[4].

A number of salts or non-ionized substances may be present in buffer mixtures. An equation (1) applicable to solutions containing a mixture of salts, acids or bases was derived by G. S. ADAIR[5]

$$\mathrm{d} \ln a_p = (v_s/RT)\,\mathrm{d}\pi - b_a\,\mathrm{d} \ln a_a^\circ - b_b\,\mathrm{d} \ln a_b^\circ - b_i\,\mathrm{d} \ln a_i^\circ \ldots . (1)$$

a_p = activity or ' effective concentration of protein '
v_s = volume of solvent per mol of protein
π = osmotic pressure
b_a = ' excess coefficient ' for substance a, referred to later
a_a° = activity of substance a in the dialysate

Similar terms, with subscripts b, c, i may be applied to the substances b, c and i.

If substance a be a strong electrolyte with two ions, it may be convenient to introduce the mean activity of the ions $a_\pm$

$$b_a\,\mathrm{d} \log a_a^\circ = 2\,b_a\,\mathrm{d} \log a_\pm^\circ$$

The ' excess coefficient ' is defined by Formula 2.

$$b_a = (m_a - m_a^\circ)\,(1/m_p) \qquad \ldots . (2)$$

m_a = molality of substance a in the protein solution L
m_a° = molality of substance a in the dialysate L°
m_p = molality of protein in L

It may be noted that the activities of salts, acids or bases in L° can be measured or calculated from molalities by a number of methods that are not applicable to protein solutions.

If we are considering a series of dilute phosphate buffers, with pH values close to the isoelectric point of the protein, the activities of the individual ions in L and L° are nearly equal, and it may be possible to apply a simplified form of equation 1 expressed in terms of ionic activities instead of mean activities.

$$\mathrm{d} \log \gamma_p = - b_H\,\mathrm{d} \log a_H^\circ - b_t\,\mathrm{d} \log a_t^\circ \qquad \ldots . (3)$$

γ_p = activity coefficient = a_p/m_p for solutions where m_p is constant.
b_H = hydrogen ions combined with protein.
a_H° = activity of hydrogen ions in L°
b_t = corrected total excess coefficient for all ions excluding hydrogen ions.
a° = sum of activities of all ions excluding hydrogen ions.

$$b_t = (m_t - m_t^\circ - e_i)\,(1/m_p) \qquad \ldots . (4)$$

m_t and m_t° = sums of molalities of diffusible ions excluding hydrogen ions in L and L°

The term e_i is a correction for the osmotic effects of the excess of ions inside the membrane, calculated in accordance with Donnan's theory. This correction is extremely small in the region of the isoelectric point.

Formula 3 is less exact than formula 1, but it is more easily applied to experimental data in the form now available.

Table I

Ox Carbon monoxide Haemoglobin. Dialysate NaCl 0·10 M, Na_2HPO_4 0·015 M, NaH_2PO_4 0·005 M. *Ionic Strength* 0·15. CO_2 *in solution after dialysis* 0·0003 M.
π (*Osmotic pressure*) 395 mm Hg at 1·0°C. E (*membrane potential*) — 0·9 millivolts at 1·0°C .m_p (*protein molality*) 0·007548 (36·649 g protein in 100 ml solution).

	Protein Solution	*Dialysate*
*p*H *at* 1°C	7·2384	7·2550
Density at 1°C	1·0981	1·0071
H_2O g/ml	0·7247	0·9985
Sodium molality	0·1297	0·1356
Chloride molality	0·1138	0·0998
Phosphate molality	0·0264	0·0198
PO_4 *mean valence*	1·7428	1·7500

The measurements summarized in *Table I* show the effects of a high concentration of haemoglobin, comparable with the red blood corpuscle, on the membrane equilibrium of sodium, chloride and phosphate ions.

In systems of this type, the differences $m_a - m_a^\circ$ are directly proportional to m_p if m_a° be kept constant. In practice it may be essential to use a high concentration of protein to obtain differences that are significant.

Some of the properties of haemoglobin in the medium specified in *Table I* are as follows— The membrane potential E and the net charge were both negative, the valence being $-1{\cdot}4$ for a molecular weight of 67,000. The isoelectric *p*H, where E = 0, was estimated as $6{\cdot}9 \pm 0{\cdot}15$. The number of hydrogen ions combined per molecule of haemoglobin was $+4{\cdot}0$, and the isoionic *p*H, where $b_H = 0$, was estimated as $7{\cdot}7 \pm 0{\cdot}15$ for 1·0°C. This figure is appreciably higher than most published values. The correction term e_i in formula 4 was estimated as 0·00022 molal from the membrane potentials. The activity ratio a_H/a_H° for hydrogen ions was 1·039.

The differences between the charge and the number of hydrogen ions combined suggests that anions are associated with the protein.

Table II

Effect of haemoglobin on activity coefficients and the excess of ions.

γ/γ° *ratio for* NaCl	0·958
γ/γ° *ratio for* Na, HPO_4 *and* H_2PO_4	0·928
m_t *total molality of ions in L*	0·2699
m_t° *total molality of ions in L°*	0·2552
Total excess in L	0·0147
Theoretical excess in L	0·0002
Corrected excess in L	0·0145
b_t *excess coefficient*	1·9000
b_H *excess of H ions*	4·0000
$-\,db_H/dpH$ *(approx)*	8·0000
a_t° *total activity of ions in L°*	0·1800
$-\log a_H^\circ = p\mathrm{H}^\circ$	7·2550

Table II shows the effects of the protein on the mean activity coefficients of the salts. These coefficients are independent of assumptions concerning hydration or liquid junction potentials. The ratio γ/γ° for NaCl $= \dfrac{m_{Na}^\circ\, m_{Cl}^\circ}{m_{Na}\, m_{Cl}}$

The ratio γ/γ° for sodium phosphates is an approximation, computed by taking the mean valence of the anions as 1·75.

The most important terms included in *Table II* are the excess coefficients b_t and b_H, required for the application of formula 3, and the total activity of ions a_t°. The total activity was calculated from the total molality m_t° on the assumptions that sodium dihydrogen phosphate has the same activity coefficient as sodium chloride, and disodium phosphate the same activity coefficient as sodium sulphate.

The experimental data in *Table I* refer to ox haemoglobin which is highly soluble. A few experiments made on supersaturated solutions of horse haemoglobin and ammonium phosphate buffer mixtures showed an excess of salts inside the membrane, comparable with results obtained from more soluble species of haemoglobin.

A series of measurements of the type exemplified in *Table I* is required for the integration of formula 3. *Table III* gives a series of values of $p\mathrm{H}^\circ$, a_t° and b_t for 20 per cent solutions of sheep haemoglobin, dialysed against ammonium phosphate buffer mixtures at 0°C. The ionic strength of the buffer mixtures was varied by altering the total phosphate, keeping the percentage of each salt constant at 50 per cent of $(NH_4)_2\,HPO_4$ and 50 per cent of $NH_4H_2PO_4$. The

total activity a_t° was estimated by assuming that these salts have similar activity coefficients to ammonium sulphate and ammonium chloride respectively.

The figures marked b_t (*obs*) may be subject to correction. A value of $b_t = 2{\cdot}3$ instead of 2·7 for *p*H 7·08 was obtained in an experiment made at a later date, using different analytical methods. Analytical data used for estimating the excess coefficients in *Table III* were summarized by G. S. ADAIR and M. E. ADAIR[6]

Table III

Total excess coefficients for CO *Haemoglobin* (*sheep*) *at* 0°*C*. I = *ionic strength of buffer mixture with* 50 *per cent* $(NH_4)_2\,PO_4$ + 50 *per cent* $NH_4H_2PO_4$. $m^\circ_{PO_4}$ = *molality of total phosphate in the dialysate.* m_t° = sum $m^\circ_{PO_4} + m^\circ_{NH_4}$

Ionic strength	*p*H°	$m^\circ_{PO_4}$	m_t°	a_t°	b_t *obs.*	b_t *calc.*
0·01	7·11	0·0050	0·0126	0·011	+ 2·1	+ 2·3
0·02	7·08	0·0098	0·0243	0·019	+ 2·7	+ 3·0
0·04	7·04	0·0197	0·0502	0·037	+ 4·6	+ 4·1
0·07	6·95	0·0344	0·0859	0·057	+ 4·4	+ 5·1
0·31	6·80	0·1396	0·3560	0·175	+ 9·3	+ 8·7
2·09	6·30	1·0450	2·6130		− 32·1	

The last column headed b_t (*calc*) gives values for b_t computed by an empirical formula with two constants. The first constant, $k_1 = 22{\cdot}2 \pm 5$. The second constant k_2 was estimated as 3·4.

$$b_t\,(calc) = k_1\,(a_t^\circ)^{\frac{1}{2}} - k_2\,a_t^\circ \qquad \ldots.(5)$$

On the assumption that formula 5 is valid over the range from $a_t^\circ = 0{\cdot}00$ to 0·17 an estimate of the effects of sodium, ammonium or phosphate ions over the same range can be made by formulae 6 and 7

$$d \log \gamma_p\,(\text{salt effect}) = - b_t\,d \log a_t^\circ \qquad \ldots.(6)$$

$$\log \gamma_p\,(\text{salt effect}) = - 0{\cdot}4343\,[2\,k_1\,(a_t^\circ)^{\frac{1}{2}} - k_2\,a_t^\circ] \qquad \ldots.(7)$$

The first effect of salts is to diminish γ_p in accordance with formula 7. In a molar solution, where b_t is negative, salts tend to increase the

activity of the protein. The excess coefficient for water, b_w, determined by the formula $b_w = -(55{\cdot}51/m_t^\circ)\, b_t$ is then positive and it follows that

$$\text{d ln } \gamma_p \text{ (salt)} = +(b_w\, m_t^\circ /55{\cdot}51)\ \text{d ln } a_t^\circ \qquad \ldots.(8)$$

$$\text{d log } \gamma_p \text{ (salt)} = K_a\, \text{dm}_t^\circ + \text{dy} \qquad \ldots.(9)$$

$$\text{dy} = b_w\, (m_t^\circ/55{\cdot}51)\ \text{d log } \gamma_t^\circ \qquad \ldots.(10)$$

$K_a = 0{\cdot}4343\ (b_w/55{\cdot}51)$, a constant over a range where b_w is constant. In the molar solution of ammonium phosphates, $b_t = -32$ and $b_w = +680$, and therefore $K_a = +5{\cdot}3$.

In comparing measurements of activity coefficients with measurements of solubilities, the following equations may be found useful. All terms in equation 11 refer to a saturated aqueous solution of the protein. All terms in equation 12 refer to a saturated solution of the protein which contains inorganic salts.

$$(m_p)_w(\gamma_p)_w \text{ (solution)} = (m_p')_w(\gamma_p')_w \text{ (solid phase)} \qquad \ldots.(11)$$

$$m_p\gamma_p \text{ (solution)} = m_p'\gamma_p' \text{ (solid phase)} \qquad \ldots.(12)$$

If the measurements of solubilities S_w and S be expressed in g protein per 100 g of water it follows that

$$\log (S_w/S) = \log \gamma_p - \log \gamma_p' + k \qquad \ldots.(13)$$

The term k, which may be relatively small, is given by formula 14.

$$k = \log\, [(m_p')_w(\gamma_p')_w/m_p'(\gamma_p)_w] \qquad \ldots.(14)$$

Haemoglobin crystals in equilibrium with water vapour contain from 41 to 44 per cent of water[7] and it may be assumed that in the solid phase the protein is approximately $0{\cdot}02 \pm 0{\cdot}002$ molal.

On the hypothesis that the solid phase is like an 'ideal' mixed

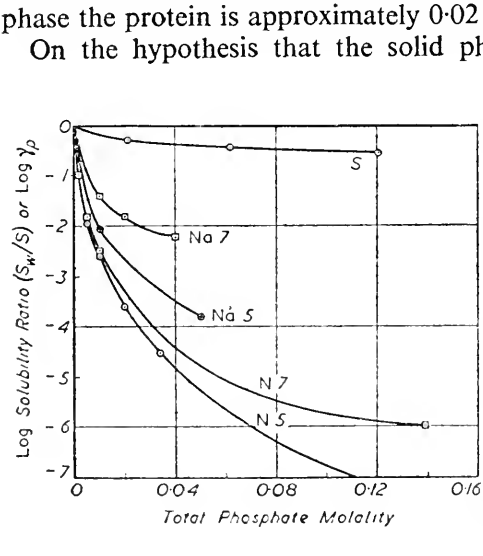


Figure 1. *Effects of salt concentration on activity coefficients of haemoglobin. Curve* Na7 *ox carboxyhaemoglobin in sodium phosphate buffers containing* 75% *of the disodium salt; in curve* Na5 *buffers contain* 50% *of the disodium salt. Curve* N7 *sheep carboxy haemoglobin in ammonium phosphate buffers containing* 70% *of diammonium salt; in curve* N5 *buffers contain* 50% *of diammonium salt. Curve S shows solubility ratios of horse oxyhaemoglobin in potassium phosphate buffers* pH 6·8.

crystal, the term γ_p' should be close to unity, and under the same conditions, curves representing the effects of salts on solubilities and on γ_p should be the same.

The experimental results available are summarized in *Figure* 1. The upper curve S gives values for the solubility ratio determined by E. J. COHN and A. M. PRENTISS[3]. The four lower curves give values for log γ_p (salt effect) calculated by formula 7. These curves may need modification, when more complete data are available, but there seems to be sufficient evidence to justify the conclusion that salts cause large alterations in the term γ_p', the activity coefficient of the protein in the solid phase, because the curves for log γ_p (salt effect) are much lower than the curve for the solubility ratio.

This conclusion is supported by the observation that there is a net excess of salts in the solid phase, when crystals of haemoglobin are equilibrated with 0·01 molar ammonium phosphates[6].

Received September 1948

REFERENCES

[1] ADAIR, G. S. *Ann. Rev. Biochem.* 6 (1937) 186
[2] COHN, E. J. and EDSALL, J. T. *Proteins, Amino Acids and Peptides* New York 1943
[3] JOSEPH, N. R. *J. biol. Chem.* 126 (1938) 389
[4] BJERRUM, N. *Z. phys. Chem.* 104 (1923) 406
[5] ADAIR, G. S. *Trans. Far. Soc.* 31 (1935) 98
[6] — and ADAIR, M. E. *Bio-chem. J.* 28 (1934) 1230
[7] KATZ, J. R. *Hoppe-Seyl. Z.* 95 (1915) 1
[8] COHN, E. J. and PRENTISS, A. M. *J. gen. Physiol.* 8 (1927) 619

A Rapid and Accurate Method for the Measurement of the Osmotic Pressure of Haemoglobin

G. S. ADAIR

A description is given of a simple toluene osmometer adapted for rapid measurements of osmotic pressures of dilute protein solutions. Determinations of osmotic pressures of 0·24 *to* 3 *per cent horse CO haemoglobin solutions at* 1·0°*C have not yielded evidence of dissociation of the molecule. The mean molecular weight determined was* 66,400 *; the mean deviation was* 400.

SIR JOSEPH BARCROFT[1] was greatly interested in the suggestion that the degree of aggregation of haemoglobin depends on the salt concentration. In 1920 he asked me to attempt to measure the osmotic pressure of haemoglobin solutions so that we might obtain direct experimental evidence as to the relationship between particle size and salt concentration. At that time we had no facilities for carrying out experiments at a low temperature, and as haemoglobin tends to denature at room temperature, we tried to devise osmometers that allowed osmotic equilibrium to be reached rapidly.

In the simplest method, a modification of that described by S. P. L. SÖRENSEN[2], the protein was contained in a cylindrical collodion membrane of 6 mm diameter and 5 to 15 cm length, which was fitted to a capillary tube of 0·7 mm bore. The membrane was immersed in a large volume of salt solution, and readings of the rate of osmosis or dV/dt were made with the column of protein solution adjusted at different heights above the level of the salt solution.

The osmotic pressure π_s in cm solution was computed by formula 1.

$$dV/dt = \tfrac{1}{4}\,\pi d^2\, dh/dt = -(h - c - \pi_s)\,AQ \qquad \ldots.(1)$$

d = diameter of capillary — h = height of column of protein
c = correction for capillarity — A = area of membrane
Q = permeability constant — V = volume of protein solution

Mercury manometers, or a compensating device in which air was compressed by mercury, were used if the pressures were greater than 70 cm water.

This work has been described in a thesis[3] but it was not published elsewhere. Experiments made subsequently at the Low Temperature

Station, Cambridge, where the protein solutions could be equilibrated for many days at 0°C, confirmed the value of 66,000 for the molecular weight of haemoglobin.[4]

The osmometers used for these experiments were essentially similar to that shown in *Figure* 1. *T* denotes a glass tube graduated in mm, h = height of haemoglobin solution, *M* a collodion membrane of diameter 1·1 cm and length 8 cm, which is tied by rubber string to a piece of semipressure tubing into which a 5 mm glass tube G_2 is inserted. W = level of buffer in tube of 35 mm diameter.

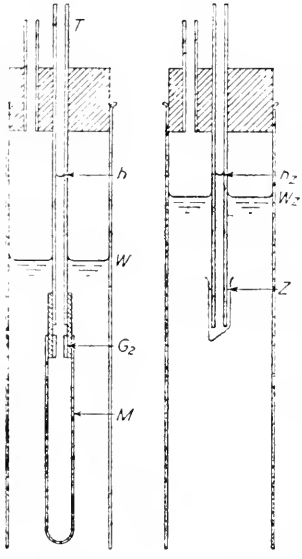


Figure 1. *Osmometer and capillarity measurement for small pressures at* 0°*C.*

Membranes are made from dry pyroxylin dissolved in absolute alcohol and anhydrous ether, and their permeability is adjusted by the addition of varying amounts of ethylene glycol. The texture of the membranes is of significance. Thick membranes may slowly adsorb protein and give pressures which fall slowly over long periods.

The diameter of the osmometer tube is 1·3 mm for pressures greater than 10 cm solution. For more dilute protein solutions osmometers of 3·3 mm bore are used so that the correction for capillarity is smaller. *Figure* 1 also shows the method now used for the determination of capillarity. *Z* is a small open tube containing the protein solution, h_z and W_z denote the levels of the protein solution and of the buffer used as dialysate. With osmometers of 3·3 mm bore, capillarity readings indicate an apparent equilibrium within one hour but then fall slowly at a rate of approximately 0·1 mm per day for about 6 days. If the membrane is of suitable permeability and surface area, the time required for capillarity effects to reach equilibrium may be much longer than the times required for osmosis and the diffusion of salts.

Recently, rapid and accurate measurements of the osmotic pressure of haemoglobin have been made with toluene manometers in which the capillarity effects usually reach equilibrium in a few seconds. Paraffin, alcohol or toluene manometers have been used in osmometers designed by Weber[5], Oakley[6], Bourdillon[7], Bull[8] and Scatchard *et al.*[9] The experiments to be recorded have been made with toluene osmometers simplified by omission of taps and joints.

Capillarity effects at an oil-water interface within a glass tube may

cause errors. In this work it was found that reproducible results could be obtained if the toluene-buffer interface was in the form of a hanging drop of toluene. The surface area is large and the volume relatively small, and errors caused by temperature fluctuations are minimized. The expansion of toluene is compensated by the contraction of water between 0°C and 4°C. *Figure* 2 shows the simplest type of toluene manometer, adapted for the measurement of pressures up to 15 cm toluene. A graduated pyrex capillary tube of 0·5 mm internal diameter is fused to a flattened bulb of 16 mm diameter. R_1 is a soft rubber connection ; G_1 a glass tube 3 cm long, which contains buffer solution in the upper part ; M a collodion membrane as in *Figure* 1. L=toluene meniscus ; W = meniscus level of dialysate ; B = lowest point of toluene-buffer interface. This interface is in contact with glass at the level B_1, at about 5 mm above B.

The diameter of the outer tube containing buffer is important because of capillarity effects at the meniscus W. There are measureable variations (0·2 mm) even if the tube be as wide as 35 mm. A diameter of 45 mm is advisable to obtain capillarity readings constant within 0·1 mm.

In order to prepare the manometer tube for use, a rubber tube, fitted with a screw-clamp and closed at one end with a small glass rod, is filled with buffer and connected to the osmometer below the bulb. By turning the screw, buffer is forced slowly to the level B_1. The device shown in *Figure* 2*a* is then put on to the top of the graduated end of the manometer tube. R_3 is a rubber bung, G_3 a short, wide glass tube. Water is put in G_3 to the level W^1 and a layer of toluene coloured with Sudan III added up to the level T^1. The screw-clamp is manipulated to draw toluene down the capillary and then to drive air bubbles upwards until all the air is expelled. The buffer-toluene interface is of the form shown in *Figure* 2. Toluene and water are then removed from G_3, and R_3 and G_3 are replaced by a short rubber tube which can be closed when necessary with a screw-clamp.

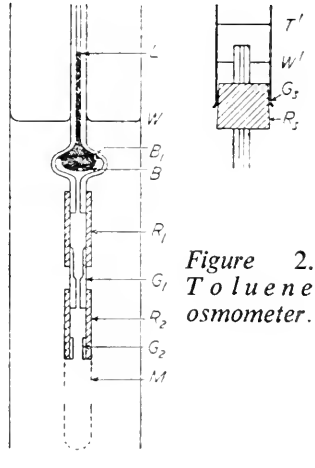


Figure 2. Toluene osmometer.

Before the membrane which contains the protein is connected to the osmometer, the rise of toluene due to capillarity is determined. The osmometer is immersed to the appropriate depths in buffer solution contained in the tube of 45 mm diameter and left for at least

2 hours at 0°C to reach temperature equilibrium. The levels L, W and B are then measured.

A membrane containing protein which has been dialysed against the buffer solution is then connected to the osmometer. Screw-clamps on the tubes R_1 and R_2 may be used to keep the volume of buffer in the bulb approximately constant, during operations of attaching and detaching rubber tubes or connections to the membrane below the bulb.

The rate of osmosis may be sufficient to eliminate 50 per cent of an initial deviation from the equilibrium pressure within half an hour. The osmometer is left overnight at 0°C to reach equilibrium.

After equilibrium has been attained, the osmotic pressure of the protein π_t, expressed in cm toluene, may be calculated by formula 2 from readings of L, W and B. The capillarity is redetermined at the end of the experiment.

$$\pi_t = (L - W) - (W - B)\,\mathrm{k} + \mathrm{q} - \mathrm{cap} \qquad \ldots.(2)$$

$\mathrm{k} = (\rho_b - \rho_t) / (\rho_t - \rho_a)$. ρ_b, ρ_t and ρ_a denote densities of buffer, toluene and air. q = a correction term to allow for differences in densities of buffer and of protein solution. Cap. = correction for capillarity.

The apparatus shown in *Figure* 2 can be modified by the addition of U tubes and T pieces so that the manometer can be connected to the dialysate by opening a screw clamp.

Experimental data recorded in *Tables I* and *II* show to what degree the measurements are reproducible. These data refer to carbon monoxide haemoglobin prepared from horse blood and dialysed against phosphate buffer Na_2HPO_4 0·03M, NaH_2PO_4 0·01M. The final *p*H was 7·38 at 0°C.

The third column in *Table I* gives molecular weights, computed by formula 3.

$$M = 170{,}330\ (273 + t/273)\ (C/\pi)\ \phi$$
$$\phi = 1/(1 - 0{\cdot}02853\ C + 0{\cdot}0002109\ C^2) = \textit{osmotic coefficient} \ldots.(3)$$

t = temperature. C = g haemoglobin per 100 ml solution.*

The mean molecular weight is 66,400 ; the mean deviation is 400. *Table II* shows the relationship between observed osmotic pressures and values calculated on the assumption that the molecular weight is 66,400 in dilute solutions.

Diffusion and ultracentrifugal measurements[11] have yielded evidence for the dissociation of horse haemoglobin at concentrations below 0·8 per cent at a temperature of or above 20°C. The measurements of osmotic pressure at either 0·1°C or 1·0°C recorded in *Table II* show

* The formula for the osmotic coefficient ϕ was derived from unpublished data for concentrated haemoglobin solutions by the method of Adair and Robinson.[10]

Tables I and II
C = *g* CO *Haemoglobin per* 100 *ml Solution*
π = *Osmotic Pressure in mm* Hg
M = *Molecular Weight*

I			*II*		
C	π	M	C	π *observed*	π *calculated*
3·028	8·50	66,500	0·532	1·39	1·38
3·015	8·43	66,600	0·509	1·32	1·33
2·140	5·81	66,800	0·508	1·32	1·32
2·025	5·54	66,800	0·504	1·31	1·31
2·010	5·46	65,500	0·496	1·32	1·29
0·976	2·55	66,500	0·494	1·27	1·28
0·978	2·59	66,000	0·260	0·67	0·67
			0·250	0·63	0·65
			0·244	0·68	0·63
			0·242	0·64	0·62

no evidence for dissociation. The mean difference between observed and calculated pressures is 0·015 mm mercury or 0·2 mm toluene. It is possible that the discrepancy between the two sets of results is accounted by the difference of temperatures.

REFERENCES

[1] BARCROFT, J. *The Respiratory Function of the Blood* Cambridge 1914
[2] SÖRENSON, S. P. L. *C. R. Lab.* Carlsberg 12 (1917) 262
[3] ADAIR, G. S. *Thesis* King's College, Cambridge (1922–23)
[4] —— *Proc. roy. Soc. A* 108 (1925) 627 109 (1925) 292
[5] WEBER, H. H. and STÖVER, R. *Biochem. Z.* 259 (1933) 269
[6] OAKLEY, H. B. *Trans. Far. Soc.* 31 (1935) 136
[7] BOURDILLON, J. *J. biol. Chem.* 127 (1939) 617
[8] BULL, H. B. *J. biol. Chem.* 137 (1941) 143
[9] SCATCHARD, G., BATCHELDER, A. C. and BROWN, A. *J. Amer. chem. Soc.* 68 (1946) 2320
[10] ADAIR, G. S. and ROBINSON, M. E. *Biochem. J.* 24 (1930) 1864
[11] SVEDBERG, T. and PEDERSEN, K. O. *The Ultracentrifuge* Oxford, 1940

The Osmotic Pressure of Haemoglobin in Strong Salt Solutions

H. GUTFREUND

In the introduction previous studies on the size and molecular dissociation of mammalian haemoglobin in solutions are summarized. The influence of dissociation phenomena and of solvent-solute and solute interaction phenomena on the osmotic pressure of solutions is discussed, and the procedure of differentiating between the two effects is described.

Data of measurements of osmotic pressures of solutions of horse and human haemoglobin in phosphate, sodium chloride and lithium chloride solutions of varied concentrations are recorded. The results of these experiments show that high salt concentrations increase the interaction phenomena at high protein concentration and cause dissociation of horse and human haemoglobin at low protein concentration.

THE results of osmotic pressure measurements described by G. S. ADAIR[1-5] showed that the molecular weight of haemoglobin from man, horse, ox and sheep is 66,700, or four times the equivalent weight calculated from the iron contents of these proteins. Studies of the osmotic pressure of adult and foetal goat haemoglobin[6], foetal sheep haemoglobin[7] and foetal human haemoglobin (GUTFREUND, unpublished observations) showed that these proteins too have a molecular weight of 66,000–67,000.

Recent careful reinvestigation of the sedimentation constants of horse, adult and foetal human and adult and foetal sheep haemoglobin (CECIL and GUTFREUND, unpublished observations) showed these to be almost identical. This indicates that not only the molecular weight but also the shape of haemoglobins from these species is the same. The sedimentation constant is dependent on the molecular weight, shape and hydration, but the latter (0·3 g per g of protein) is not likely to differ significantly from one haemoglobin to another. The similarity of size and shape of various haemoglobins is in marked contrast with the great differences observed in their chemical composition, number and type of free amino groups as well as in their physiological, solubility and electrophoretic behaviour.

The dissociation of horse haemoglobin in very dilute solutions was first observed by A. TISELIUS and D. GROSS[8] from measurements of the diffusion constant and T. SVEDBERG and K. O. PEDERSEN[9] reported

dissociation of both horse and human haemoglobin in dilute solutions from ultracentrifugal observations. Only for foetal sheep haemoglobin have results of osmotic pressure measurements on haemoglobin solutions shown signs of dissociation on dilution[7], and some preliminary ultracentrifugal studies (Gutfreund, unpublished observations), showed that molecules of this protein tend to dissociate into four sub-units. There is certainly a marked difference between haemoglobins from different sources in their tendency to dissociate on dilution ; with adult sheep haemoglobin no dissociation effect could be detected on ultracentrifugal examination of solutions containing 0·1 per cent protein ; at this concentration a marked drop in the sedimentation constant can be observed for horse and human haemoglobin.

Solvents of different compositions have been used to study the dissociation of haemoglobin ; among the most prominent of these are aqueous solutions of urea and guanidine. The osmotic pressure of protein solutions of finite concentration, however, does not only depend upon the molecular weight of the protein ; this point will be discussed in greater detail later in this paper. It is very difficult to determine the haemoglobin concentration really accurately in the presence of large quantities of urea or guanidine. Another medium which is reported to cause dissociation of haemoglobin molecules in solution, namely concentrated aqueous sodium chloride, was therefore chosen for a preliminary investigation to differentiate between the effect of protein dissociation and solvent-solute interaction on the osmotic pressure of protein solutions.

EXPERIMENTAL PROCEDURE

The osmotic pressure of haemoglobin solutions was measured in the simple osmometer described by G. S. ADAIR[2]. The protein concentration, after equilibration, was in each case determined by the micro-Kjeldahl procedure using all the precautions recommended by A. C. CHIBNALL, M. W. REES and E. F. WILLIAMS[10] as well as their value of 16·8 per cent as the nitrogen contents of haemoglobin. The variations of the nitrogen contents of haemoglobins from various species is within the experimental error of the methods employed. The effect of large amounts of chloride on the nitrogen estimation was checked by digesting ammonium sulphate with sulphuric acid and catalyst in the same manner as the haemoglobin samples, and with similar quantities of sodium chloride and lithium chloride. It was found that the results were not affected by the addition of chlorides.

The osmotic pressure was recorded in cms of water, calculated, after correction for capillarity and density of the solution, from direct

measurements of the height of solution in the capillary tube. All measurements recorded here were carried out at 3–4°C.

The horse and human haemoglobin samples were prepared by laking red cells, after repeated washing in 1 per cent sodium chloride solution, with ether and separating the stroma by centrifugation. These stock solutions of horse and human haemoglobin were, after prolonged dialysis against distilled water, equilibrated with 0·2M phosphate (0·1M Na_2HPO_4 and 0·1M KH_2PO_4; this mixture will be referred to as 0·2M phosphate throughout this paper) before dialysis against any of the other salt solutions used during this investigation.

RESULTS AND DISCUSSION

The results of all the osmotic pressure measurements of horse and human haemoglobin in solutions of varied composition, salt and protein concentration are recorded in *Tables I—VI*. C is the concentration of dry haemoglobin in grams per 100 ml of solvent, and P is the osmotic pressure in cms of water. The apparent molecular weight, M, can be calculated from each measurement by the equation

$$M = 10\ RT/\frac{P}{C} \qquad \ldots.(1)$$

where R is the gas constant and T the absolute temperature.

At 3°C $$M = 234000/\frac{P}{C}$$

and for $M = 66700$, which is the most accurate estimate for the molecular weight of haemoglobins (from iron analysis) $\frac{P}{C} = 3{\cdot}5$. Discussion of the data presented here will be concerned with the examination of the deviations from the value of $\frac{P}{C} = 3{\cdot}5$.

Insertion of the computed values for the molecular weight from each measurement would be misleading as changes in the value for P/C can have other causes besides variation of the molecular weight of haemoglobin. If zero overall surface charge of the colloidal solute and thus equal concentration of the non-colloidal components in the dialysate and the solution are assumed, deviations from the ideal solution law

$$P = m\ RT \qquad \ldots.(2)$$

(where m is the molar concentration of the protein) at finite protein concentration are due to

1 Solute-solvent interaction (solvation)
2 Solute interaction (van der Waals and Coulomb forces)
3 Change of entropy of the solution due to presence of large molecules (this effect becomes very marked for asymmetric particles).

Equation 2 is only valid at zero concentration and becomes

$$P = m\,RTg \qquad \ldots.(3)$$

at finite concentrations ; g is the osmotic coefficient and is dependent upon the protein concentration, other conditions (solvent composition and temperature) being kept constant.

Table I

The Osmotic Pressure of Solutions of Horse Haemoglobin in 0·2M Phosphate

C	*P*	*P/C*
0·65	2·5	3·84
0·81	3·1	3·82
1·11	3·9	3·51
1·24	4·7	3·79
1·65	5·7	3·46
1·78	6·8	3·82
2·17	8·3	3·82
2·54	8·9	3·40
2·98	11·2	3·76
3·52	13·4	3·80
3·90	14·6	3·74
4·89	19·6	4·00
6·06	23·9	3·94
8·01	34·2	4·27
8·89	38·7	4·36

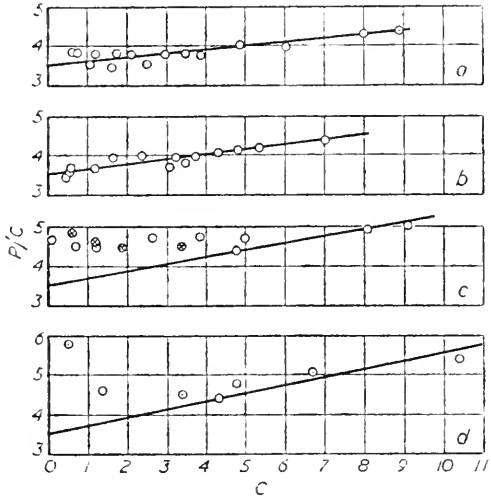
Table II

The Osmotic Pressure of Solutions of Human Haemoglobin in 0·2M Phosphate

C	*P*	*P/C*
0·47	1·6	3·40
0·56	2·0	3·57
0·60	2·2	3·66
1·29	4·7	3·64
1·66	6·5	3·92
2·39	9·5	3·98
3·09	11·3	3·65
3·25	12·8	3·93
3·47	13·2	3·80
3·75	14·9	3·96
4·34	17·6	4·06
4·83	19·9	4·12
5·40	22·6	4·18
7·01	30·6	4·36

To obtain correct values for M from equation 1 the usual procedure is to determine the osmotic pressure over a series of protein concentrations and plot P/C against C and use the value for P/C obtained from extrapolation to zero concentration for the computation of M. The value for g due to any of the three causes given above tends to unity as C approaches zero. For haemoglobin therefore the plot of P/C against C should extrapolate to $P/C = 3{\cdot}5$. *Figures* 1*a* and 1*b*,

which show a graphic representation of the data obtained from osmotic pressure measurements on solutions of horse and human haemoglobin respectively in 0·2 M phosphate, demonstrate the order of accuracy and type of plot obtained. The slope of the horse haemoglobin plot is the same as that obtained by plotting Adair's data for sheep haemoglobin on the same scale[5]. The slope of the human haemoglobin plot is slightly larger, in agreement with some data given by G. S. ADAIR[4].



Plot of P/C against C, where C is the concentration of haemoglobin in grams per 100 *ml of solvent and P is the osmotic pressure in cms of water.* a *Horse haemoglobin solutions in* 0·2 *M phosphate.* b *Human haemoglobin solutions in* 0·2 *M phosphate.* c *Horse haemoglobin* (⊙) *and human haemoglobin* (⊗) *solutions in* 0·2 *M phosphate* + 1 M *sodium chloride.* d *Horse haemoglobin solutions in* 2 M *sodium chloride.*

Table III

The Osmotic Pressure of Solutions of Horse Haemoglobin in 0·2*M Phosphate and* 1*M NaCl*

C	*P*	*P/C*
0·15	0·7	4·65
0·76	3·4	4·47
1·28	5·7	4·46
2·68	12·6	4·70
3·91	18·4	4·70
4·82	20·8	4·32
5·02	23·5	4·67
8·12	39·5	4·86
9·23	46·2	5·01

Table IV

The Osmotic Pressure of Solutions of Human Haemoglobin in 0·2*M Phosphate and* 1*M NaCl*

C	*P*	*P/C*
0·68	3·3	4·85
1·23	5·6	4·56
1·92	8·6	4·47
3·42	15·3	4·47

G. S. ADAIR studied the influence of high concentrations of sodium chloride upon the osmotic coefficient of concentrated (20 per cent) haemoglobin solutions[4] ; he showed that it was considerably increased. If only interaction phenomena or the entropy of the solution are responsible for the increase in the osmotic coefficient, then P/C should still extrapolate to 3·5 for $C = 0$. *Figure* 1*c* shows the plot of the data from *Tables III* and *IV* obtained from measurements on solutions of horse and human haemoglobin in 0·2M phosphate + 1M sodium chloride. The points on this graph corresponding to protein concentrations of 5 per cent and above could be extrapolated to $\frac{P}{C} = 3{\cdot}5$ for $C = 0$ and the slope of this line is steeper than that obtained for either of the two haemoglobins in phosphate solution. None of the points below protein concentrations of 5 per cent would fall on this line of extrapolation and it must therefore be assumed that some other effect is taking place which increases the osmotic coefficient for dilute solutions, and this cannot be due to any of the three causes given above. The only possible explanation for large osmotic coefficients in the more dilute solutions can be found in progressive dissociation of horse and human haemoglobin molecules on dilution in 1M sodium chloride solutions.

A further series of experiments was carried out on horse haemoglobin in 2M sodium chloride. The data recorded in *Table V* are plotted in *Figure* 1*d*. Again the data obtained for the more concentrated haemoglobin solutions could be extrapolated to $P/C = 3{\cdot}5$, giving an even steeper slope than that obtained from data on solutions in 1M sodium chloride. In the more dilute haemoglobin solutions

Table V

The Osmotic Pressure of Solutions of Horse Haemoglobin in 2M NaCl

C	P	P/C
0·55	3·2	5·81
1·39	6·4	4·60
3·41	15·3	4·48
4·36	19·3	4·41
4·77	22·9	4·78
6·71	34·1	5·07
11·40	62·1	5·45

Table VI

The Osmotic Pressure of Solutions of Horse Haemoglobin in 2M LiCl

C	P	P/C
2·03	10·0	4·91
4·38	22·1	5·04
5·41	29·0	5·35

the results are very similar in the two cases, though the dissociation effect may be slightly more marked in 2M sodium chloride.

Three measurements of the osmotic pressure of horse haemoglobin in 2M lithium chloride are recorded in *Table VI.* Some preliminary experiments with this electrolyte were undertaken on account of its large activity coefficient as compared with that of sodium chloride in two molar solutions. The results so far obtained, though quite incomplete, indicate even greater interaction effects at high haemoglobin concentrations and also greater dissociation effects in more dilute solutions of horse haemoglobin. It would be premature to draw any conclusions from this, but more detailed studies on haemoglobin in lithium chloride solutions of varied concentrations as well as in concentrated solutions of several strong electrolytes of varied activity coefficients may yield valuable results.

It is evident from the above results that differences of osmotic pressures of proteins in solutions of varied composition, must be interpreted with great caution. A complete set of data comprising measurements over a wide range of protein concentrations in each solvent is essential even if there is no danger of interference through partial pressure of excess diffusible electrolytes. The results and discussion given here form necessarily only a preliminary report. Much more detailed studies on these lines should enable one to carry out a thermodynamic analysis of intermolecular forces (interaction energies) and of the intramolecular forces holding the subunits of the haemoglobin molecules together. The former could be co-related with the change of average intermolecular distance on change of protein concentration and thus afford an interesting study of long range forces in solutions of varied composition.

Received September 1948

REFERENCES

[1] ADAIR, G. S. *Proc. Camb. phil. Soc. (Biol.)* 1 (1924) 75
[2] — *Proc. roy. Soc. A* 108 (1925) 627
[3] — *Proc. roy. Soc. A* 109 (1925) 292
[4] — *Proc. roy. Soc. A* 120 (1928) 573
[5] — *Proc. roy. Soc. A* 126 (1929) 16
[6] MCCARTHY, E. F. *J. Physiol.* 80 (1933) 206
[7] — and POPJÀK, G. *Nature, Lond.* 159 (1947) 198
[8] TISELIUS, A. and GROSS, D. *Kolloidzschr.* 66 (1934) 11
[9] SVEDBERG, T. and PEDERSEN, K. O. *The Ultracentrifuge* Oxford, 1940
[10] CHIBNALL, A. C., REES, M. W. and WILLIAMS, E. F. *Bio-chem. J.* 37 (1943) 354

The Ultraviolet Spectral Absorption of Haemoglobins Inside and Outside the Red Blood Cell

E. M. JOPE

The ultraviolet spectral absorption of haemoglobins has been studied in the high concentrations found in red blood cells : Beer's Law is obeyed in the Soret band region up to 38·8 *per cent haemoglobin concentration. The effects of denaturation and drying on the ultraviolet spectral absorption of haemoglobins are described and the recombination of haem and globin is discussed. These spectral absorption studies have been extended to intact red blood cells. Continuous spectral light sources have been used and the haemoglobin bands in the ultraviolet appear at wavelengths identical with those in simple solution. The Soret absorption band is present and of expected intensity and an extra absorption band at* 378 *mμ has been observed in human red cells. Red cells have been studied both as suspensions and as individual cells using an ordinary glass train, and later a reflecting microscope to project an image of the red cell on to the spectrograph slit. Spectrograms of cross-sections of red cells so obtained have been analyzed on a recording densitometer. The differences between cytoplasmic and nuclear absorption in frog red cells are shown. A difference in the ultraviolet spectral absorption between human adult and foetal haemoglobin, which does not extend to sheep or rat, has been observed.*

THE study of the spectral absorption in the ultraviolet has considerable value as a means of obtaining information about haemoglobins, and I have set out here to discuss some of the potentialities of such studies for illuminating the chemical behaviour of the haem-protein compounds, and to show what can be learnt of their biological behaviour when the study is extended to intact living material. SIR JOSEPH BARCROFT saw clearly the value of spectral observations, using them whenever possible, and recent developments in equipment and expansion of the study of absorption spectra enable us to extend the usefulness of such observations.

The spectral absorption of the haem-proteins may be considered in two distinct groups ; one, in the ultraviolet, is due to the aromatic amino-acids of the protein and is dealt with towards the end of this paper ; the other, a system of bands in the near ultraviolet and

visible, is due to the haem group (*Figure* 1). These two absorbing parts of the molecule exert their effects in a large measure independently of each other.

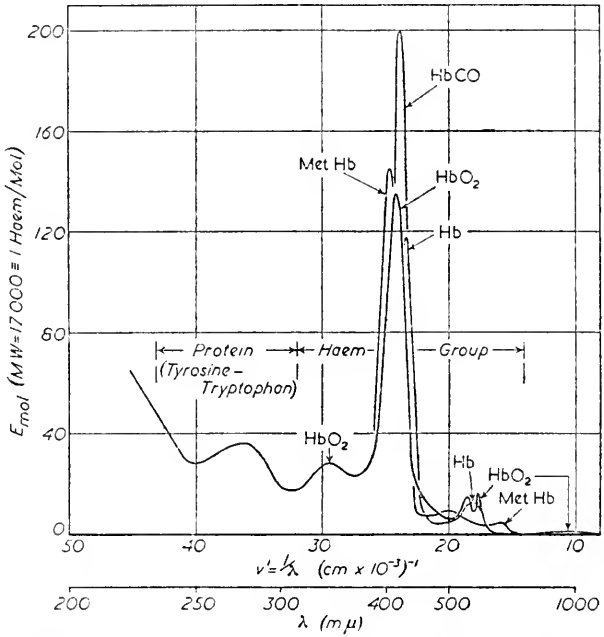


Figure 1. The ultraviolet and visible spectral absorptions of haemoglobins.

The band system due to the haem groups is responsible for the colour of haemoglobins, and consists of several bands in the visible, of molar extinctions of the order 10 to 20 $\times$ 10^3*, and a very intense band of molar extinction of the order 100 to 200 $\times$ 10^3 in the extreme violet at about 400 to 425 mμ. This is called the γ, or Soret band[1], and is an essential property of the 16-membered porphyrin ring of conjugated double bonds. Both the visible and Soret bands depend in wavelength position upon the valency of the central iron atom of the haem, on combination with O_2 or CO, and on changes in the double bond systems of the side-chains conjugated to the 16-membered central ring, as for instance in protoporphyrin with its vinyl side chains, and mesoporphyrin with its ethyl side chains in those positions[2]. The bands due to the haem are also dependent to some extent upon

* For convenience in comparisons molar extinctions of haem—protein compounds are usually stated in terms of molecular units containing 1 haem group and 1 atom of Fe, usually associated with a protein unit of molecular weight about 17,000.

the nature of the protein carrier, and possibly upon the manner of linkage between the protein and the haem. Thus the visible and Soret absorption bands of mammalian red cell haemoglobins lie at slightly shorter wavelengths than do those of the corresponding muscle haemoglobins (*Table I*). This is probably not due to the different molecular weights of the red cell haemoglobins (67,000) and those from muscle (17,000) as no changes can be detected in the spectral absorption of red cell haemoglobins under conditions such as strong salt concentration or high haemoglobin dilution when many haemoglobin molecules are known to split into smaller units[3,4]. On the other hand the Soret band wavelengths and molar extinction coefficients of red cell haemoglobins remain remarkably constant over a great range of species—adult and foetal in man, sheep, pig, and rat, and adult in horse, ox, rabbit, frog, salamander, and many birds—representing a wide variety of amino-acid composition and molecular shape of protein. The relation between the nature of the protein and the wavelength and intensity of these absorption bands due to the haem group may prove to be related to the nature of the linkage between the haem and the protein, and its further study may help to clarify this problem. These Soret band data are summarized in *Table I.*

Table I

Wavelengths (in mμ) of Soret bands of haemoglobins of red cells and muscle

	HbO_2	HbCO	MetHb	Hb
Red cell haemoglobins: Mammalian, amphibian, and avian (Jope, unpublished)	414·5	420	406	425
Muscle haemoglobins: horse heart and man (Theorell 1934)	418	424	407	435

As a preliminary to the spectral study of haemoglobins in biochemical and biological systems it has been necessary to accumulate data on variations in their spectral absorption with controllable

changes in environment, such as salt and hydrogen ion concentration, or concentration of haemoglobin itself, and to observe the effects of drying and of denaturation.

Denaturation of methaemoglobin by alkali, heat or strong urea has no effect upon the peak wavelength of the Soret band. The effect of denaturation upon the spectral absorption of HbO_2 may be divided into three stages, the first in which the Soret band peak changes to about 410 mμ (*Figure* 2), the solubility in the isoelectric region remaining, however, fairly high and some measure of reversible combination with oxygen being retained; the second in which the Soret band shifts to 406 mμ, that of methaemoglobin, but the visible bands remain as in HbO_2, and the final stage which appears to yield denatured methaemoglobin. The intermediate stage resembles in many ways the usual recombined haem-globin product (see p. 218). The first stage occurs on prolonged standing of HbO_2, especially in dilute solution, or in strong salt concentrations, and at room temperature. These are conditions in which electrovalent links and hydrogen bondings in proteins are weakened. D. L. DRABKIN and J. H. AUSTIN[5] observed what may be a related effect in the visible spectrum of HbO_2 on standing in dilute solution.

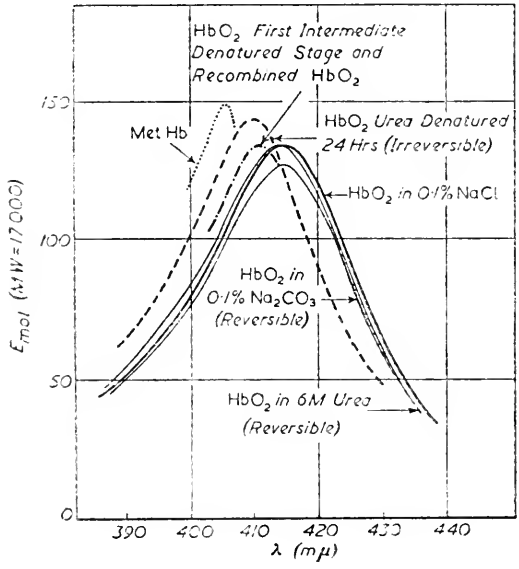


Figure 2. *Spectral absorption in the Soret band region of haemoglobins, showing effect of denaturation.*

Changes in hydrogen ion concentration have little effect upon the Soret bands of the haem proteins. The HbO_2 Soret band remains constant in wavelength but is reversibly depressed by about 5 per cent

on changing the *p*H from neutrality to the region of *p*H 9–10. On standing for 24 hours at room temperature at such alkalinity the first intermediate denaturation state noted above is observed[6]. In more alkaline solutions the HbO_2 becomes rapidly denatured (as methaemoglobin), as it does also on the acid side of *p*H 5·5. The Soret bands of HbCO[6] and of methaemoglobin[7, 8] remain entirely unchanged both in wavelength and in intensity up to at least *p*H 9, and the visible bands of all these compounds are insensitive to *p*H changes so long as the protein is not denatured, with the exception of the 635 mμ band of methaemoglobin[9, 6]. which changes over the range *p*H 6–10.

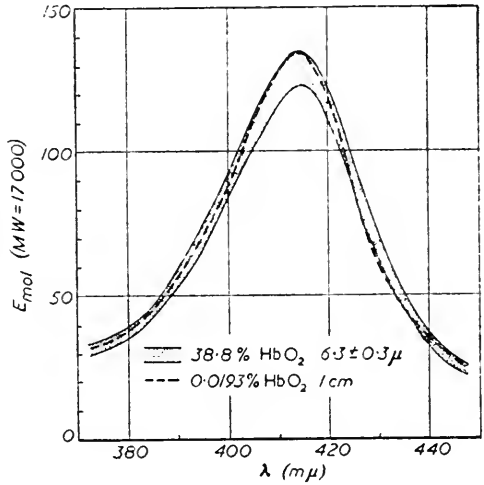


Figure 3. *Spectral absorption of human adult oxyhaemoglobin in the Soret band region, showing adherence to Beer's law from* 0.193 *per cent to* 38.8 *per cent oxyhaemoglobin.*

Within the red blood cell the concentration of the haemoglobin is about 35 per cent, and in extending these spectral studies to intact red cells the spectral absorption of haemoglobin at these high concentrations must be known. DRABKIN and AUSTIN[9] have shown that in its visible spectral absorption HbO_2 obeys Beer's law up to these high concentrations, and I have been able to show that it obeys Beer's law in the Soret band region also in concentrations from 0·019 per cent to 38·8 per cent (*Figure* 3). The high molar extinction of this band requires the use of very thin layers when concentrations as high as 38·8 per cent are used : $E_{(414{\cdot}5\ m\mu)}$ for a 5μ layer of a 38·8 per cent HbO_2 solution is 1·5. Split mica spacers were used between quartz plates to obtain these thin layers, and the large range for the limiting curves of the spectral absorption of the strong solution (*Figure* 3) is due to the uncertainty of measurement of the mica spacer thickness. It must also be remembered that the refractive index changes at the quartz/solution interfaces are somewhat different for the 0·019 per cent

and the 38·8 per cent solutions, especially in the region of an intense absorption band, so that light losses by reflexion are expected to be a little greater with the stronger solution. This adherence to Beer's law at such high protein concentrations is not so surprising when it is remembered that it represents a concentration of the absorbing haem groups of only about 4 per cent, and that they are probably carefully protected from close proximity to one another by their positioning on the protein carrier molecules and by the sheath of bound water which appears to be carried by the whole molecule[10].

It has been found possible to prepare dried uniform films of HbO_2, HbCO, and methaemoglobin about 3–10 μ thick which exhibit these visible and Soret bands, and also the farther ultraviolet bands, at the same wavelengths as do these compounds in solution. Quantitative extinction coefficient data are rather more difficult to interpret owing to the different light losses at the quartz/solution and quartz (or air)/dried protein film interfaces, which cannot be accurately allowed for. With HbO_2 or HbCO the film must not be dried by reduced pressure unless precautions are taken to keep the O_2 or CO pressures above the levels required for keeping the $Hb + O_2 \rightleftharpoons HbO_2$ and the $Hb + CO \rightleftharpoons HbCO$ equilibria almost completely to the right, otherwise the Hb produced may be gradually oxidised to methaemoglobin and in any case a mixed absorption spectrum will result. It is therefore probable that only in the case of methaemoglobin, and possibly of HbCO, that the spectral absorption of the haem protein can easily be studied in the condition of close-packed molecules with the sheath of bound water removed[10]. This bound water must have been removed in the lyophil dried preparations with Fe contents of 0·31–0·33 per cent [11]. It is only in such preparations, with haem contents of about 10 per cent, that the haem groups of adjacent molecules might be brought into sufficiently close proximity for any interaction leading to changes in spectral absorption to be expected, and comparisons of muscle and red cell haemoglobins under these conditions might yield interesting results.

With this background of spectral absorption data on haemoglobins under simple known conditions it is possible to proceed profitably to the study of spectral absorption in intact cell structures containing the pigment. There have been reports that the Soret absorption band at 414·5 mμ is suppressed so as to be undetectable in intact red blood cells[12, 13] and this has been used[14] as a basis for arguing that HbO_2 exists in the red cell in a form chemically different from that in solution, combined for instance with stromatin. D. KEILIN and E. F. HARTREE[15], while confirming the phenomenon of the absence of the Soret absorption band in the spectral absorption of red cell sus-

pensions, suggested that this effect was purely optical. P. A. COLE and F. S. BRACKETT[16] had published data showing two points in the Soret band region of the spectral absorption of a single human red cell, which indicated that the HbO_2 as it existed in the intact red cell was in fact absorbing strongly in this region, and the same is true of the more recent data of B. THORELL[17]. The failure to observe the Soret band in ordinary suspensions of red cells is in fact due to the scattering of light by the cells, and by using different optical systems collecting light over a larger angle E. J. ROBINSON[18] and D. L. RUBINSTEIN and H. M. RAVIKOVICH[19] have shown the presence of a strong Soret absorption band in suspensions of intact red blood cells. In the region of this strong absorption band the cells tend to behave as semi-opaque particles, scattering the light considerably, and sufficient of this scattered light which has not traversed a haemoglobin path reaches the spectrograph split in the usual spectrographic system to obscure completely the absorption in this region from light which has passed through the red cells. Scattering of light tends to increase with the length of the light path[20] and I have reduced this ratio of scattered light to light transmitted by the cells by allowing the red cells to settle on to a horizontal quartz plate, giving a layer of mean thickness about 1·3 red cells, or of the order of 3 μ. Spectrograms taken by the moving plate method[21, 22] with the arrangement shown in *Figure* 4 show that when the cells required for such a layer are distributed before settling throughout a layer of saline 0·5 cm thick no Soret band absorption can be detected (*Figure* 5), but that after settling, which takes about $\frac{1}{2}$ hour, an intense Soret band is observed (*Figure* 6). *Figure* 7 shows a record made from *Figure* 6 on a Siegbahn recording photometer. Examination of these settled layers under a microscope shows that the red cells settle fairly evenly, and spectrograms have also been made from blood smears, both wet and dried.

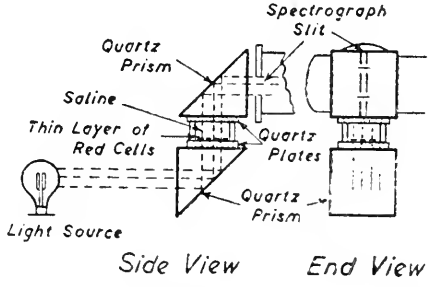


Figure 4. *System for spectrography of settled red cell suspensions.*

The absorption curve of a suspension of human red cells is not quite flat through the Soret band region as illustrated by Keilin and Hartree[15], but shows a slight rise on the long wave side of the Soret

band (*Figure* 8), the observed curve being probably due to the superposition of the variations with wavelength of both absorption and refraction within the red cells, and reflexion and scattering from their surfaces. The slight changes in the curve as a result of alterations in the size and internal haemoglobin concentration of the red cells due to suspending them in varying strengths of saline may be noted (*Figure* 8).

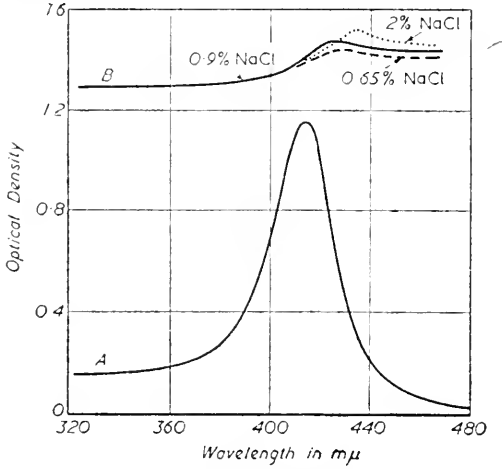


Figure 8. *Spectral absorption of a solution of human haemoglobin of a concentration of* 0·03 *per cent, in* 0·5 *cm. layers* (*Curve A*) *and spectral absorption of human red cells suspended in* 0·65 *per cent,* 0·9 *per cent and* 2 *per cent sodium chloride* (*Curve B*).

Although the wavelength of the Soret absorption peak remains unchanged in solutions up to 38 per cent, and also in the red cells themselves, examination of the spectrograms of these suspensions of red cells obtained using a tungsten ribbon filament lamp as a continuous spectral source shows the presence of an extra absorption band at 378 mμ in human red cells (*Figures* 6 and 7) which cannot be detected in the spectrograms from strong solutions of haemoglobin prepared from these cells (*Figure* 9). This extra band is quite sharp and represents about 2–3 per cent of the Soret band intensity. It has not been possible so far to detect this band in any preparation of red cell ghosts—the insoluble remains after haemolysis—and it was thought to be due to some optical effect such as a reflexion spectrum from the red cell interface. But the presence of this extra band at 378 mμ was confirmed in examining the central region of a single human red cell (see below), and its wavelength could not be shifted by altering the refractive index change at the interface by suspending the cells in different strengths of saline, or even in 60 per cent dextrin. On the other hand it is difficult to see what compound with such an absorption spectrum could be present in red cells in sufficient amounts, and yet remain undetectable after haemolysis. This problem is at present under further investigation.

Figure 5. Spectrogram of a suspension of human red blood cells before settling, showing no Soret band absorption.

Figure 6. Spectrogram of a settled suspension of human red cells in the Soret band region, showing the Soret absorption band at 414·5 mμ and a faint extra band at 378 mμ.

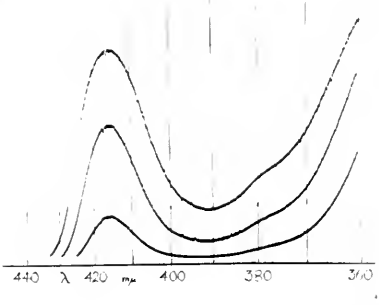


Figure 7. Record from a Siegbahn photometer of a spectrogram of a settled suspension of human red blood cells in a layer 1·3 cells thick.

Figure 9. Spectrogram of a 35 per cent solution of HbO_2 in a 5μ layer, showing Soret band at 414·5 mμ but no band at 378 mμ.

It was clearly desirable to check the validity of the data given by the study of red cell suspensions and layers by extending the study to individual red cells. In the work which has been published up to the present time upon the study of the ultraviolet spectral absorption of individual cell structures by microscopic methods investigation has been concentrated upon absorption intensities at certain wavelengths, the assumption being made that each substance exhibits the same spectral absorption under biological conditions within the cell structure as in simple solution. This was inevitable so long as line sources of light were used to examine the cell structures, giving a limited number of points on the absorption curve, usually insufficient to give absorption peak wavelengths with any accuracy. In order to be able to interpret these intensity data with certainty, however, it is necessary to show for instance that the wavelengths of absorption peaks are the same for substances within the cell structures, approximating to living conditions, as they are in simple solutions. This can only be done satisfactorily using light sources giving a continuous spectrum over the whole wavelength range studied. The method described above using settled suspensions of red cells and a continuous source has given valuable information on the Soret band of red cells, necessary for the interpretation of the microspectrophotometric data on intact cells[17] and has indicated a hitherto unsuspected absorption band at 378 mμ which could not have been detected by the usual techniques for examining intact cell structures[23, 24, 17, 16]. This suspension technique was further extended to the shorter ultraviolet, using a hydrogen discharge tube as a continuous source, with valuable results, showing the absorption band systems of the tryptophan and phenylalanine of the intracellular protein of human red cells, both adult and foetal, to be at the same wavelength position as in simple solution (see below, p. 217 and *Table II*).

In order to check the results indicated by the suspension technique on individual red cells, DR. HOLIDAY and I devised the necessary microspectrographic system for use with a continuous light source. The most convenient method is to illuminate the object in the microscope with a continuous spectrum and place the spectral analyzer *after* the microscope, the image from the latter being focussed on the spectrograph slit. This is the opposite of the usual arrangement as used by T. CASPERSSON and others in which the microscope is illuminated from a monochromator, giving a sequence of monochromatic photographs or photocurrent readings. Caspersson's series of monochromatic micrographs or readings are made one after the other over a considerable period of time, whereas by the technique described here these are recorded at all wavelengths simultaneously, thus giving comparable

pictures of the cell absorption at any one time. Our system, of course, requires microscopic equipment with a considerable degree of achromatism. We have found in practice that it is possible to choose glass condensers and objectives which are sufficiently achromatic over the wavelength region of the Soret band (*e.g.* 370–440 mμ) to give at any rate preliminary results of some value. Further down in the ultraviolet, however, the dispersion of quartz changes much more rapidly with wavelength than does that of glass in this region; even quartz-fluorite 'achromats' have at optimum wavelength a range of only about 15 mμ over which their focussing remains adequately constant [25]. More recently it has been possible to overcome this difficulty by using in collaboration with Dr R. BARER (Department of Human Anatomy, Oxford), a reflecting microscope[26, 27] which is of course completely achromatic, made by Dr C. R. BURCH of Bristol University. This instrument has an aspherical large mirror and spherical small one, and the condenser we used was a replica of the objective : this system gives a numerical aperture of 0·65. The reflecting surfaces were aluminized.

Two complementary systems have been used by us for spectral analysis of the image of the cell structures given by the microscope illuminated from a continuous source. In the first the moving plate method[21, 22] was used to record the spectrogram of a small portion of the image of the cell structure isolated by a short spectrograph slit, a system analogous to the standard spectroscopic eyepiece. By this means we have shown up the Soret band in the cytoplasm of individual human red cells and shown up also the existence of the extra band at 378 mμ observed earlier by the suspension methods. This technique is particularly adapted to the revealing of such finer gradations in spectral absorption[28, 30].

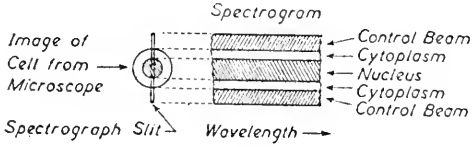


Figure 10. *Diagram of image from microscope projected on to slit of spectrograph and resulting spectrogram.*

In the second system the image from the microscope was again focussed on the spectrograph slit, and as this slit is in focus on the spectrograph plate a spectrogram was obtained of the cross-sections of the cell-structure transmitted by the slit (*Figure* 10). *Figure* 11 shows a spectrogram of a cross-section across a frog red blood cell in the Soret band region, produced by this technique using the reflecting microscope and an overrun tungsten filament lamp : the Soret band

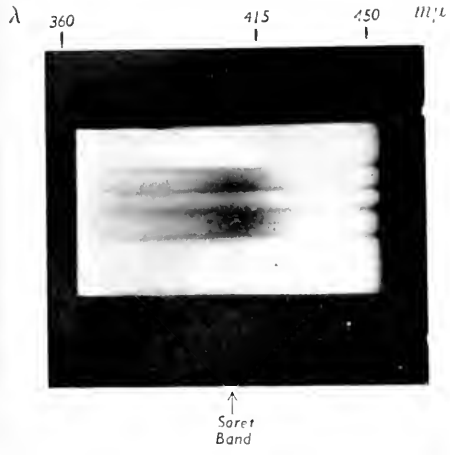


Figure 11. Spectrogram in the Soret band region of a cross section of a frog red blood cell, showing Soret band absorption strong in the cytoplasm and absent in the nucleus.

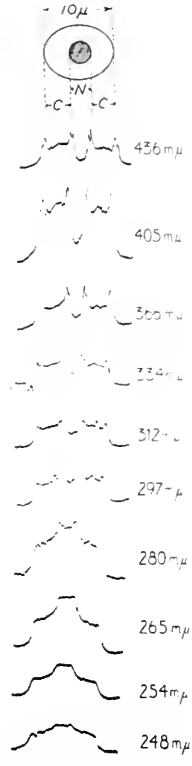


Figure 12. Microphotometer records at various wavelengths across the spectrogram of a frog red blood cell obtained from a reflecting microscope and quartz spectrograph.

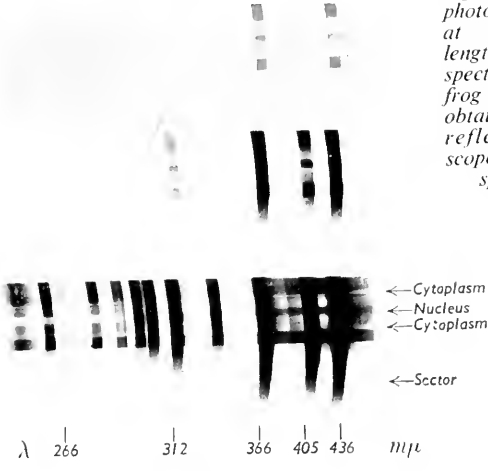


Figure 13. Spectrogram of a frog red cell using a mercury arc (three different exposure times) with a rotating sector in the lower part of the field for density calibration. This spectrogram is a negative, i.e. greater blackening represents greater transmission of the object.

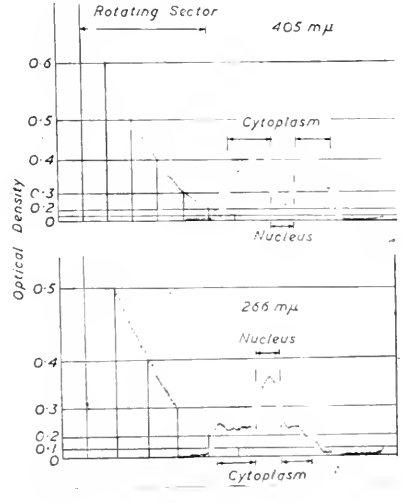


Figure 14. Photometer records across a spectrogram of a frog red blood cell, with rotating sector for density calibration in the lower part of the field.

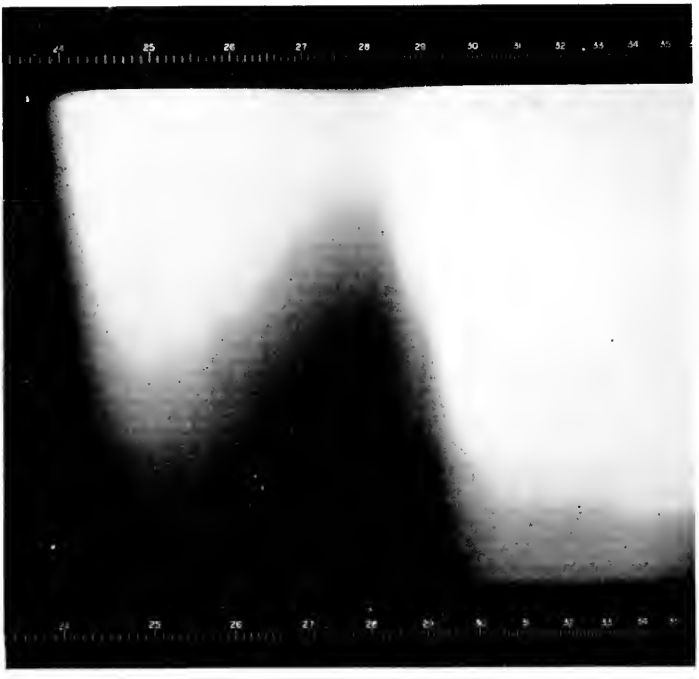


Figure 15. *Spectrogram in the ultraviolet of a preparation of native human globin, showing tryptophan band at* 290 *mμ and the phenylalanine bands in the range* 250–270 *mμ. Hydrogen arc as light source.*

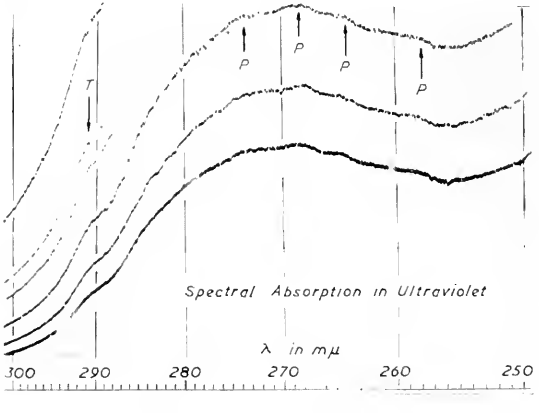


Figure 16. *Photometer record across a spectrogram of a settled suspension of human red blood cells, in the ultraviolet, showing the tryptophan* (*T*) *and phenylalanine* (*P*) *bands. Layer* 1·3 *cells thick.*

absorption can be clearly seen in the cytoplasm and is absent in the nucleus. From these spectrograms horizontal microphotometric plots can be made of density against wavelength for given parts of the cell structure, or a series of vertical plots can be made giving absorption cross-sections of the cell across the line of the slit at chosen wavelengths. *Figure* 12 shows an example of this type of plot, made in this case on a spectrogram of a frog red blood cell using the reflecting microscope and a mercury arc source, the plots being taken at the Hg line wavelengths. Although this is a line spectrum, the lines represent simultaneous exposures of the object. Continuous spectra have to be obtained in the further ultraviolet using a hydrogen arc. In *Figure* 12 the strong absorption of the cytoplasm and low absorption of the nucleus in the Soret band (405 mμ) can be seen. This picture changes completely below about 280 mμ, when the nucleoprotein absorption of the nucleus becomes apparent down to 248 mμ. The cytoplasm absorption remains fairly high in this region owing to absorption by the protein part of the haemoglobin. The peaks at the outer edges of the cytoplasm and at the cytoplasm/nucleus interfaces are due to the total reflexion in these regions[17, 31]. In an attempt to make the density measurements more quantitative a rotating sector has been incorporated into the system giving in the lower half of the control beam an image of the slit of graded blackening representing object optical densities of 0·3 to 2·3 (*Figure* 13). This is then scanned on the same photometer record as the cross-section of the cell structure and can be used to derive the cell densities directly (*Figure* 14). This system has of course rigid requirements in evenness of illumination over the area of field imaged across the spectrograph slit. B. THORELL[17] and P. A. COLE and F. S. BRACKETT[16] use a stepped sector in a similar manner. *Figure* 14 shows the photometer records from a spectrogram such as *Figure* 13 of a frog red cell, at two wavelengths, 405 mμ, where the haemoglobin of the cytoplasm is absorbing, and at 266 mμ where the nucleoprotein of the nucleus is absorbing.

Having dealt with the Soret band absorption of haemoglobins in solution and in red cell structures there remains to be treated the further ultraviolet absorption of the protein, due to the aromatic amino acids, tyrosine, trypophan and phenylalanine. First, by a quantitative study of the spectrum of native globin it has been possible to clarify the confusing tryptophan analyses given in the literature[32]: Haemoglobins contain just over 1 per cent tryptophan, and globins prepared from these appear from many analyses (usually by Voisenet technique) to contain more than 2 per cent of tryptophan. By a spectrophotometric analysis by the method of Holiday[33, 34] on native human and horse globin preparations it has been possible to show

tyrosine/tryptophan ratios of about 3:1, agreeing more closely with some of Block's more recent chemical analyses[32] and also more closely with the usual haemoglobin tyrosine/tryptophan ratio. It would have been difficult to see how the tryptophan could suddenly rise in the preparation of globin from haemoglobin unless the 67,000 unit split into two unequal parts and one only remained in solution. The haem group absorption unfortunately makes such a spectrophotometric analysis difficult on haemoglobins themselves.

The spectral absorption of tryptophan shows a prominent peak at 288 mμ. In some polypeptides, such as gramicidin, this band appears also at 288 mμ, but in most tryptophan-containing proteins, it appears at a considerably longer wavelength, 291 mμ in most cases. The cause and significance of this wavelength shift will be discussed in detail elsewhere by Dr Holiday and myself. Three interesting exceptions concern us here—human foetal haemoglobin, many mammalian muscle haemoglobins and preparations of globins from mammalian red cell haemoglobins. In human foetal haemoglobin this band appears at 289·8 mμ and it is particularly remarkable that other mammalian foetal haemoglobins, sheep and rat, show this band in the usual protein position of 291·0 mμ (*Table II*).

Table II

Tryptophan absorption band wavelengths in haemoglobins (in mμ)

Species	*Adult*		*Foetal and new born*	
	In solution	*In red cell*	*In solution*	*In red cell*
Red Cell Haemoglobins:				
Man	291·0	291·0	289·8	289·8
Sheep	291·0	291·0	291·0	291·0
Rat	291·0	291·0	291·0	291·0
Horse	291·0	291·0	—	—
Ox	291·0	—	—	—
Pig	291·0	—	—	—
Frog	291·0	291·0	—	—
Salamander	291·0	—	—	—
Chicken	291·0	—	—	—
Muscle Haemoglobins:				
Horse heart	290·0	—	—	—
Whale	290·0	—	—	—

This peculiarity of human foetal haemoglobin persists in the intact red cells, as has been shown by the settled suspension method described above. As judged by this band position, a very specific criterion, the haemoglobin at birth in man is largely of foetal type (shown also by crystal form, this volume, p. 269) and it should be possible to obtain some estimate of the survival of the foetal type of haemoglobin after birth, though no very accurate estimates of the proportion of foetal haemoglobin could be made on such a difference, 1·2 mμ. But this small but definite difference between human adult and foetal haemoglobins has the great advantage for cytological research that it is a property observable without disturbing the system chemically, and it should be possible to use the micro-spectroscopic techniques described above to distinguish between adult and foetal haemoglobin within red cell structures, and so learn more of the process of red cell generation in late foetal and early post-natal life.

These results on the tryptophan band wavelength are summarized in *Table II.* They were obtained by the moving plate method, and an example of such a spectrogram is illustrated in *Figure* 15. This is of a native human globin, chosen because it shows clearly the phenylalanine band system as well as the tryptophan band. There appears to be much less phenylalanine in foetal than in adult human haemoglobin. Tyrosine is responsible for much absorption at these wavelengths, but does not show prominent fine structure. *Figure* 16 shows a microphotometric record across a spectrogram such as *Figure* 15, obtained from a settled suspension of adult human red cells : the tryptophan (T) and phenylalanine (P) bands can be seen.

When globins are prepared from haemoglobins by the HCl-acetone method of M. L. ANSON and A. E. MIRSKY[35], the resulting globin shows the tryptophan band at positions varying between about 289 and 290 mμ. After dialyzing this against phosphate *p*H 7·4 buffer the material remaining in solution shows this band nearer 290 mμ, and is 'native' according to the criterion of J. L. CRAMMER and A. NEUBERGER[36], who studied spectroscopically the change in *p*K of the protein tyrosine on denaturation of egg albumin. The product before dialysis contains much material denatured according to this criterion. If the globin soluble at *p*H 7·4 is recombined with haem the product, although native according to the tyrosine *p*K criterion, still gives a tryptophan band at 290 mμ. It also gives Soret bands of the HbO_2 and HbCO (after reduction with ferrous tartrate) respectively at 406 mμ and 416·5 mμ instead of the normal 414·5 mμ and 420 mμ[37]. This recombined haem-globin is not therefore identical with the native haemoglobin from which the globin was prepared, but resembles the second intermediate HbO_2 denaturation state described above. By

fractionation of the globin soluble at *p*H 7·4 with 50 per cent saturated ammonium sulphate the globin remaining soluble shows the tryptophan band still at 290·0 mμ, but on recombination with haem this shifts to 290·6 mμ, much closer to the native haemoglobin wavelength of 291·0 mμ. Moreover the HbCO Soret band from this fractionated recombined material lies at 418 mμ, again closer to the native 420 mμ. But the HbO_2 Soret band appears at 411 mμ, perhaps because of the effect of the ferrous tartrate necessary to produce Hb for combination with O_2. (This product probably resembles the first intermediate denaturation state discussed above, p. 208.) These details supplement those summarized by J. WYMAN[38]. This recombined haemoglobin must then be approaching the original haemoglobin in its properties and it is hoped to study it further. It appears possible to prepare a recombined haem-muscle globin very closely resembling the original muscle haemoglobin[39].

Such methods as those described here and those used by Thorell[17, 31] for working with individual red cells, when based upon an adequate study of the spectral absorption of the compounds involved, seem to show some promise of diagnostic value in haematology as well as illuminating biochemically the inter-relationships of nucleic acid, protein and haem in the natural process of haemoglobin production. The immediate problems are largely technical—the development of sufficiently powerful sources of continuous spectrum in the ultra-violet and the building of more reflecting microscopes of adequate performance. These matters are receiving attention and meanwhile considerable advances can be made with existing resources. A more detailed account of the micro-spectroscopic methods used in the above work (pp. 213, 214) is being published.[40]

I wish to thank the Medical Research Council for a personal grant, Dr E. R. Holiday for continual interest and discussion throughout this work, Mr J. R. P. O'Brien for help in the earlier part of the work, Dr R. Barer for work with the reflecting microscope, Dr Kuhn for aluminizing its surfaces, my wife for discussion and for materials, Mr M. W. Rees and Mr K. Schmid for muscle haemoglobins, the Bernhard Baron Memorial Research Laboratory, Queen Charlotte's Hospital, the Obstetrics Department, the London Hospital, and Professor J. D. Boyd for supplies of human foetal blood, Mr J. C. Kendrew for sheep foetal haemoglobin, Mr E. A. Johnson for help with illustrations, and Mr T. Hudson for technical assistance.

Received October 1948

REFERENCES

[1] SORET, J. L. *Arch. Sci. phys. nat.* 61 (1878) 322
[2] DRABKIN, D. L. *J. biol. Chem.* 146 (1942) 605
[3] GUTFREUND, H. Unpublished work, 1946, quoted in BOYES-WATSON, DAVIDSON and PERUTZ[10]
[4] — This volume, p. 197 and personal communication
[5] DRABKIN, D. L. and AUSTIN, J. H. *J. biol. Chem.* 98 (1932) 719
[6] JOPE, E. M. *Thesis*, Oxford, 1941
[7] HOLDEN, H. F. *Aust. J. exp. Biol. med. Sci.* 14 (1936) 291
[8] — *Ibid.* 15 (1937) 43
[9] DRABKIN, D. L. and AUSTIN, J. H. *J. biol. Chem.* 112 (1935) 67
[10] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. F. *Proc. roy. Soc. A* 191 (1947) 111, 124
[11] JOPE, E. M., JOPE, H. M. and O'BRIEN, J. R. P. *Bio-chem. J.* 37 (1943) ix and unpublished observations
[12] MACALLUM, A. B. and BRADLEY, R. C. *Science* 71 (1930) 341
[13] ADAMS, G. A., BRADLEY, R. C. and MACALLUM, A. B. *Bio-chem. J.* 28 (1934) 482
[14] — *Bio-chem. J.* 32 (1938) 646
[15] KEILIN, D. and HARTREE, E. F. *Nature, Lond.* 148 (1941) 75
[16] COLE, P. A. and BRACKETT, F. S. *Rev. Sci. Instrum.* 11 (1940) 419
[17] THORELL, B. *Acta med. scand.* Suppl. cc. (1947)
[18] ROBINSON, E. J. *Amer. J. Physiol.* 133 (1941) 428
[19] RUBINSTEIN, D. L. and RAVIKOVICH, H. M. *Nature, Lond.* 158 (1946) 952
[20] EVANS, D. S. *Phil. Mag.* Ser. 7 35 (1944) 300
[21] HOLIDAY, E. R. *J. Sci. Instrum.* 14 (1937) 166
[22] — In the press
[23] CASPERSSON, T. *J.R. micr. Soc.* 60 (1940) 8
[24] — *Soc. exp. Biol. Sympos. I* (1947) 127
[25] FOSTER, L. V. and THIEL, E. M. *J. opt. Soc. Amer.* 38 (1948) 689
[26] BURCH, C. R. *Proc. R. phys. Soc. Lond.* 59 (1947) 41
[27] BARER, R. *Brit. Science News* 1 (1948) 11
[28] HOLIDAY, E. R. *Bio-chem. J.* 39 (1945) 1 xv
[29] BERENBLUM, I., HOLIDAY, E. R. and JOPE, E. M. *Brit. Med. Bull.* 4 (1947) 326
[30] HOLIDAY, E. R. and JOPE, E. M. *Cancer Research* 6 (1946) 702
[31] THORELL, B. *Cold Spr. Harb. Monogr.* 12 (1947) 247
[32] BLOCK, R. J. and BOLLING, D. *The Amino Acid Composition of Proteins and Foods* Springfield, Illinois, 1945, pp. 114–5
[33] HOLIDAY, E. R. *Bio-chem. J.* 30 (1936) 1795
[34] — and OGSTON, A. G. *Bio-chem. J.* 32 (1938) 1166
[35] ANSON, M. L. and MIRSKY, A. E. *J. gen. Physiol.* 13 (1930) 469
[36] CRAMMER, J. L. and NEUBERGER, A. *Bio-chem. J.* 37 (1943) 302
[37] JOPE, E. M. Quoted in RIMINGTON and FULTON *Bio-chem. J.* 41 (1947) 619
[38] WYMAN, J. *Advances in Protein Chemistry* 4 (1948) 434
[39] DRABKIN, D. L. *J. biol. Chem.* 158 (1945) 721
[40] BARER, R., HOLIDAY, E. R. and JOPE, E. M. In the press.

VI

BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS

Methaemoglobinaemia

H. BARCROFT, Q. H. GIBSON and D. C. HARRISON

This article contains some account of work done on methaemoglobinaemia in Belfast from 1943 to 1946, and includes a partial review of the literature up to the present. The effect of ascorbic acid in idiopathic methaemoglobinaemia is described and discussed as well as the results of investigations of the enzyme systems in the erythrocytes normally responsible for methaemoglobin reduction.

THE work done in Belfast on methaemoglobinaemia began as the result of a remarkable coincidence arising from some experiments on the effect of ascorbic acid on polycythaemia vera carried out by J. DEENY[1]. These experiments had led Deeny to believe that ascorbic acid was of benefit in this condition and he continued to look out for suitable cases on whom the treatment could be tested. In this way he came across two brothers both of whom were deeply cyanosed and who had abnormally high red cell counts. On treating one of the brothers with large doses of ascorbic acid, he found that in about three weeks the skin colour had gradually changed from a deep slaty-blue almost to normal. As the effect was striking, he brought the brothers to the laboratory in Belfast to supplement his clinical observations with objective evidence of the changes in their blood. There, investigation showed the blood of the brothers to contain methaemoglobin : about 40 per cent of the total pigment in the untreated case, and about 8 per cent in the treated one.

On going into the literature it was found that methaemoglobinaemia is a very rare disease, particularly as a familial condition, and, in fact, no familial cases had been described as occurring in Great Britain. The effect of ascorbic acid, however, had been discovered by C. LIAN, P. FRUMUSAN and M. SASSIER[2], and used by them in the treatment of their two cases. Although it was clear from the literature that the normal mechanisms for the reduction of methaemoglobin (MHb) depended on the enzymes in the red cells and probably on the oxidation of glucose and lactate[3], it was not possible to form a clear picture either of the abnormality leading to the accumulation of MHb in our cases, or of the action of ascorbic acid in improving their condition. Since we had the good fortune to have access to these cases, it seemed worth while to carry out work on some of these problems and attempt to devise a theory fitting the cases into a general picture of methaemoglobinaemia.

As a start, the effect of ascorbic acid on the untreated brother was observed. The clinical aspects have been reported[4]. There was a striking change in skin colour of the ear at various intervals after the beginning of treatment. The principal changes in the blood are summarized in *Figure* 1. These results were discussed by BARCROFT, GIBSON, HARRISON

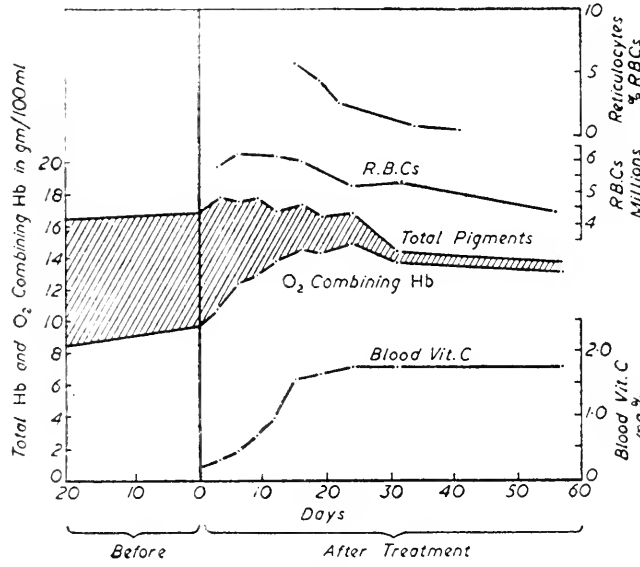


Figure 1.

and MCMURRAY[5]. The decline in MHb concentration in the blood took place comparatively slowly. There was no evidence of increased blood destruction during treatment, and it seemed likely that regeneration of haemoglobin from MHb was taking place.

Next, the reduction of MHb by ascorbic acid was investigated[6]. The rate of reaction varied a good deal in different experiments and was increased catalytically by trace metals in the solutions, as well as by the ferri-ferrocyanide system and by some dyestuffs. The reaction appeared to be bimolecular, and was rather slow even with fairly high concentrations of ascorbic acid. Extrapolation from the *in vitro* results to the concentrations found in the blood of the cases during treatment gave a theoretical rate of MHb disappearance of about the order observed *in vivo*, if it was assumed that dehydroascorbic acid formed during MHb reduction was at once reduced, say, by glutathione, in the red cells.

The comparatively sluggish reaction between MHb and ascorbic acid, as compared with the rapid enzymatic reduction with glucose and lactate reported by Cox and Wendel[3] for dogs, suggested that ascorbic acid does not normally play an important rôle in MHb reduction *in vivo*, and prompted investigations into the enzymes in the red cells of the cases, as well as a more detailed study of the reactions taking place in normal red cells during the reduction of artificially produced MHb.[7] The main conclusions regarding the normal mechanism of reduction are summarized in column A of *Figure* 2. The general idea advanced was that the limiting factor in the reduction of MHb in normal cells is its reaction with coenzyme factor I (diaphorase I), and that triosephosphate and lactate are the principal substrates concerned. It was possible to assay the coenzyme factor I

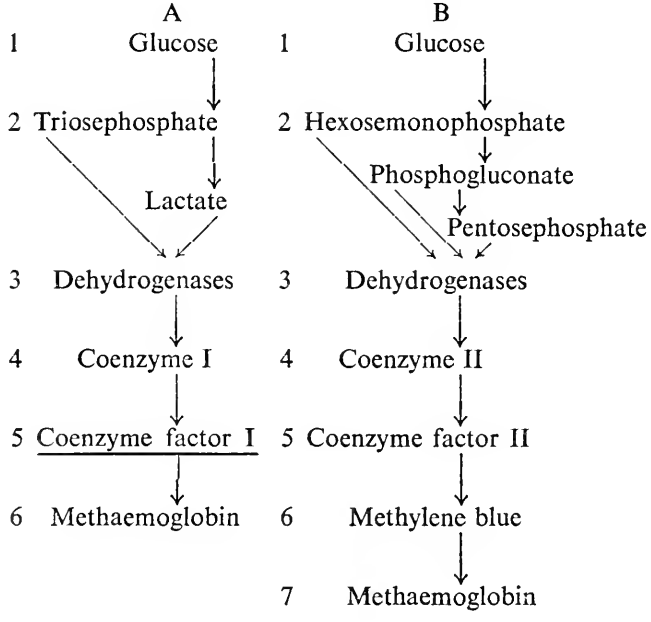


Figure 2. *Diagrammatic scheme of the reactions taking place in the reduction of* MHb *in erythrocytes. Column* A *reactions occurring in the absence of methylene blue ;* B *in the presence of methylene blue. In cases of idiopathic methaemoglobinaemia where there is a deficiency of coenzyme factor I, the reactions in column* A *do not occur, and* MHb *reduction is very slow.*

activity of the erythrocytes and demonstrate a deficiency in the cells from the cases (*Figure* 3). This deficiency appears to explain the

difference in behaviour between the normal and pathological cells in the presence and absence of glucose and lactate (*Figure* 4). The figure shows the effect of adding these substrates to washed MHb-containing red cell suspensions. Whereas the rate with normal cells is increased about fivefold, scarcely any change takes place with cells from the cases.

Two minor points of some interest in connection with the coenzyme factor I activity of the cells are the following. First, in the assay experiments haemolysis increased the apparent activity very substantially, the activity per unit quantity of haemoglobin being about six times the highest activity observed in intact cells. This difference may be due to the close packing of the haemoglobin molecules in the

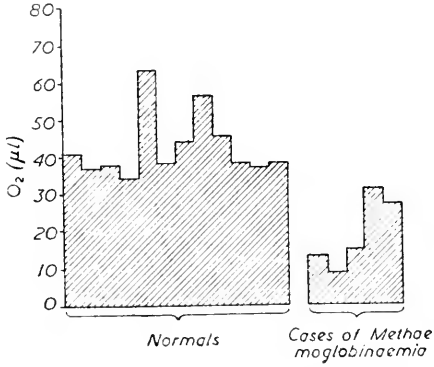


Figure 3. *The hatched rectangles represent the results of assays of coenzyme factor I in the blood of twelve consecutive normal persons and in five cases of methaemoglobinaemia. The last three cases were patients described by Gibson and Harrison* (1947). *The blood of the last two cases contained considerably less* MHb *than that of the first three.*

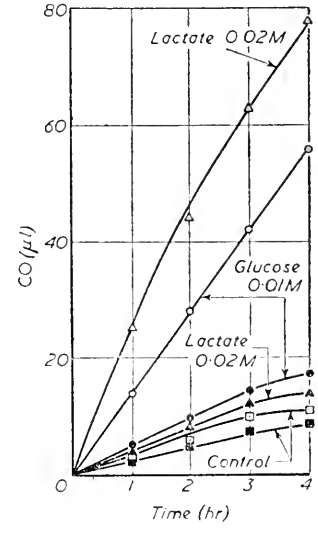


Figure 4. *Reduction of* MHb *by erythrocytes from a normal person and from a case of idiopathic methaemoglobinaemia. The ordinate gives the amount of* MHb *reduced, expressed in μl.* CO *absorbed by* 2·0 *ml of a cell suspension containing* 15·6 *g./*100 *ml total pigments, incubated at* 37°. *The clear symbols refer to the normal, the shaded symbols to the case.*

erythrocytes. Second, F. B. STRAUB[8] has isolated a protein from heart muscle whose chemical behaviour is similar to that of coenzyme factor I[9]. He has shown that the prosthetic group of this protein is flavine-adenine-dinucleotide. One of our patients was given riboflavin in large dosage (20 mg daily for a week) in the hope that synthesis of coezyme factor might be favoured by a high concentration of the vitamin in his blood. The level of MHb in the blood was not influenced by this treatment[10]. The riboflavin was given intramuscularly, so that the possibility of impaired intestinal absorption may be ruled out.

It has been known for some time that certain dyestuffs capable of undergoing reversible oxidation and reduction greatly accelerate the rate of MHb reduction *in vivo*. This effect was also investigated, and it was suggested that in the presence of methylene blue, the sequence of reactions in the breakdown of glucose may follow the scheme of F. DICKENS[11] (see *Figure* 2, column B) and involve coenzyme factor II. The cells from the cases appeared to be quite normal so far as this series of reactions is concerned. It was concluded that coenzyme II dehydrogenases could play only a subsidiary part in the normal mechanism for the reduction of MHb, presumably because coenzyme factor II did not react sufficiently rapidly with it. The observations of M. KIESE and W. SCHWARTZKOPFF[12] on the effect of various combinations of inhibitors on the reduction of MHb in intact red cells suggest that the residual reduction in the absence of added substrate may be due to reactions involving coenzyme II dehydrogenases, whose contribution would then be about one-fifth of that of coenzyme I dehydrogenases. This work and other work by Kiese[13, 14] on the mechansim of MHb reduction in normal cells was carried out in Germany during the recent war and was unknown to the Belfast workers. Where they overlap, the two sets of experimental findings agree very closely. In addition Kiese in detailed studies[13] succeeded in fractionating an enzyme system capable of reducing MHb, and showed that a further factor corresponding to coenzyme factor was required besides the dehydrogenases and pyridine nucleotides.

There are two main ways in which MHb may accumulate in the blood : *a* It may be formed at a rate in excess of the reducing capacity of the normal enzyme systems in the erythrocytes ; *b* there may be some fault in the reducing mechanisms. The first group would include the toxic methaemoglobinaemias such as those arising in the course of treatment with sulphonamides, and also the interesting cases of enterogenous cyanosis described by B. J. STOKVIS[15], A. A. H. VAN DEN BERGH[16] and R. L. M. WALLIS[17]. E. H. FISHBERG[18] has recently described a case of this kind, in which benzoquinoneacetic acid was excreted in the urine. This acid was shown to be capable of forming

MHb *in vitro*. The present cases seem to belong to the second group since there is a definite deficiency in the reducing mechanisms in the erythrocytes. The condition appears to be due to an inborn error of metabolism, probably inherited as a recessive character[5, 19].

If it is supposed that the accumulation of MHb in the blood of these cases is due solely to an enzyme deficiency, then one must also suppose that MHb is constantly being formed in the blood of normal persons. It seemed that measurement of the rate of MHb disappearance from the blood of these cases *in vitro* would give an estimate of the rate of MHb formation *in vivo*, for as the amount of MHb in the blood of the patients was more or less constant, the rates of disappearance and formation must have been equal. The rate of disappearance was of the order of 0·8 per cent of total pigment per hour. The measurements, however, were made under definitely unphysiological conditions, since the cells were suspended in buffered saline, not in serum, and were equilibrated with a gas mixture consisting of 80 per cent N_2 and 20 per cent CO. (The uptake of CO was used as a measure of MHb reduction.) This rate agrees well with the estimate of the activity of the coenzyme II dehydrogenases mentioned earlier, but seems rather high when considering the effect of ascorbic acid, for, smoothing out the curve of MHb disappearance from the blood of the case observed during treatment with ascorbic acid, and calculating on the same basis as before, the greatest effect of treatment is to produce an increased rate of MHb removal of the order of 0·1 per cent of total pigment per hour. It seems rather improbable that so small a relative change in the rate of removal of MHb should produce a fall in equilibrium concentration of MHb from 43 per cent of total pigment to 6 per cent. Certainly it would imply a delicate balance between rates of removal and of formation, since a small percentage change in one relative to the other would lead to large swings in the MHb level in the blood. The rate of MHb disappearance noted above is much higher than that observed by R. F. SIEVERS and J. B. RYON[20] who found no significant decrease in MHb concentration on allowing whole blood from a methaemoglobinaemia patient to stand for 24 hours at room temperature. The conditions in their experiments, however, could not exclude the possibility of methaemoglobin formation continuing *in vitro*. The experiments were carried out in the presence of oxalate, which is known to interfere with glycolysis.[21] Further work is needed to settle this point, which is obviously important in relation to the metabolism of blood pigments in normal persons.

The therapeutic limitation of the effect of ascorbic acid is rather clearly brought out by the calculation of its activity just quoted ; it can be of value only where the enzymatic mechanisms are deficient.

The rate of MHb removal by normal red cell enzymes is of the order of 5 per cent of total pigment per hour, and the additional contribution of 0·1 per cent per hour to be expected from ascorbic acid is negligible. Thus in drug methaemoglobinaemias there is no reason to suppose that ascorbic acid will be beneficial. In a case of enterogenous cyanosis, however, Fishberg[18] has demonstrated a specific effect of ascorbic acid in causing the disappearance of benzoquinoneacetic acid from the urine of her patient and an immediate rise in the O_2 combining power of the blood. In this case the effect of ascorbic acid seems to be linked with its activity as a component of the oxidizing enzyme system responsible for the complete catabolism of tyrosine.

From the point of view of oxygen transport, one would expect to find some evidence of reduced exercise tolerance in the more severe cases of methaemoglobinaemia, but except for the two cases of Lian *et al*[2] described as dyspnoeic and the case of Sievers and Ryon[20], such evidence is notably lacking. K. HITZENBERGER'S[22] case with 40 per cent MHb was a heavy labourer, while the second of the two cases of Deeny *et al*[4] was a member of a hockey team. Gibson and Harrison[19] described two further cases with 25–30 per cent MHb, neither aware of any physical disability and both employed as farm labourers. This finding is the more striking in that the presence of methaemoglobin leads to a shift in the dissociation curve to the left, demonstrated experimentally by R. C. DARLING and F. J. W. ROUGHTON[23], and observed in the naturally occurring condition by Hitzenberger[22] and Gibson and Harrison[19]. Associated with the shift to the left is a change in form of the curve from sigmoid to hyperbolic which theoretically should interfere still further with oxygen transport. There appears to be room for a detailed physiological study of oxygen transport in these cases and of the compensatory mechanisms which are brought into play.

Received September, 1948

REFERENCES

[1] DEENY, J. *Brit. med. J.* 2 (1940) 864
[2] LIAN, C., FRUMUSAN, P. and SASSIER, M. *Bull. Soc. méd. Hóp. Paris.* 55 (1939) 1194
[3] COX, W. W. and WENDEL, W. B. *J. biol. Chem.* 143 (1942) 331
[4] DEENY, J., MURDOCK, E. T. and ROGAN, J. J. *Brit. med. J.* 1 (1943) 721
[5] BARCROFT, H., GIBSON, Q. H., HARRISON, D. C. and MCMURRAY, J. *clin. Sci* 5 (1945) 145

[6] GIBSON, Q. H. *Bio-chem. J.* 37 (1943) 615
[7] — *Bio-chem. J.* 42 (1948) 13
[8] STRAUB, F. B. *Bio-chem. J.* 33 (1939) 787
[9] CORRAN, H. S., GREEN, D. E. and STRAUB, F. B. *Bio-chem. J.* 33 (1939) 793
[10] HARRISON, D. C. Unpublished
[11] DICKENS, F. *Bio-chem. J.* 32 (1938) 1626
[12] KIESE, M. and SCHWARTZKOPFF, W. *Arch. exp. Path. Pharmak.* 204 (1947) 267
[13] — M. *Bio-chem. Z.* 316 (1944) 264
[14] — *Arch. exp. Path. Pharmak.* 204 (1947) 288
[15] STOKVIS, B. J. *Ned. Tijdschr. Geneesk.* 2 (1902) 678
[16] VAN DEN BERGH, A. A. H. *Dtsch. Arch. klin. Med.* 83 (1905) 86
[17] WALLIS, R. L. M. *Quart. J. Med.* 7 (1913) 73
[18] FISHBERG, E. H. *J. biol. Chem.* 172 (1948) 155
[19] GIBSON, Q. H. and HARRISON, D. C. *Lancet* (1947) (2) 941
[20] SIEVERS, R. F. and RYON, J. B. *Arch. intern. Med.* 76 (1946) 299
[21] LOHMANN, K. and MEYERHOF, O. *Biochem. Z.* 273 (1934) 60
[22] HITZENBERGER, K. *Wien. Arch. inn. Med.* 23 (1932) 85
[23] DARLING, R. C. and ROUGHTON, F. J. W. *Amer. J. Physiol.* 137 (1942) 56

Ferrihaemoglobin in Normal Blood

W. N. M. RAMSAY

The paper is a review of published work in the field covered by the title. Possible methods of formation and removal of ferrihaemoglobin in normal erythrocytes are discussed. Gasometric determinations of 'inactive haemoglobin' are described and compared with spectrophotometric determinations of ferrihaemoglobin. The conclusion is reached that at the most only a small proportion of the so-called 'inactive haemoglobin' can be accounted for as ferrihaemoglobin.*

THE extraordinary ability which ferrohaemoglobin possesses, of combining with molecular oxygen to form a covalent compound, is apt to overshadow the fact that ferrohaemoglobin is an oxidizable substance and that oxygen might reasonably be expected to function as an oxidizing agent. This aspect of the reaction was studied some years ago by J. BROOKS[1], [2], who found that the rate of oxidation to ferrihaemoglobin appeared to follow a course which was unimolecular with respect to unoxygenated ferrohaemoglobin. He also found the rate of oxidation to be proportional to a function of the pressure of oxygen, which increases in value as the pressure increases. Since raising the oxygen pressure tends to increase the rate of oxidation (through the oxygen pressure factor), and simultaneously to decrease it (by converting a larger proportion of the other reactant, ferrohaemoglobin, to the apparently inert oxygenated form) it should be possible, by making experiments at different oxygen pressures, to find a point of balance between the opposing tendencies where the rate of oxidation to ferrihaemoglobin is maximal. Brooks found indeed such a point. Under the conditions of his experiments, at *p*H 5·69, it was near 20 mm. Although these experiments were done at a *p*H which hardly falls within the mammalian physiological range, there seems to be no reason to expect that his results would not hold at least qualitatively at *p*H 7·4. If that be granted, it seems clear that the normal erythrocyte contains a mechanism which must tend to produce ferrihaemoglobin, especially on the venous side of the circulation, where there is much unoxygenated ferrohaemoglobin and yet still a quantity of available oxygen.

* The terms ferrihaemoglobin and ferrohaemoglobin are used throughout this paper in place of methaemoglobin and haemoglobin[6].

The coin, however, has tails as well as heads. When ferrihaemoglobin does occur in red cells, as under pathological and experimental conditions, it has been repeatedly shown to be rapidly removed. As long ago as 1930 O. WARBURG, F. KUBOWITZ and W. CHRISTIAN[3] showed that ferrihaemoglobin can be reduced by an enzyme system in red cells which oxidizes glucose to gluconic acid. Although it may be objected that this is a reaction which seems to have no great physiological significance, the same criticism cannot be levelled at the recent work of Q. H. GIBSON[4], who has made a detailed study of the reduction of ferrihaemoglobin generated in red cells by the action of amyl nitrite in phosphate-saline solution. He has found that the reduction is brought about by the triose-phosphate and lactate dehydrogenating systems. The rate of removal of ferrihaemoglobin from the blood of experimental animals was investigated by W. W. COX and W. B. WENDEL[5]. They induced ferrihaemoglobinaemia in dogs, to the extent of 25–60 per cent of the total blood pigment, by the injection of nitrobenzene, aniline, acetanilide and a number of other compounds. The rate of disappearance of ferrihaemoglobin was independent of the causal agent, and was the same in a test-tube as in the living animal. It was, however, influenced by temperature, and while at 38°C the rate of reduction corresponded to 11 per cent of the total pigment per hour, at 20°C it was only about one-quarter of this. The fact that the rate was constant and independent of the concentration of ferrihaemoglobin in each particular experiment seems to indicate that it was limited by the amount of some other necessary factor throughout the range of ferrihaemoglobin concentrations studied.

It is thus clear that red cells are amply provided with mechanisms for the reduction of ferrihaemoglobin as well as for its formation by the oxidation of ferrohaemoglobin. The accumulation of the ferric compound in the blood of experimental animals or pathological subjects may be due either to an increased rate of oxidation or to a decreased rate of reduction, so that the state of balance between the two tendencies in the normal erythrocyte assumes an importance which, as far as the author is aware, has not previously been emphasized.

Until fairly recently the assumption that the whole of the blood haemoglobin was normally in the ferrous state was implicit, if not actually stated. In recent years, however, several independent lines of work have suggested that this belief may not be wholly accurate. For example, T. G. KLUMPP[7], who does not seem to have been directly interested in ferrihaemoglobinaemia and made no pronouncement on this aspect of the subject, published a careful comparison of

the total iron of blood with its carbon monoxide capacity, which he called ' oxygen capacity determined by the carbon monoxide method '. He used an accurate volumetric method for his iron determinations and the usual Van Slyke technique, which is well known to give highly reproducible results, for his carbon monoxide estimations. He found that the carbon monoxide capacity of his specimens was usually 2–5 per cent less than that calculated on the assumption that the whole of the blood iron was present as ferrohaemoglobin. This not only suggests the inaccuracy of such an assumption, but at the same time imposes a maximum concentration for ferrihaemoglobin, which in any given specimen could not be greater than the discrepancy between the carbon monoxide capacity and the iron content. Other evidence leading to a similar conclusion is contained in the introduction to the paper of Cox and Wendel[5], who stated that ferrihaemoglobin was not demonstrable spectroscopically (by means of the absorption band of acid ferrihaemoglobin at 631 mμ) in normal blood specimens from nearly a dozen species. The method used was devised by Wendel[5], and will detect ferrihaemoglobin in a mixture with oxyhaemoglobin when the mixture contains only 4 per cent of the former pigment. This again indicates that there cannot be more than 4 per cent ferrihaemoglobin present in the total pigment of normal blood, so that the whole question turns out to be one of rather delicate analysis. In fact, the present author feels that the critical investigation of the analytical methods involved may well prove in the end to be the most important aspect of the whole matter.

More specific evidence has been provided by making use of the fact that although ferrihaemoglobin does not combine with oxygen or carbon monoxide, reducing agents such as $Na_2S_2O_4$ or alkaline solutions prepared from $TiCl_3$ will readily convert it to ferrohaemoglobin, which does combine with these gases. D. D. VAN SLYKE and A. HILLER[9] described a method for the determination of ferrihaemoglobin as the difference in the carbon monoxide capacities before and after reduction with $Na_2S_2O_4$; some years later this technique was applied by E. AMMUNDSEN[10] to the examination of 82 normal blood specimens, with the interesting result that in 40 per cent of these there appeared to be a significant difference between the two carbon monoxide capacities. Occasionally reduction caused an increase of as much as 2·5 vols CO per cent, or 12–15 per cent of the total pigment, but much more commonly the increase was less, only about 0·5–1 vol per cent. The author discussed the possibility that the difference might be due to the presence of ferrihaemoglobin, but preferred, in the absence of further evidence, to use the phrase ' inactive haemoglobin ', since it was possible that some compounds other than

ferrihaemoglobin might be measured by this method. This work was repeated and greatly extended by F. J. W. ROUGHTON, R. C. DARLING and W. S. ROOT[11]. The amounts of ' inactive haemoglobin ' which they found were not generally as large as those noted by Ammundsen, but a most interesting additional observation was made : that if the bloods were allowed to stand in the laboratory, the inactive haemoglobin disappeared ; that is to say, the carbon monoxide capacity gradually increased until it attained the value initially observed only after reduction with hydrosulphite. The oxygen capacity was also observed to increase in blood specimens which were allowed to stand in air, but these authors did not make any estimates of the oxygen capacities after reduction.

An unfortunate defect of the carbon monoxide method, at any rate in theory, is its lack of specificity. Carbon monoxide will combine not only with ferrohaemoglobin, but also with sulphhaemoglobin, choleglobin and several ferrohaemochromogens. A more specific technique, however, has been a possibility since J. B. CONANT, N. D. SCOTT and W. F. DOUGLASS[12] discovered that ferrihaemoglobin could be estimated from the increase in oxygen capacity resulting from reduction with an alkaline titanous tartrate solution. The original method required relatively large volumes of blood and was tedious to operate, but the present author was fortunate in being able to simplify the technique without sacrificing accuracy[13]. The modified method was applied to the analysis of 38 normal human blood specimens, and significant differences were found between the apparent oxygen capacities before and after reduction in 21 of these. The author then believed it likely that the differences were due to the presence of ferrihaemoglobin. The largest difference noted amounted to 7 per cent of the total pigment present, but most of the values lay between 1·5 per cent and 3 per cent. A number of differences of the order of 1 per cent were observed, but these were not considered at that time to be large enough to be statistically significant. Allowing the specimens to stand in the laboratory caused an increase in apparent oxygen capacity up to the value initially obtained after reduction (*cf.* Roughton *et al.*[11]), and in a later paper on horse and sheep bloods[14] a close correspondence was noted between the oxygen capacity after reduction with titanium and the total iron concentration. This last observation demonstrates the absence from normal bloods of haemochromogens and other haemoglobinoid pigments which do not combine with oxygen even after reduction, and hence justifies the use of the carbon monoxide technique employed by the other workers.

The accuracy of all gasometric methods depends on several factors, among which we may mention the technique for the determination

of the quantity of gas, the technique used for the saturation of the pigment solution with the gas, and the fact that the system under investigation must be presumed to be stable during the time normally allowed for the experiment. The precision of the actual Van Slyke analysis is beyond question, but the mere fact that both oxygen and carbon monoxide capacity tend to increase in drawn blood exposed to the atmosphere throws doubt on the validity of the earlier stages of gasometric determinations of ferrihaemoglobin. The complexity of the matter is indicated by some experiments carried out by the author[14], which show that although fresh blood may, under certain experimental conditions, take up to an hour to attain an apparently maximal oxygen capacity, the same blood, if deoxygenated *in vacuo* immediately after oxygenation, may be reoxygenated in a few minutes. This effect cannot be due to the presence of carbon dioxide, since evacuation of fresh, unoxygenated blood does not result in an increased rate of oxygen uptake. Treatment of the fresh blood with a reducing agent does, however, change the system in such a way that the pigment takes up its maximum quota of oxygen from air in a few minutes. These experiments were done with horse blood, which has such a high sedimentation rate that the technique of the rotating tonometer cannot be used for saturating it with oxygen. The apparatus which was used is probably less efficient than the usual one, and it seems possible that most of these effects might be masked by the use of a more efficient oxygenator. Conant, Scott and Douglass[12], however, found it necessary to aerate blood in a tonometer for only five minutes after reduction with titanous tartrate. Other interesting observations have been made on the carbon monoxide technique by S. KALLNER[15], who repeated successfully the experiments of Ammundsen, using as she did a CO pressure equivalent to about 25 mm Hg, in accordance with the recommendation of Van Slyke and Hiller[9]. He noted also the disappearance of the difference between the CO capacities before and after reduction as the blood stood in air, and then repeated the whole series of experiments, using 150 mm CO for the saturation instead of 25 mm. This time the initial CO capacity was equal to the value formerly obtained only after reduction, and neither reduction nor exposure to the atmosphere caused any further increase. Kallner himself, without further evidence, attributed the results to the fact that fresh blood contained CO_2, which in some way inhibited the uptake of CO, whereas the blood which was allowed to stand had lost its CO_2. Although the verification of this theory, or its replacement, if necessary, by some alternative must await the results of further investigation, it does seem that we do not yet know sufficient about the uppermost regions of the equilibria between ferrohaemoglobin

and oxygen or carbon monoxide to justify the application of the gasometric methods to the determination, or even the detection, of such small proportions of ferrihaemoglobin as may reasonably be expected to occur in normal blood.

It is interesting, then, to turn to analytical methods which are based on different properties of ferrihaemoglobin: the spectrophotometric methods. It is true that these methods demand that the solutions used for the evaluation of the original spectrophotometric constants must have the requisite degree of purity, which may be difficult to ensure, but as the actual analysis may not require any further chemical action on the specimen, or at worst only reactions which proceed rapidly to completion, the results are perhaps easier to interpret than those of gasometric analyses. Several methods have been based on the fact that the high extinction coefficient of ferrihaemoglobin in the region of 631 mμ is changed to a very low one by the addition of KCN (see, for example, K. A. EVELYN and H. T. MALLOY[16]; H. O. MICHEL and J. S. HARRIS[17]). W. D. PAUL and C. R. KEMP[18] have applied the method of Michel and Harris to blood samples from 20 blood donors, all of which appeared to contain ferrihaemoglobin, although the quantities found ranged only from 0·2 per cent to 0·9 per cent of the total pigment present. Other analysts, such as for example Q. H. GIBSON and D. C. HARRISON[19] have reported similar results, which stand in marked contrast to those of the gasometric experiments. Although it is true that contamination of the original oxyhaemoglobin standard with ferrihaemoglobin would lead in the end to low results, and furthermore that many of the methods used have been based on constants published by one set of workers[20, 21, 22], so that any error in the original would be repeated through all the applications, the original figures show great consistency with a number of different pigment preparations. This in itself makes it unlikely that the oxyhaemoglobin preparations were appreciably contaminated with ferrihaemoglobin. B. L. HORECKER[23], who gives similar but not identical figures, points out that different instruments may have different effective widths of slit, and such differences will undoubtedly be reflected in the results obtained. Sufficient attention has perhaps not always been paid to this point, but the good agreement of results from different laboratories suggests that in this connection, at any rate, it has not been important.

Spectrophotometric evidence of a rather different kind has been given by D. L. DRABKIN and C. F. SCHMIDT[24], who made examinations of arterial blood in dogs and men. They used a special cuvette only 0·07 mm in depth, and were thus able to make spectrophotometric observations on undiluted specimens within a few seconds of

the moment when the blood left the living circulation. In fact, for certain purposes they were able actually to include the cuvette in the circulation of the living, nembutalized dog. Total pigment was determined in three different ways : as ferrihaemoglobin cyanide, after treatment with ferricyanide and KCN ; as oxyhaemoglobin after saturation with oxygen at one atmosphere pressure ; and as ferrohaemoglobin after reduction. If the original specimens had contained ferrihaemoglobin, the determination as oxyhaemoglobin would have been lower than the other two, while these should have given identical results. In point of fact the three values agreed within 0·5 per cent in all three of the cases published in detail. This indicated that none of these specimens could have contained as much as 0·5 per cent of the total pigment in the ferric form. It is a pity that more figures are not available by this technique, which might indicate a real, but small difference between arterial and venous blood. It would be unwise at present to suggest any such difference, especially as in all cases the analytical methods are stretched very near their limits.

The work so far described suggests strongly that although there may be traces of ferrihaemoglobin in normal blood, the gasometric methods give results which, for reasons which are not yet altogether clear, are usually much too high ; and this in spite of the fact that the complete gasometric analysis consumes an appreciable time, during which ferrihaemoglobin will tend to disappear. In 1946 an invaluable comparison of the gasometric method and a spectrophotometric method was published by Van Slyke, Hiller, Weissiger and Cruz[25]. The gasometric technique employed was a modification of earlier techniques designed to eliminate as many potential sources of error as possible. The pressure of CO used for all saturations was of the order of 150 mm and it was found possible to reduce the time required for saturation to as little as 1·5 to 2 minutes. The spectrophotometric method used was that of Horecker and Brackett[26], in which ferrihaemoglobin is determined from the fall in extinction coefficient at 800 mμ which results from the addition of KCN. The most stringent precautions against analytical errors seem to have been taken, and the two methods were compared in 19 cases. In all of these the gasometric method detected some ' inactive haemoglobin ', usually of the order of 1 per cent of the total pigment. In almost all the cases the spectrophotometrically determined ferrihaemoglobin was much lower than this (the mean was 0·4 per cent of the total pigment), and in several it was not possible to detect any. Previous observations, such as the gradual disappearance of ' inactive haemoglobin ' from drawn blood, were confirmed, but although the CO pressure was as high as that used by Kallner[15], this did not eliminate entirely the ' inactive

haemoglobin ', as it did in Kallner's experiments. The experimental error of the latter author, however, seems to have been a little higher than that of Van Slyke *et al*,[25] and indeed was of much the same order as the amounts of ' inactive haemoglobin ' detected in the more recent work.

It seems almost beyond doubt that many normal blood specimens do contain minute traces of ferrihaemoglobin. This, however, does not show the tendency to disappear that one would expect from the work of Gibson[4], and the possibility does not yet seem to have been excluded that it may be an artefact arising as a result of dilution or lysis of the cells. It seems also quite certain that the greater part of the so-called ' inactive haemoglobin ' detected by the gasometric methods is not ferrihaemoglobin.

In conclusion, it may not be amiss to point out that the work reviewed in this paper bears directly on a physiological topic of no small importance which was the subject of much pioneering research by the late Sir Joseph Barcroft : the percentage saturation of the arterial haemoglobin with oxygen, and the relation of this to the tension of oxygen in arterial blood. Certain work suggested that arterial oxygen tension was as much as 20–30 mm (for references, see article by D. L. Drabkin in this book) lower than would be expected on physical and chemical grounds, and Roughton, Darling and Root[11] seem to have been the first to point out that the presence of small amounts of inactive haemoglobin in fresh blood, which disappeared during the course of *in vitro* experimentation, would go far to account for the discrepancy.

Received August 1948

REFERENCES

[1] Brooks, J. *Proc. roy. Soc. B.* 109 (1931) 35
[2] — *Ibid* 118 (1935) 560
[3] Warburg, O., Kubowitz, F. and Christian, W. *Biochem. Z.* 221 (1930) 494, 227 (1930) 245
[4] Gibson, Q. H. *Bio-chem. J.* 42 (1948) 13
[5] Cox, W. W. and Wendell, W. B. *J. biol. Chem.* 143 (1942) 331
[6] Coryell, C. B., Slott, F. and Pauling, L. *J. Amer. Chem. Soc.* 59 (1937) 633
[7] Klumpp, T. G. *J. clin. Invest.* 14 (1935) 351
[8] Wendel, W. B. *J. clin. Med.* 24 (1938) 96
[9] Van Slyke, D. D. and Hiller, A. *J. biol. Chem.* 78 (1928) 807
[10] Ammundsen, E. *J. biol. Chem.* 138 (1939) 563
[11] Roughton, F. J. W., Darling, R. C. and Root, W. S. *Amer. J. Physiol.* 142 (1944) 708
[12] Conant, J. B., Scott, N. D. and Douglass, W. F. *J. biol. Chem.* 76 (1928) 223
[13] Ramsay, W. N. M. *Bio-chem. J.* 38 (1944) 470
[14] — *Ibid.* 40 (1946) 286
[15] Kallner, S. *J. Physiol.* 104 (1945) 6

[16] EVELYN, K. A. and MALLOY, H. T. *J. biol. Chem.* 126 (1938) 655
[17] MICHEL, H. O. and HARRIS, J. S. *J. Lab. clin. Med.* 25 (1940) 445
[18] PAUL, W. D. and KEMP, C. R. *Proc. Soc. exp. Biol. Med. N.Y.* 56 (1944) 55
[19] GIBSON, Q. H. and HARRISON, D. C. *Bio-chem. J.* 39 (1945) 490
[20] AUSTIN, J. H. and DRABKIN, D. L. *J. biol. Chem.* 112 (1935) 67
[21] DRABKIN, D. L. and AUSTIN, J. H. *Ibid.* 112 (1935) 51
[22] — — *Ibid.* 112 (1935) 105
[23] HORECKER, B. L. *J. biol. Chem.* 148 (1943) 173
[24] DRABKIN, D. L. and SCHMIDT, C. F. *J. biol. Chem.* 157 (1945) 69
[25] VAN SLYKE, D. D., HILLER, A., WEISSIGER, J. R. and CRUZ, W. O. *J. biol. Chem.* 166 (1946) 121
[26] HORECKER, B. L. and BRACKETT, F. S. *J. biol. Chem.* 152 (1944) 669

The Biosynthesis of Haem

C. RIMINGTON

The problem of the chemical mechanism by which the haem pigments, so important in the life of the cell, are synthesized presents a challenge to the biochemist. Experience shows that the organism can readily and rapidly synthesize the haem nucleus but until recently nothing was known concerning the intermediate steps involved ; even the intervention of protoporphyrin at the penultimate stage is conjectural.

In spite of the chemical possibility of different position isomers of porphin derivatives, all natural ferriprotoporphyrins belong to the isomeric series derivable from aetioporphyrin III. Nevertheless, series I porphyrins occur naturally in excreta and, in much larger quantities, in certain pathological conditions having a genetical basis. Numerous attempts have been made to represent the biosynthesis of haems from simple pyrrole derivatives but each encounters the difficulty of explaining the predominance of one position isomer. To overcome this difficulty, Rimington postulated an enzymic theory.

The various suggested mechanisms of haem synthesis are briefly reviewed and the modern evidence discussed which is based on the use of isotopically labelled glycine and acetate. Both these materials appear to be building stones for the porphyrin ring of haem. Experiments by a group of workers, still in progress, on pigment synthesis by C. diphtheriae *are reported and whilst the intracellular haems of this organism appear to be built up from glycine (labelled N), the coproporphyrin III produced in the medium would not appear to be derived from this precursor as the isotopic N abundance is considerably lower even than that of the total cell mass. It is emphasized that proto- and copro- porphyrins may well derive from different synthetic processes as even the interconvertibility of these pigments is held in serious doubt.*

No greater challenge to the biochemist exists than the problem of the biosynthesis of haemoglobin and of the haem systems of the cell. The latter are of essential importance for the life of nearly every type of cell while haemoglobin has been the subject of intense study and is indispensable to the vertebrates.

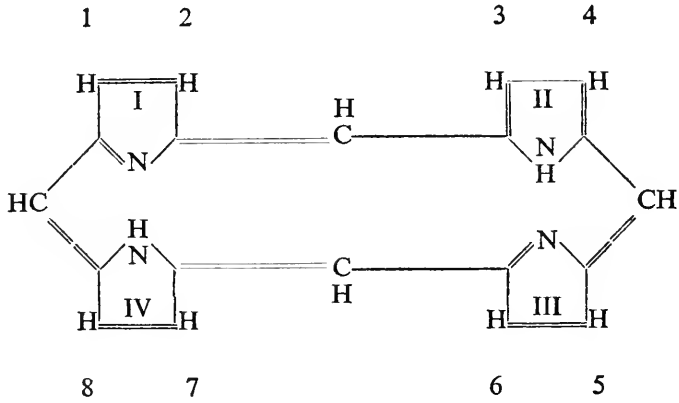
Until very recently all that could be said about the steps by which haemoglobin was synthesized was that protoporphyrin was probably

a precursor of haematin. It was found to be present in bone marrow, reticulocytes, the developing chicken's egg, *etc.*

All experience has gone to show that the organism can readily and rapidly synthesize the porphyrin nucleus of haemoglobin. There is no conservation of its constituents as there is of iron.

Little information has been gained from the study of excretion products ; in fact, the recognition of two isomeric series of porphyrin derivatives as a result of Fischer's work has only made the problem more difficult.

All porphyrins may be regarded as derivable from porphin, acid or alkyl groups taking the place of any or all of the 8 hydrogen atoms in the β positions of the pyrrole rings. Fischer's formula for porphin together with a key indicating the constitution of the more important porphyrins is reproduced below.



Porphyrin	*Positions of substituent groups*			
	·CH_3	·$CH{=}CH_2$	·$CH_2{\cdot}CH_2{\cdot}COOH$	·$CH_2{\cdot}COOH$
Protoporphyrin IX	1, 3, 5, 8	2, 4	6, 7	
Coproporphyrin I	1, 3, 5, 7		2, 4, 6, 8	
,, III	1, 3, 5, 8		2, 4, 6, 7	
Uroporphyrin I			2, 4, 6, 8	1, 3, 5, 7
,, III			2, 4, 6, 7	1, 3, 5, 8

In normal urinary porphyrin both coproporphyrins I and III are present but no haem or natural prosthetic group has been discovered belonging to the I series. All natural ferriprotoporphyrin derivatives

are of protoporphyrin IX (isomeric series III). On the supposition that the porphyrin ring is built up from pre-fabricated pyrrole units, containing substituent groups in the β-positions, all four position isomers would be chemically and biologically possible but no representatives of isomers II and IV have ever been found in nature.

That there does exist a relationship between coproporphyrin production and the synthetic events of erythropoiesis seems to follow from the finding of increased urinary coproporphyrin excretion by animals during regeneration following blood withdrawal. There is no evidence of the normal degradation of haemoglobin passing through a porphyrin stage.

In order to explain the excretion of coproporphyrin I and of its increase during erythropoietic activity, I postulated in 1938[1] an enzymic system in the bone marrow A $\begin{matrix} \nearrow \text{P III} \\ \searrow \text{P I} \end{matrix}$ which visualized the possible synthesis from precursor materials 'A' of porphyrins belonging to either isomeric series (P III and P I) but suggested that the events leading to one of these products were specifically accelerated thus making that isomer the main and the other the by-product.

But we must approach more closely to the mechanism of synthesis and the sequence of chemical events. Some possible systems for such studies may be arranged as follows :

Bone marrow in intact animal.
Bacteria, especially *C. diphtheriae*.
Yeast.
Yeast press juice.
Porphobilinogen-containing porphyria urines.
Maturing reticulocytes (Watson).
Nucleated erythrocytes.

Before dealing with the uses to which these systems have been put and, in particular, with the latest contributions based on the use of isotopes, I will summarize briefly the various theoretical speculations which have been put forward concerning the building up of the porphin ring.

The earliest serious suggestion which I have been able to trace is in a paper[2] by Sir Robert Robinson in the *Proceedings of the University of Durham Philosophical Society* for 1928. Although based upon the old Küster formula for haematin, this scheme is of historical interest. Denoting $-CH_3$ by Me and $-CH_2{\cdot}CH_2{\cdot}COOH$ by S, condensation is envisaged between two aliphatic chains followed by combination with ammonia and further elimination of water as cyclization occurs

(see *Figure* 1). Although no experimental support has been adduced in favour of this scheme, it remains an attractive piece of chemical speculation.

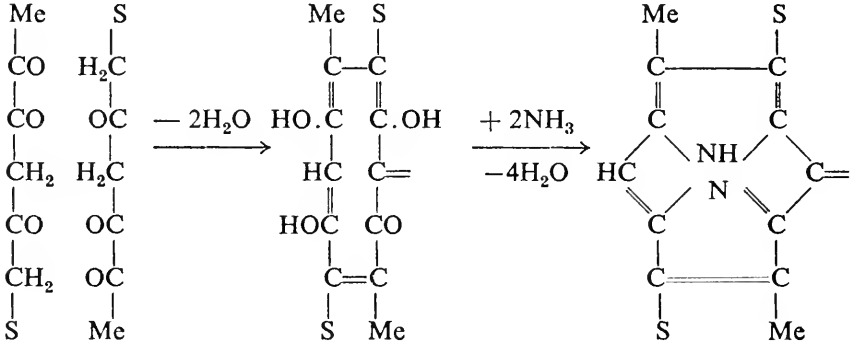


Figure 1. Suggested biosynthesis of haem pigments[2]

Other authors have generally assumed that pyrrolic precursors or building stones are first built up from some such likely raw material as proline or pyrrolidone carboxylic acid and may have postulated certain arrangements of combination in order to explain the appearance in nature of the I and III series but not the II or IV series of pigments. Efforts to explain the non-appearance of the latter on structural grounds lead into bewildering complexity without any really acceptable solution.

K. DOBRINER and C. P. RHOADS[3] pictured synthesis of the porphyrin ring from dipyrromethenes *A* and *B* together with formaldehyde (*Figure* 2) but even so, were unable to explain the non-appearance of the theoretically possible type II isomer.

A somewhat different scheme was proposed by W. J. TURNER[4] in 1940 on the basis of the study by A. H. CORWIN and J. S. ANDREWS[5, 6] of the aldehyde synthesis of dipyrrylmethenes. These authors showed that a tripyrrylmethene is almost certainly formed as an intermediate but that by fission, which could occur in either of two ways, dipyrrylmethenes are the final result. Turner visualized a possible porphyrin synthesis as shown in *Figure* 3. A fact of much significance relative to this hypothesis is the known occurrence of a compound of similar structure—the bacterial pigment prodigiosin (*Figure* 4). Nevertheless Turner's hypothesis would again imply the possible synthesis of porphyrins of isomeric type II. The production of the simple pyrrole units might occur, according to Turner, by degradation of tryptophan (*Figure* 5).

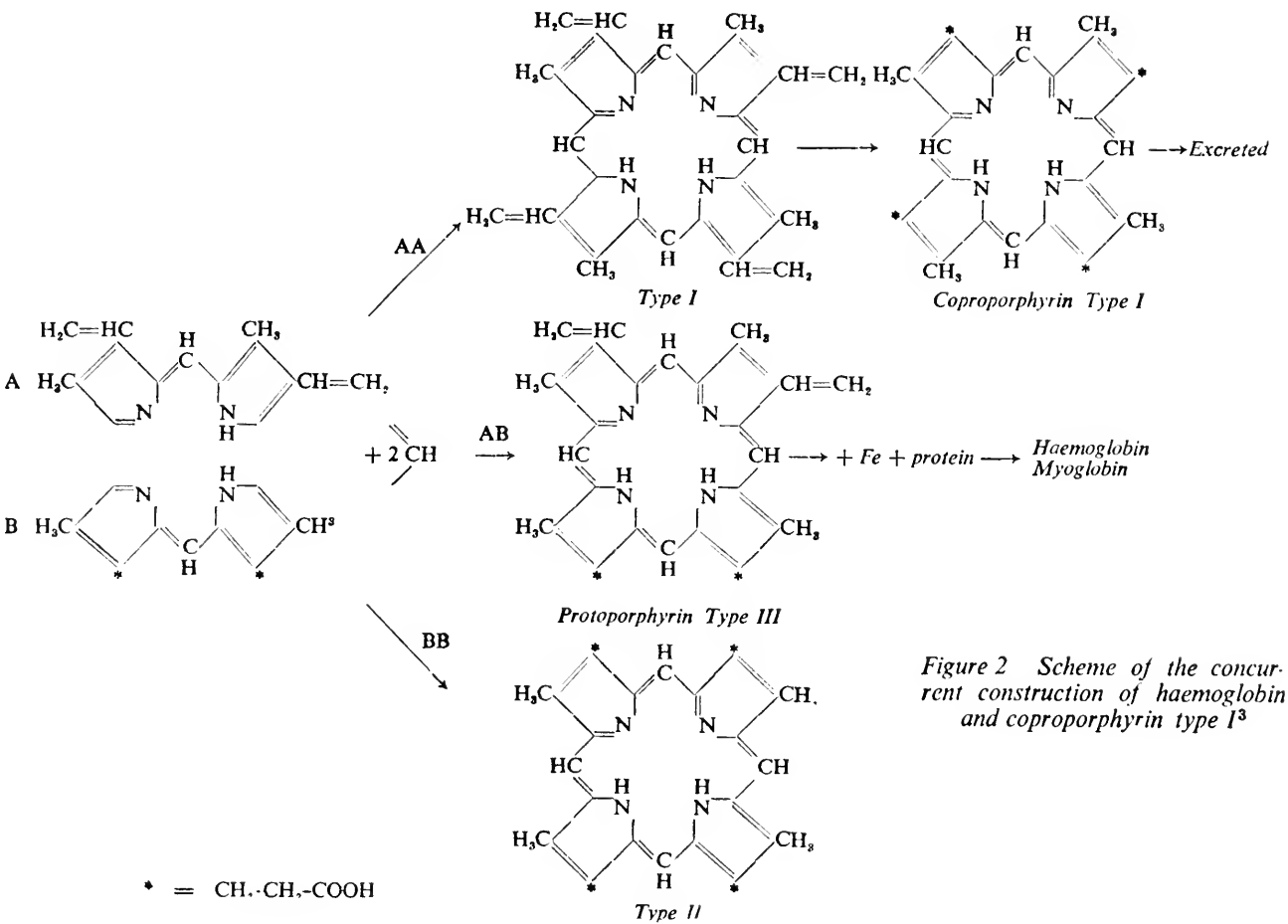


Figure 2 Scheme of the concurrent construction of haemoglobin and coproporphyrin type I[3]

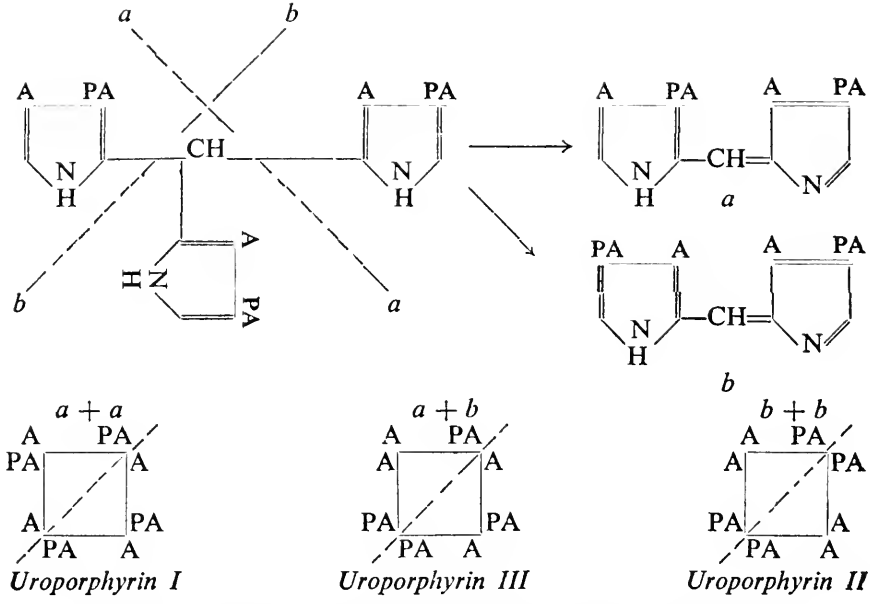


Figure 3. Scheme for the biosynthetic production of porphyrin isomers[4]

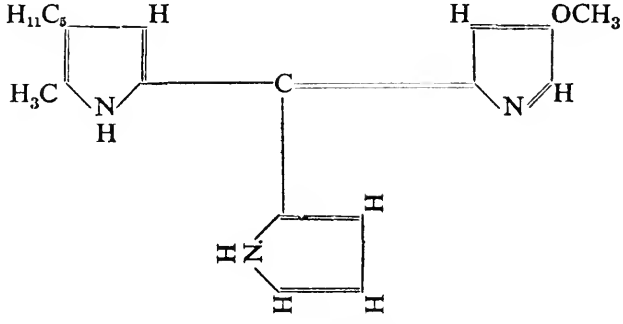


Figure 4. Prodigiosin

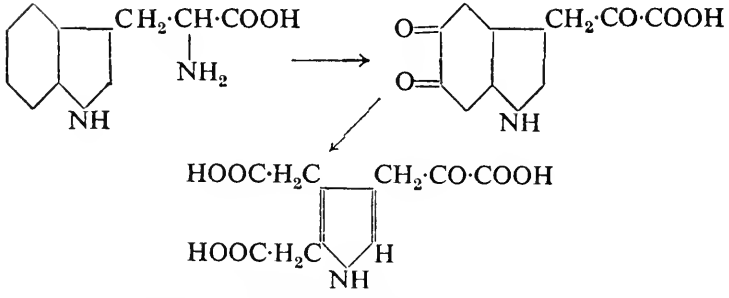
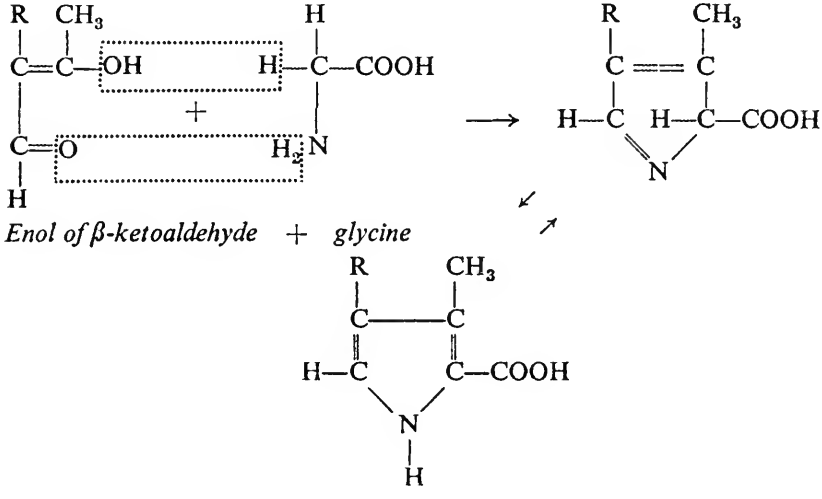


Figure 5. Suggested origin of pyrrole units[4]

At this point chemical theorizing stopped. The first decisive experimental evidence concerning the building stones of the porphyrin ring came from D. SHEMIN and D. RITTENBERG[7] who found that in man, after a large quantity of N_{15} labelled glycine had been taken, the N_{15} abundance in the haemin isolated from the blood was sufficiently great to denote a specific utilization of glycine in the synthesis. The same school found that deuterium of deuteroacetate also appeared in the blood haemin, presumably in the side-chains. More recently still[8] they have utilized the nucleated erythrocytes of the duck's blood and shown that N_{15} glycine is incorporated into haemin *in vitro*.

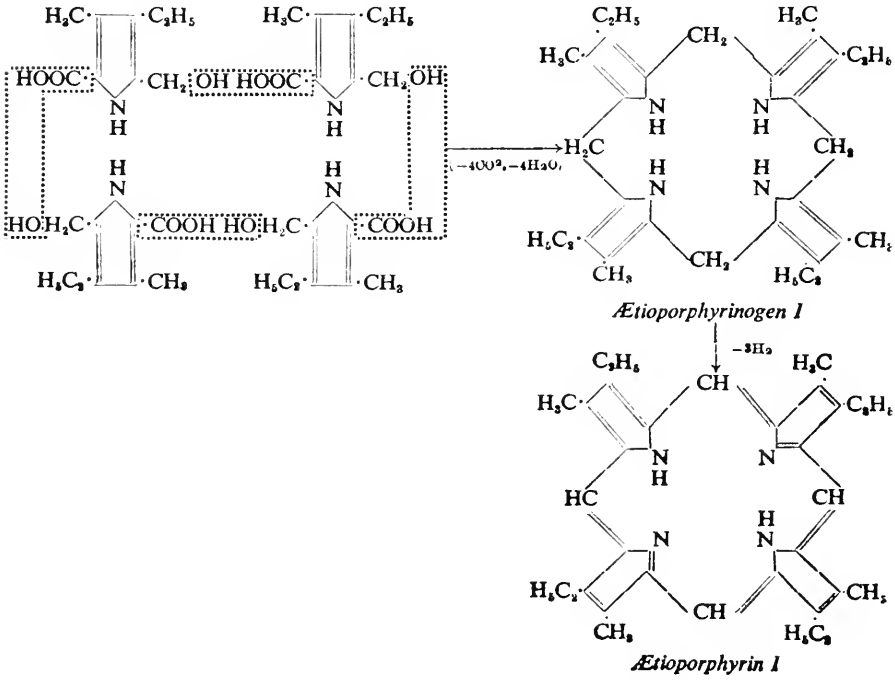
Referring to an observation of H. FISCHER and E. FINK[9] on a chemical reaction between formylacetone and glycine, Shemin and Rittenberg[7] suggest that the pyrrole ring may arise *in vivo* from condensation between glycine and a β-ketoaldehyde, as follows :



The carbon atoms of the methane bridges in the porphyrin structure would thus be derived from the carboxyl groups of the amino acid. Recent experiments with C_{14} labelled glycine have shown, however, that the carboxyl carbon atoms do not appear in haemin[10] although the methylene carbons do[11] and there has been a tendency to interpret the earlier evidence as an indication that not glycine itself but some simpler substance derived from it is the real building stone of the pyrrole ring.

I do not think this view is correct. The alternative would be, of course, that at some stage in porphyrin formation the carboxyl groups of the glycine molecules are eliminated and I find strong support for

this suggestion in the reaction mechanism studied by W. SIEDEL and F. WINKLER[12] during the war. These workers discovered a new method for porphyrin synthesis based upon the great reactivity of pyrroles bearing as α-substituents a carbinol and a carboxyl group. Four such units combined, even on standing in neutral methanolic solution, to form a porphyrin, the α-carboxyl groups being simultaneously eliminated as CO_2 thus :



Some combination also took place in such a way as to lead to formation of the series II isomer but, from the point of view of the present discussion, the important fact is that here we have an example of the spontaneous elaboration of a simple pyrrole carboxylic acid into a porphyrin in which the carboxyl carbon, though necessary to the reaction, does not itself appear.

One might postulate from the present evidence that the glycine first combines with materials in such a way as to give rise to an hydroxymethyl pyrrole carboxylic acid, a porphyrin being subsequently formed with loss of CO_2. Whether the β side chains are fully elaborated before or after the latter step we do not know but the way seems open to examine these points experimentally. In my laboratory we are endeavouring to obtain confirmation of the elimination of CO_2 from carboxyl-labelled glycine during the biological synthesis.

I would like now to refer to some experiments which a group* in London of which I am a member have carried out with the *Corynebacterium diphtheriae* as the synthesizing system. This organism has long been known to produce porphyrin in its culture fluid and Ch. GRAY and L. B. HOLT[13] have characterized the pigments as mainly coproporphyrin III together with a small quantity of uroporphyrin I and traces of an unidentified porphyrin. We have succeeded in growing the organism on a purely synthetic medium with good yields of pigment. As is well known, the quantity of iron in the medium greatly affects both porphyrin and toxin production. With minimal iron, the porphyrin production is high, that of intracellular haems low and with abundant iron these proportions are reversed. W. A. RAWLINSON and J. H. HALE[14] have examined the intracellular haems and find the greater part to be ferriprotoporphyrin IX, identified with the prosthetic group of cytochrome *b*, but there is also present a dichroic haem somewhat resembling chlorocuoro (spirographis) haem, which they consider to be the prosthetic group of the *a* cytochromes.

Our runs were performed with high and low iron and normal and N_{15} labelled glycine. The results are shown in Table I.

Table I

Utilization of N-Labelled Glycine by C. Diphtheriae

		Atom % excess N_{15}		
		Bacterial cell N	*Intracellular haem* N	*Coproporphyrin* N
Low Fe	*Normal glycine*	Nil	—	0·023
	Isotopic glycine (5·584 *atom* % *excess*)	0·903	—	0·489
High Fe	*Normal glycine*	0·008	lost	—
	Isotopic glycine (5·584 *atom* % *excess*)	0·863	1·247	0·511

* Hale, J. H., Rawlinson, W. A., Gray, Ch., Holt, L. B., Rimington, C., Smith, W.

Although we have obviously much more to do before we can visualize the mechanism of synthesis of these pigments and their interrelation, one to another, the results of this initial experiment do suggest certain conclusions, namely

1 The system is a good one for the study envisaged ; the small quantity of haem generally obtainable is the most adverse feature.
2 The high relative abundance of N_{15} in the bacterial protein is surprising and would at first sight lead one to suspect a high degree of randomization but as preliminary figures* indicate that the glycine content of the bacterial growth is of the order of 10 per cent, this need not be so.
3 The figure for the intracellular haem in run 4 indicates that glycine N is specifically utilized (in part at least) for the synthesis of protohaem by this system, thereby confirming Shemin and Rittenberg's findings for man, rat and duck bloods.
4 The much lower relative abundance of N_{15} in the coproporphyrin appearing in the medium than in the intracellular haem argues against any assumption that the former is derived from the latter by further transformation. Rather does it indicate a separate synthesis of coproporphyrin.
5 Since the coproporphyrin figure is considerably lower even than that of the total cell mass (in which glycine constitutes roughly 10 per cent) it is difficult to see how any specific utilization of glycine N can have occurred in the synthesis of the coproporphyrin.

We have succeeded also in isolating the uroporphyrin I produced in these experiments and, although the quantities are very small, it should be possible by dilution to get at least approximate figures for their relative N_{15} abundance. Such results promise to be of much interest. Whilst exercising the greatest caution at this stage, I would point out that to jump to the conclusion that the molecule of coproporphyrin is synthesized from the same structural units as is protoporphyrin may be an unwarrantable assumption. Shemin and Rittenberg's work refers only to protoporphyrin and recent work has even cast serious doubt upon the interconvertibility of the one pigment to the other[15].

We are attacking this problem from several different angles and hope eventually to lay bare the details of the steps by which these various porphyrin pigments are synthesized in the living cell. One can not doubt that light will then be thrown upon the pathological disorders of metabolism such as congenital and acute porphyria.

Received July 1948

* Kindly supplied by Dr. C. E. Work using a chromatographic method.

REFERENCES

[1] RIMINGTON, C. *Lab. Carlsberg Sér. chim.* 22 (1938) 454
[2] ROBINSON, R. *Proc. Univ. Durham phil. Soc.* 8 (1928) 14
[3] DOBRINER, K. and RHOADS, C. P. *J. clin. Invest.* 17 (1928) 95
[4] TURNER, W. J. *J. Lab. clin. Med.* 26 (1940) 323
[5] CORWIN, A. H. and ANDREWS, J. S. *J. Amer. chem. Soc.* 58 (1936) 1086
[6] — — *Ibid.* 59 (1937) 1973
[7] SHEMIN, D. and RITTENBERG, D. *J. biol. Chem.* 166 (1946) 621
[8] SHEMIN, D., LONDON, I. M. and RITTENBERG, D. *Ibid.* 173 (1948) 799
[9] FISCHER, H. and FINK, E. *Hoppe-Seyl. Z.* 280 (1944) 123
[10] GRINSTEIN, M., KAMEN, M. D. and MOSSE, C. V. *J. biol. Chem.* 174 (1948) 767
[11] ALTMAN, K. I., CASERETT, G. W., MASTERS, R. E., NOONAN, T. R. and SALOMON, K. *Proc. Fed. Amer. Soc. exp. Biol.* 7 (1948) 2
[12] SIEDEL, W. and WINKLER, F. *Liebigs Ann.* 554 (1943) 162
[13] GRAY, Ch. and HOLT, L. B. *J. biol. Chem.* 169 (1947) 235
[14] RAWLINSON, W. A. and HALE, J. H. *Bio-chem. J.* 42 (1948) xlvii
[15] WATSON, C. J., PASS, I. J. and SCHWARTZ, S. *J. biol. Chem.* 139 (1941) 583

Disturbances of Haemoglobin Synthesis in Lead Poisoning

A. VANNOTTI

This paper presents evidence that in lead poisoning there is an inhibition of haemoglobin synthesis because the iron is prevented from entering the porphyrin ring. This inhibition is localized in the bone marrow, and particularly in the cytoplasm of the erythroblast. On the other hand lead poisoning does not disturb the synthesis of the haem of cytochrome C. This is evidence that the mechanism and site of haem synthesis are different for haemoglobin and for the cellular haems. Increase of protoporphyrin in the blood results from an inhibition of haemoglobin synthesis, which may be caused either by the toxic action of lead, or by toxi-infectious disturbances of haemoglobin synthesis, or by iron-deficiency anaemia.

Lead poisoning is characterized by the appearance of an anaemia and by a notable increase of porphyrin in the blood and in the urine. This anaemia is due not only to a decrease in the production of haemoglobin, but also to the toxic action of lead on the formation of erythrocytes, which thus exhibit notable morphological modifications and undergo an increased haemolysis, especially at the beginning of the poisoning. The increase of porphyrin production is especially found in the bone marrow and in the blood in the form of protoporphyrin ; this increase leads to the elimination of a porphyrin III, *i.e.* a porphyrin corresponding to that of haemoglobin[1, 2].

We studied the mechanism of the poisoning[3] and concluded that the presence of protoporphyrin was due to an inhibition of haemoglobin synthesis in the bone marrow. C. RIMINGTON[4] also came to the conclusion that lead inhibited the incorporation of iron into the molecule of protoporphyrin during haemoglobin synthesis (see also R. KARK and A. P. MEIKLEJOHN[5]).

However, other hypotheses have been put forward to explain the presence of porphyrin. Thus, J. WALDENSTRÖM[6] and BJÖRKMAN thought that the appearance of this pigment was related to a disturbance of the metabolism of cytochrome and other haem-containing respiratory enzymes. Again, P. MARTINI[7] thought that the protoporphyrin was eliminated from the organism prematurely, before being combined with iron to form haemoglobin.

We should like to present briefly here our researches which led to the conclusion that lead inhibits the synthesis of haemoglobin. We studied in man and in rabbit the metabolism of iron in lead poisoning, making use especially of non-haemoglobin iron in the serum and later of radioactive iron. We often found that the percentage of non-haemoglobin iron increased during lead poisoning and the fact that phenylhydrazine produced increased haemolysis showed clearly that the increase of non-haemoglobin iron was more marked and of longer duration than in the normal animal (rabbit. See *Figure* 1). The increase of iron after haemolysis can be explained either by an insufficient storage of iron in the spleen during the poisoning, or by the inability of the bone marrow in lead-poisoned animals to make use of iron. However we were able to eliminate the first possibility by comparing the results of haemolysis due to lead poisoning in normal animals with those in splenectomized animals[14].

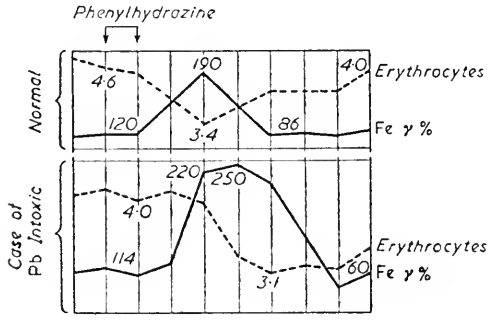


Figure 1

In this connection it is interesting to note that if we carry out a splenectomy and a very strong blockade of the reticulo-endothelial system, and if we then induce lead poisoning, anaemia rapidly appears but is not accompanied by an increased production of protoporphyrin. The disturbances of the metabolism of non-haemoglobin iron were also pointed out by J. E. KENCH, A. E. GILLAM and R. E. LANE[8] who, like ourselves, found increased values of iron in the bloodstream. Finally, by means of radioactive iron, we were able to observe in lead poisoning, after intravenous injection of radioactive iron lactate, a notable increase of iron in the serum, a more rapid and considerable storage of iron in the liver than in the normal subject, and a significant accumulation of iron in the bone marrow (see *Figure* 2). These experiments confirm the hypothesis that lead does not interfere with the passage of the iron through the storage organs, but that it merely inhibits the utilization of iron for the synthesis of haemoglobin in the bone marrow.

Examination by fluorescence microscopy shows that in lead poisoning the bone marrow and especially the erythroblasts are most rich in porphyrin. By separating by means of centrifugation (according to the method of Claude) the cells of the reticulum, the nuclei of the cells in the bone marrow, and their cytoplasm, we were able to observe

that the greater part of the protoporphyrin is in the cytoplasm ; the nuclei contain only one third of it, while the reticulum seems to contain no porphyrin. We are thus inclined to think that it is especially in the erythroblast that protoporphyrin is formed, and that the synthesis of haemoglobin probably takes place in the cytoplasm of the erythroblast. In fact, a similar analysis made by means of radioactive iron again showed that the greatest quantity of iron in the cell is to be found in the cytoplasm. Histochemical analysis of the bone marrow permitted us to observe that lead does not appear in the erythroblast.

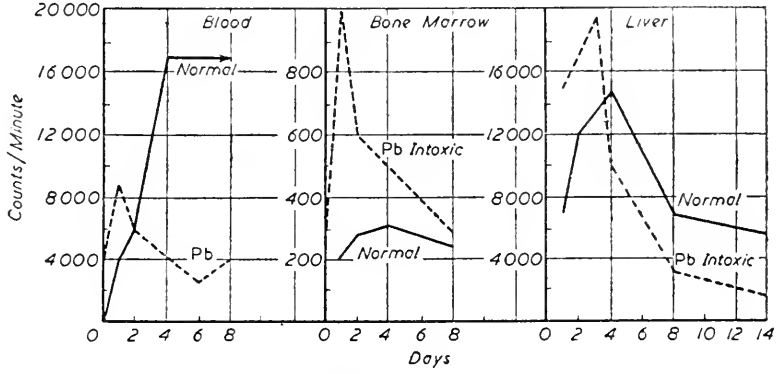


Figure 2

Therefore we may conclude that in lead poisoning the metal has a direct action, either on the erythroblast, or more probably on the transport system of iron from the reticulo-endothelial system to the erythroblast ; in this way it inhibits the synthesis of the haemoglobin and provokes the formation of protoporphyrin. We were never able to observe the presence of important quantities of protoporphyrin in the other organs, even in the liver or the musculature.

These observations made us wonder whether lead could inhibit not only the synthesis of haemoglobin but also that of the other haems, particularly the cellular ones. In the course of experiments on lead poisoning of the rabbit, our collaborator A. PRADER[9] followed the activity of non-haemoglobin iron, of haemoglobin, and of cytochrome C which is a cellular haemin closely resembling haemoglobin in chemical structure (protoporphyrin + iron + protein). The researches of Prader can be summarised in *Table I.*

Thus we must conclude that in lead poisoning, although we can regularly observe a considerable decrease of haemoglobin, the cytochrome C is not decreased, but on the other hand invariably increases. Indeed we can observe a change in the effect on the cytochrome

concentration as poisoning proceeds. During the first two or three weeks of poisoning, Prader was unable to find a significant increase of the pigment ; it was only from the third week that he observed a considerable increase in the cytochrome C content, particularly in the musculature (141 per cent). In the brain the increase is only 10 per cent.

Table I

	Time of poisoning	*Haemoglobin (average)*		*Non-haemoglobin iron (average)*	*Cytochrome C mgr. %*			
		before	*after*	*after*	*heart*	*musculat.*	*liver*	*brain*
Series I (5 *rabbits*)	1–2 *weeks*	67%	45%	267 μg. *per* 100 *ml.*	22·0 ±3·6	3·5 ±1·2	2·2 ±0·8	2·9 ±0·6
Series II (5 *rabbits*)	2–3 *weeks*	65%	50%	230	21·7 ±2·7	4·0 ±1·4	3·1 ±0·9	3·2 ±0·7
Series III (5 *rabbits*)	3–6 *weeks*	70%	40%	228	29·7 ±4·1	7·0 ±2·4	3·7 ±0·9	3·3 ±0·5
Mean values of cytochrome C in the normal animal :					20·0 ±2·6	2·9 ±0·6	2·1 ±0·7	3·0 ±0·7
Increase in % of cytochrome C for Series III :					48·5%	141%	67%	10%

These observations show that in lead poisoning only the synthesis of haemoglobin is injured ; *per contra*, the cellular haems (cytochrome C) do not show any reduction. We must insist on this observation, because it gives us indirectly the answer to another problem of pigmentary metabolism : that of the site of haem synthesis. If the organism used the haem synthesized in the erythroblast of the bone marrow for cytochrome C synthesis, we should observe a decrease of cytochrome C parallel to that of the haemoglobin. Prader's observations permit us to conclude that the synthesis of the cellular haems is independent of that of the haemoglobin and that, *per contra*, a notable increase of the cellular haem may correspond to a decrease of the haemoglobin. We were able to observe this fact with GOBAT in other cases of severe anaemia, and WHIPPLE noted it for myoglobin in pernicious anaemia, in which a compensation for the decreased supply of oxygen is involved ; this compensation was observed by our collaborators A. DELACHAUX and A. TISSIÈRES[10] in oxygen lack at high altitude (over 18,000 feet) and in cases where an increase of oxygen in the organism is necessary (muscular hypertrophy and hyperthyroidism according to Tissières[11] and D. L. DRABKIN[15]).

We should like to insist, with Prader, on the analogy of the clinical observations of anaemia to the appearance of protoporphyrin in the blood during lead poisoning and in acute and chronic toxi-infectious conditions. Protoporphyrinemia of essential hypochromic anaemia (iron-deficiency anaemia) was observed and described by G. E. CARTWRIGHT and his collaborators[12], and that of infective anaemia by C. J. WATSON *et al*[13]. In these cases the iron is probably prevented from participating in the synthesis of haemoglobin, partly by a toxic inhibition of the bone marrow and partly by a decrease in the iron, which cannot be transported owing to a low and altered protein content of the blood serum and which has been deposited in certain tissues by the inflammatory process. In these cases the sideremia is low ; in lead poisoning where inhibition of the employment of iron is accompanied by an increased haemolysis, the concentration of non-haemoglobin iron remains high even when that of haemoglobin decreases considerably.

These few experiments seem to show that in lead poisoning the iron cannot enter into the porphyrin ring of haemoglobin. This inhibition is localized in the bone marrow, and particularly in the cytoplasm of the erythroblast. On the other hand lead is not able to disturb the synthesis of the haem of cytochrome C.

This observation shows us with certainty that the mechanism and site of haem synthesis are different for haemoglobin and for the cellular haems. The organism can lose haemoglobin very rapidly, but it loses the cellular haems much more slowly and more rarely, and only in particular pathological circumstances. These should properly be regarded as cases of ' tissue anaemia '.

Increase of protoporphyrin in the blood results from an inhibition of haemoglobin synthesis, either by the toxic action of lead, or by toxi-infectious disturbances of haemoglobin synthesis, or by iron-deficiency anaemia. There are certain functional relationships between the concentration of haemoglobin and that of the cellular haems, governed by the respiratory necessities of the cell.

Received July 1948

REFERENCES

[1] VIGLIANI, E. and WALDENSTRÖM, J. *Dsch. Arch. Klin. Med.* 18 (1937) 182
[2] VANNOTTI, A. and IMHOLZ, A. *Z. ges. exp. Med.* 106 (1939) 597
[3] — *Porphyrine und Porphyrinkrankheiten*, Berlin, Springer (1937)
[4] RIMINGTON, C. *Carlsberg. Ser. chim. C.R. Lab.* 22 (1938) 454
[5] KARK, R. and MEIKLEJOHN, A. P. *J. clin. Invest.* 21 (1942) 91

[6] WALDENSTRÖM, J. *Acta med. scand. Suppl.* 82 (1937) 1
[7] MARTINI, P. *Verh. dtsch. Ges. inn. Med.* 280 (1933)
[8] KENCH, J. E., GILLAM, A. E. and LANE, R. E. *Bio-chem. J.* 36 (1942) 384
[9] PRADER, A. *Schweiz. med. Wschr.* (1948)
[10] DELACHAUX, A. and TISSIÈRES, A. *Helv. med. Acta* 13 (1946) 333
[11] TISSIÈRES, A. *Arch. int. Physiol.* 54 (1946) 305
[12] CARTWRIGHT, G. E., LAURITSEN, M. A., JONES, P. J., MERILL, J. M. and WINTROKE, M. M. *J. clin. Invest.* 25 (1946) 65
[13] WATSON, C. J., GRINSTEIN, M. and HAWKINSON, J. *J. clin. Invest.* 23 (1944) 69
[14] VANNOTTI, A. and DELACHAUX, A. *Iron Metabolism.* London, F. Müller (1949)
[15] DRABKIN, D. L. *Fed. Proc.* 7 (1948) 483

VII

DIFFERENCES BETWEEN ADULT AND FOETAL HAEMOGLOBIN

Foetal Haemoglobin and Rh. Antagonisms

J. H. P. JONXIS

The differences between foetal and later haemoglobin in human beings make it probable that in foetal Hb the four parts from which the Hb molecule (mol. weight 68,000) may be built up are connected more firmly with each other than in later Hb. In most other mammalia the forces which hold together the parts of the Hb molecule are greater in the adult form. In adult human beings two forms of Hb can be detected. The alkaline resistances of these two forms are only slightly different. In cases of erythroblastosis foetalis due to the Rh. antagonism the red blood corpuscles containing foetal Hb are haemolyzed, the erythrocytes which already contain later Hb being only slightly attacked.

THAT there are differences between the haemoglobins of the newborn and of adults was demonstrated for the first time in 1866 by E. KÖRBER[1]. In that year he published a paper on the differences in the resistance of foetal and normal blood to alkalis and acids. Since then other investigators[2, 3, 4] have repeated these experiments, using more or less pure haemoglobin solutions instead of whole blood.

Besides this difference there are others: various crystal forms, differences in solubility and iso-electric point, differences in amounts of amino-acids, absorption spectra, dissociation curves, and different behaviour in monomolecular layers.

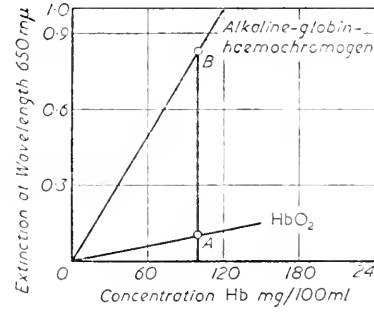


Figure 1. *The influence of the denaturation of oxyhaemoglobin on the extinction at a wavelength of* 650 *mμ.*

One of the most simple methods of distinguishing haemoglobins is the determination of differences in denaturation rate in buffer solutions of *p*H 12·7. In the course of this denaturation the red colour of oxyhaemoglobin changes into the brown of alkaline-globin-haemo-

chromogen. At a wavelength of 650 mμ there is a considerable increase in the light absorption during this denaturation (*Figure* 1), and this increase can be used for the determination of the amount of oxyhaemoglobin which has not yet been denatured. By measuring at intervals of one minute the extinction of a 0·2 per cent solution of haemoglobin to which has been added buffer of *p*H 12·7 it is possible to calculate the percentage of oxyhaemoglobin still present at different times. The values found in this way can be used to make a curve (*Figure* 2). On the horizontal axis the time is plotted in minutes, and on the vertical axis the logarithm of the percentage of oxyhaemoglobin still present. If there is only one haemoglobin present in the solution the values will form a straight line ; this means that the denaturation behaves as a monomolecular reaction. When there are two different forms of haemoglobin in the solution with different resistances to alkali the values will form two straight lines. By extrapolating back to the vertical axis the line which is formed by the values of that fraction of haemoglobin which denatures more slowly, it is possible to calculate the percentage of this more resistant haemoglobin. In human beings the foetal haemoglobin is far more resistant than the adult one. The rate of denaturation depends on the kind of haemoglobin, the *p*H, the concentration of the buffer solution and the temperature. When buffer solution is used with a *p*H of 12·7 the denaturation of later haemoglobin is rapid while that of foetal haemoglobin is slow. Thus it is possible to calculate exactly the amounts of foetal haemoglobin present in a mixture of the two forms[5, 6].

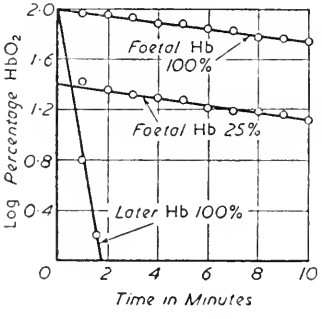


Figure 2. *Rate of alkali-denaturation of a* 0·2 *per cent solution in* 0·1 *N* NaOH *of foetal* Hb, *later* Hb *and of a mixture containing* 25 *per cent of foetal* Hb. *Temperature* 20°*C. Vertical axis : log. percentage unchanged* Hb.

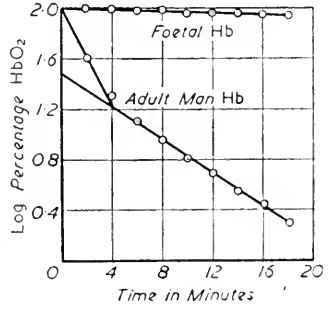


Figure 3. *Rate of alkali-denaturation at p*H 11·7 *and* 25°*C of haemoglobin from an adult man. Haemoglobin concentration* 0·2 *per cent.*

At *p*H 11·7 the haemoglobin of an adult denatures slowly. Under this condition it becomes evident that the haemoglobin of adult men is made up of two different fractions having slightly different resistances to alkali (*Figure* 3). Because the difference in denaturation rate between the two haemoglobins in the blood of human adults is only slight, the angle between the two lines representing the different rates of denaturation is small. The possibility must be considered that this phenomenon is the result of a secondary reaction. To exclude this possibility I stopped the reaction after six minutes. At this time nearly all haemoglobin of the less resistant fraction had been denatured. The denaturation-product, alkaline-globin-haemochromogen, was precipitated and the remaining undenatured haemoglobin was concentrated. This haemoglobin solution should contain only the more resistant fraction, and this proved to be the case.

The occurrence of two different haemoglobins in adults has also been demonstrated by others. R. BRINKMAN[7] determined the percentage of these two fractions in his own blood every day for a month or more. It seems that the fraction which is slightly more resistant is at times nearly absent. Subsequently over a period of a few weeks its percentage increases slowly up to about 50 per cent ; after that it once more gradually diminishes.

Although little is known about these different haemoglobins in adult men, more is known about the differences between foetal and later haemoglobin. There is an interesting difference between the behaviour of later and foetal haemoglobin when spread in a monomolecular layer on the surface of water in a trough[8, 9]. When the *p*H of the water is about the iso-electric point of the haemoglobin (concentration of the buffer solution : 3 millimolar) the later haemoglobin rapidly forms a stable monomolecular layer with a thickness of about 8Å. Foetal haemoglobin forms a stable layer but far more slowly, taking about 10 minutes to spread completely. In other words the molecule of foetal haemoglobin unfolds itself more slowly than the later form. The nature of the differences between foetal and later haemoglobin is uncertain, but is known to lie in the protein components (*i.e.* the globins). A comparison of some of the properties of foetal and later haemoglobin of different species throws some light on the nature of these differences. In human beings foetal haemoglobin is more resistant to alkali and unfolds itself more slowly to a monomolecular layer ; furthermore the oxygen dissociation curve of foetal haemoglobin is shifted to the right of the adult curve.

In most animals (*e.g.* goat, sheep, cow) the relation is just the opposite. In the sheep, goat and cow foetal haemoglobin is less resistant to alkali, the molecule unfolds itself more rapidly to a mono-

molecular layer and the dissociation curve of the foetal haemoglobin is shifted to the left. This makes it probable that a lower resistance against alkali goes with an easier unfolding of the molecule and a higher affinity for oxygen (*Table I*). The parallelism between the easy unfolding of the molecule and the low resistance to alkali may be explained by the fact that in the less resistant forms of haemoglobin the bindings are less solid between the 4 parallel layers of polypeptide chains of which the human haemoglobin molecules may perhaps be built up (by analogy with horse haemoglobin which is believed to have this structure). In the more resistant forms, the affinity for oxygen is less.

Table I

	Man		*Cow, Sheep, Goat*	
	Adult Hb	*Foetal Hb*	*Adult Hb*	*Foetal Hb*
Alkali resistance	*low*	*high*	*high*	*low*
Unfolding on trough	*rapid*	*slow*	*slow*	*rapid*
Dissociation curve	—	*shifted to the right*	—	*shifted to the left*

It is known that in many children a small proportion of later haemoglobin is already present at birth. During the following months of life the percentage of foetal haemoglobin diminishes constantly[10, 11]. I have made a number of determinations to see how long it takes for the last traces of foetal haemoglobin to disappear. The results are shown in *Figure* 4. At birth the percentage of later haemoglobin in healthy children varies between 2 and 25 per cent. The percentage of foetal haemoglobin decreases gradually: it is never found in children older than 22 weeks. There are great individual differences. In healthy children who are rapidly gaining weight the foetal haemoglobin diminishes more rapidly than in dystrophic children. When there is a considerable breakdown of haemoglobin during the first days of life the percentage of foetal haemoglobin decreases proportionately. It is clear that the breakdown of the haemoglobin in these cases occurs at the expense of the foetal haemoglobin and one can

only conclude that foetal and later haemoglobin are present in different blood corpuscles, the corpuscles filled with foetal haemoglobin being broken down more rapidly than those containing later haemoglobin. This is in agreement with the observation of Mollison who found that a part of the red blood corpuscles present at birth was broken down more rapidly than the rest.

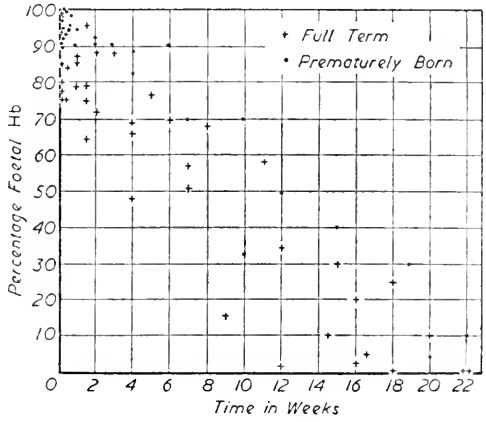


Figure 4. *The disappearance of foetal Hb from the blood in full term and prematurely born children. Vertical axis: the percentage of Hb which has still the foetal form.*

At the moment of birth prematurely born babies have less later haemoglobin than full-term babies, but even in early prematures traces of later haemoglobin can nearly always be detected. In prematurely born children foetal haemoglobin disappears later than in children born at term. Nothing is known about the site of formation of later and foetal haemoglobin. It may be suggested that later haemoglobin is formed in the bone-marrow and foetal haemoglobin in the liver, but I have never been able to find any difference between the percentages of foetal and later haemoglobin at these sites.

It is not clear how the organism can change at a given moment from the production of one form of haemoglobin to that of another. The presence of small amounts of later haemoglobin in the blood of early premature infants makes it probable that later haemoglobin is from the beginning made in small amounts at the same sites as foetal haemoglobin.

I have had the opportunity of making determinations of the percentage of foetal and later haemoglobin in some children suffering from erythroblastosis, due to Rhesus antagonism. Two of these children did not get any special treatment. At the moment of birth

both had a large amount of later haemoglobin. (This abnormality was also found in other similar cases.) In the following days there was considerable haemolysis, which concerned only the foetal haemoglobin. In one case this breakdown went so far that in a few days nearly all the foetal haemoglobin had disappeared (*Figure* 5). At the same time the later haemoglobin of this child was not attacked. On the 7th day of life only 6 gm of haemoglobin per 100 cc of blood remained. On this day the child received a blood-transfusion of 80 cc of Rh. negative blood, which produced an increase of later haemoglobin from 4·5 to 8·6 gm per 100 cc. In the following days there was a decrease of the amount of later haemoglobin from 8·6 to 6·5 gm, caused partly by a slight breakdown of the transfused blood, partly by an increase in the blood volume of 15 per cent during these days. After the 16th day there was a limited rise in the amount of foetal haemoglobin from 0·5 to 2·5 gm, this being the result of the formation of new red cells containing foetal haemoglobin.

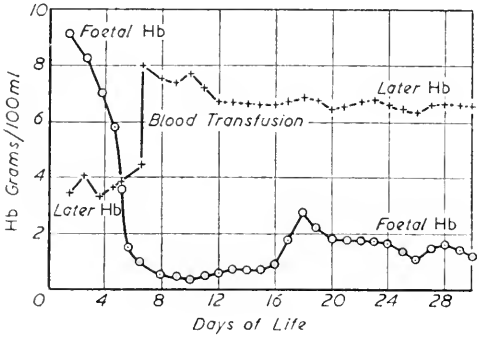


Figure 5. *The amount of foetal Hb and later Hb in* 100 *cc of blood of an untreated baby with severe erythroblastosis, caused by Rh. antagonisms during the first weeks of life.*

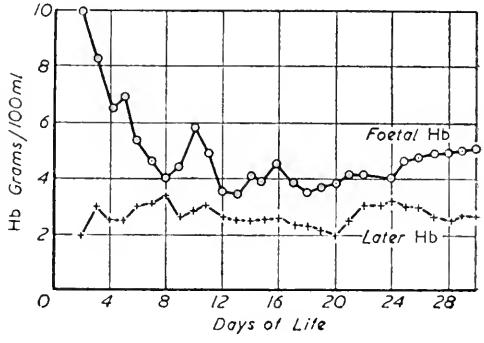


Figure 6. *The amount of foetal Hb and later Hb in* 100 *cc of blood of an untreated baby with moderate erythroblastosis caused by Rh. antagonisms during the first weeks of life.*

In the less severe case (*Figure* 6) the breakdown of the haemoglobin did not go so far and came to a stop within 5 days with more foetal haemoglobin left. It is clear that the Rhesus antibodies distinguish

between the red blood corpuscles containing foetal and later haemoglobin, only the former being haemolyzed. One might assume that this difference is the result of a higher average age of the erythrocytes filled with foetal haemoglobin, but the first case makes this hypothesis unacceptable, since all erythrocytes containing foetal haemoglobin were broken down, without the loss of any detectable amount of later haemoglobin. It is more probable that in regard to the factors which cause haemolysis, the red blood corpuscles containing foetal haemoglobin behave differently from those filled with later haemoglobin.

Received August 1948

REFERENCES

[1] KÖRBER, E. *Inaug. Dissertation, Dorpat*, 1866
[2] VON KRUGER, F. *Z. Biol.* 24 (1888) 318
[3] BISCHOFF, H. *Z. ges. exp. Med.* (1926) 54
[4] HAUROWITZ, F. *Hoppe-Seyl. Z.* (1930) 186
[5] BRINKMAN, R. and JONXIS, J. H. P. *J. Physiol.* 88 (1935) 117
[6] — — *Ibid.* 88 (1936) 162
[7] — *Ibid.* 80 (1934) 337
[8] JONXIS, J. H. P. *Doctor's Thesis, Groningen*, 1935
[9] — *Bio-chem. J.* 33 (1939) 1743
[10] — *Maandschr. Kindergeneeskunde* 5 (1936) 363
[11] *Ibid.* 6 (1937) 356

Crystallization and Solubility Studies on Human Adult and Foetal Haemoglobins

H. M. JOPE and J. R. P. O'BRIEN

The crystallization and solubility behaviour of human adult and foetal haemoglobins, carbonmonoxyhaemoglobin (HbCO), *oxyhaemoglobin* (HbO_2), *methaemoglobin* (MetHb) *and 'reduced' haemoglobin* (Hb), *have been studied under varying conditions.*

A method is given for the crystallization of adult and foetal HbCO, HbO_2, MetHb and Hb *on a scale sufficiently large for use in solubility measurements.*

Adult HbCO, HbO_2 *and* MetHb *crystals are isomorphous and belong to the orthorhombic system; foetal* HbCO, HbO_2 *and* MetHb *are also isomorphous but crystallize in a system different from that of the adult derivatives, probably triclinic.* HbCO, HbO_2 *and* MetHb, *both adult and foetal, crystallize in systems different from that of the corresponding* Hb.

Human adult haemoglobin appears from solubility, electrophoresis and ultracentrifuge data to be a homogeneous protein. Amorphous HbCO *is more soluble than crystalline* HbCO. HbCO, HbO_2 *and* MetHb, *both adult and foetal, are more soluble than the corresponding* Hb. *Adult* HbCO, HbO_2 *and* MetHb *have different types of solubility-temperature curves from those of foetal* HbCO, HbO_2 *and* MetHb. *Adult and foetal* Hb's *have very similar solubilities, which do not vary with temperature. There is, therefore, in both crystal form and solubility behaviour, a clear distinction between* Hb *on the one hand and* HbCO, HbO_2 *and* MetHb *on the other, and also between the adult and foetal haemoglobins.*

INTRODUCTION

THE possibility of obtaining human haemoglobin in crystalline form has now been recognised for a century (F. L. HÜNEFELD[1] and O. FUNKE[2] give drawings of Hb crystals), but it is only in recent years that this crystalline form has been exploited to study its behaviour and molecular structure. E. T. REICHERT and A. P. BROWN[3], G. AMANTEA[4] and C. PERRIER and P. JANELLI[5] obtained human haemoglobin crystals on a micro scale and described them but gave no photographs. F. HAUROWITZ[6] prepared crystals of human adult and foetal haemoglobins and published a photograph of adult haemoglobin crystals from salt-

free medium. A. A. GREEN, E. J. COHN and M. BLANCHARD[7] were the first to use crystalline human HbCO for solubility studies, and R. K. CANNAN and J. REDISH[8] described their methods for obtaining crystalline human HbO_2 in bulk, which they developed for purely practical transfusion purposes. While the work described below was in progress, D. L. DRABKIN[9, 10], published photographs and a crystallographic description of human HbO_2 crystals from a study on a petrographic microscope : this crystallographic description has now in some respects been superseded by x-ray data on the preparations described below (M. F. PERUTZ[11]), and the study has been extended to HbCO, MetHb and Hb, and the corresponding human foetal haemoglobin derivatives.

EXPERIMENTAL METHODS

Red blood cells (from out-dated bank blood) were separated from the plasma, washed 4 times with 1·5 per cent sodium chloride solution, haemolyzed with an equal volume of water and treated with a suspension of $C\gamma$ aluminium hydroxide to facilitate the removal of stroma. The aluminium hydroxide was removed by centrifuging and the purified HbO_2 solution dialyzed in the cold in sacs against either 65 per cent saturated $(NH_4)_2SO_4$ or 2·8M potassium phosphate *p*H 6·7, with frequent changes of the buffer until crystallization began (about 24 hours). Crystals of adult HbCO, MetHb and Hb were prepared by the same method as for adult HbO_2 crystals. HbCO was obtained by treating the red blood cells with carbon monoxide and keeping the HbCO solution always in an atmosphere of carbon monoxide. MetHb was prepared by treating HbO_2 with $K_3Fe(CN)_6$, the excess of which was removed by dialysis. Hb was prepared either by treating HbO_2 with ferrous citrate or by allowing an HbO_2 solution to become deoxygenated by bacterial action. The Hb solution and crystals were always kept in an atmosphere of nitrogen.

Foetal HbCO, HbO_2, MetHb and Hb crystals were prepared in the same way as the corresponding adult crystals but usually only 1–2 ml of blood was available and crystallization was carried out in small test tubes with cellophane tied over the end in place of the cellophane sac.

RESULTS

The crystal forms were as follows :—

Adult HbCO	...	...	orthorhombic
HbO_2	...	...	orthorhombic
MetHb	...	...	orthorhombic
Hb	...	...	monoclinic and another form whose system is still to be determined.

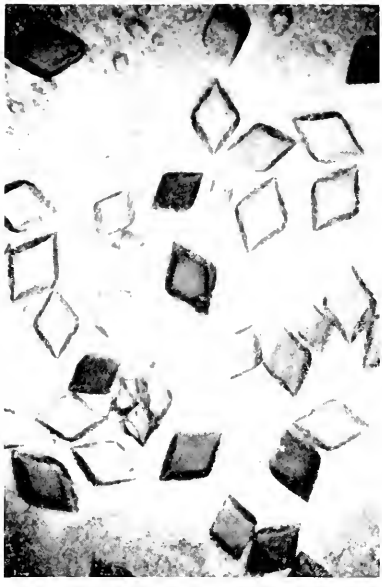


Figure 1. *Adult* HbCO *from ammonium sulphate, taken in polarized light.*

Figure 2. *Adult* HbO_2 *from potassium phosphate, p*H 6·7

Figure 3. *Adult MetHb from ammonium sulphate.*

Figure 4. *Adult MetHb from potassium phosphate, p*H 6·7

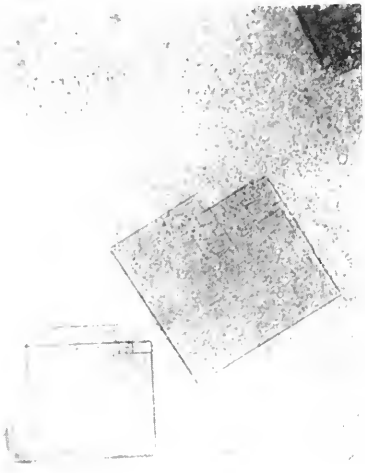


Figure 5. *Adult Hb from ammonium sulphate.*

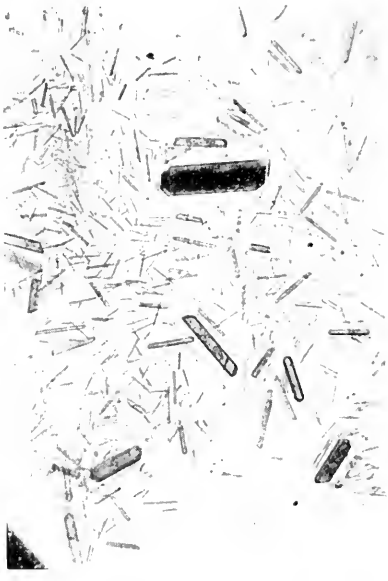


Figure 6. *Adult Hb from ammonium sulphate.*

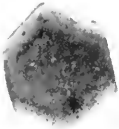


Figure 7. *Foetal* HbO_2 *from ammonium sulphate.*

Figure 8. *Foetal Hb from ammonium sulphate.*

Foetal HbCO	... ...	triclinic (or monoclinic), system still to be determined.
HbO_2	... ...	same as foetal HbCO
MetHb	... ...	same as foetal HbCO
Hb	... ...	different from foetal HbCO, HbO_2 and MetHb, possibly monoclinic

Photomicrographs of various types of crystals obtained are given in *Figures* 1 to 8.

The adult haemoglobin crystals are being studied by M. F. Perutz and the foetal crystals by J. C. Kendrew in the Cavendish Laboratory, Cambridge.

Crystals of HbO_2 prepared from blood from a patient with pernicious anaemia were orthorhombic. A specimen of HbCO which had been lyophil dried over concentrated H_2SO_4 for $5\frac{1}{2}$ hours at room temperature, followed by $\frac{1}{2}$ hour at 38°C, produced good orthorhombic crystals.

It is clear from these results that HbCO, HbO_2 and MetHb are isomorphous, both in the adult and in the foetal series, and that in both cases the crystal form is different from that of the corresponding Hb.

These differences between the Hb's and the other three corresponding derivatives and between adult and foetal haemoglobins are also strikingly apparent in the solubility studies carried out on these crystalline materials.

In solubility studies it is necessary to ensure that measurements are made only on systems which are in equilibrium, and constant values for solubilities obtained by dissolving up these protein crystals are frequently different from values obtained by salting out crystals at the same buffer concentration. It is important therefore that all comparisons are made only on results obtained in a similar manner. Moreover, amorphous material is considerably more soluble than crystalline and it is therefore important to ensure that the solid phase consists entirely of crystalline material.

Human HbCO, HbO_2 and MetHb are very soluble even in strong buffers (over 20 per cent HbCO in 2M potassium phosphate, *p*H 6·7) producing a thick solution which is difficult to filter and of a density often so similar to that of the crystals that gravitational separation is not possible. Filtration must be carried out at equilibrium temperature. Filtration was carried out here through sintered glass filters using a positive pressure of oxygen or nitrogen. Hb is less soluble and results are obtainable by gravitational separation.

Equilibration of the crystals with the buffer was carried out in cellophane sacs with frequent changes of buffer. The crystals were

then transferred to small test tubes, containing a glass bead to facilitate mixing, which were filled with CO, O_2 or N_2 for HbCO, HbO_2 and Hb, and tightly closed. The tubes were kept at constant temperature and were rotated gently by hand at intervals for a period of about 2 days, by which time constant values had usually been reached and remained so for a further week.

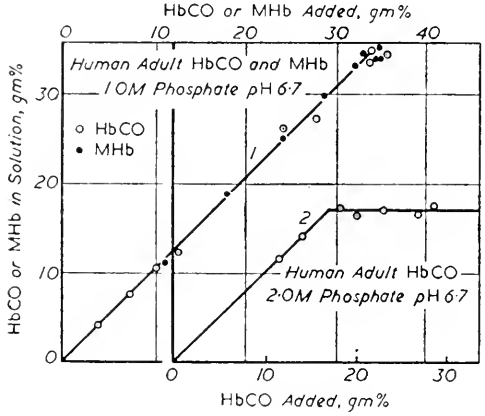


Figure 9. Solubilities of human carboxyhaemoglobin and methaemoglobin related to total haemoglobin present in liquid and solid phases.

In *Figure* 9 the concentration of HbCO in solution in potassium phosphate buffer *p*H 6·7, 1M at 0°C (curve 1) and 2M at 22·5°C (curve 2) is plotted against increasing quantities of solid phase. Data are also given for MetHb in 1M phosphate (curve 1). In 2M phosphate the curve is linear until a constant solubility is reached at 17 per cent HbCO.

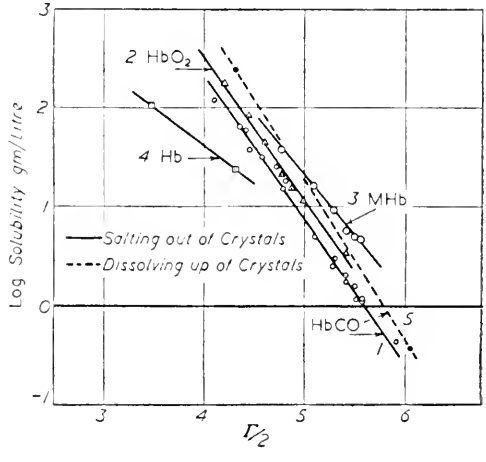


Figure 10. Variation of log solubility of human adult haemoglobins with ionic strength of phosphate buffers pH 6·7.

In *Figure* 10 curve 1 the solubility of HbCO is plotted against ionic strength of potassium phosphate buffer at *p*H 6·7 and 0°C. The data are represented by Cohn's equation ($\log S = \beta - K_s^1 \, {}^{r}/_{2}$) for ionic strengths ${}^{r}/_{2} = 4$ to 6. The curves for HbO_2, MetHb and Hb (curves 2–4) have been plotted for comparison. Curve 4 (Hb) was obtained by dissolving up crystals in buffer, curves 1–3 by salting out crystals with strong phosphate buffer. These show that Hb is very much less soluble than the other three haemoglobin derivatives HbCO, HbO_2 or MetHb, whose solubilities lie fairly close together.

Figure 10 shows also that higher solubility values are obtained by approaching the equilibrium from the undersaturated side, *i.e.* by dissolving up crystals in buffer (HbCO curve 5), than by approaching the equilibrium from the supersaturated side, *i.e.* by salting out crystals by addition of strong buffer (HbCO, curve 1). The difference between curve 5 and curve 1 is considerable, and the true equilibrium value will probably be somewhere between them. The difference does not seem to be due to insufficient time being given for the true equilibrium to be reached as values remained constant for as long as a week after the completion of the experiment. There seems, moreover, to be a tendency to overshoot the equilibrium, higher values being obtained by approaching from below than by approaching from above. This is remarkable.

There is a difference also in the equilibrium values for amorphous and for crystalline material, and this may be in some way concerned with the above observation. Amorphous HbCO was dissolved in phosphate buffer to give a solution in equilibrium with amorphous solid phase. On standing the amorphous HbCO crystallized and the amount in solution decreased, the amorphous material being considerably more soluble than the crystalline.

The variation with *p*H of solubility of HbCO in potassium phosphate buffer ${}^{r}/_{2} = 4{\cdot}6$ at 0°C appears to be slight between *p*H 6·5 and *p*H 7·1 (*Figure* 11).

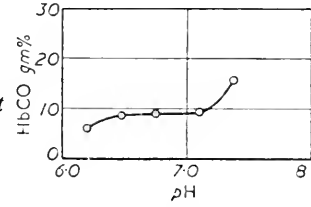


Figure 11. *Variation with p*H *of human adult* HbCO *solubility.*

The solubility of the haemoglobin derivatives, adult HbCO, HbO_2, MetHb and foetal HbCO and Hb, in 2M potassium phosphate buffer *p*H 6·7 at different temperatures is plotted in *Figure* 12 (curves 1–6) against temperature. Hb, both adult and foetal (curves 4 and 6) has

a much lower solubility than the other three haemoglobin derivatives HbCO, HbO_2 and MetHb. For both adult and foetal haemoglobins also the shape of the Hb curve is almost horizontal, with zero temperature coefficient, by contrast with the curves for HbCO, HbO_2, and MetHb which vary greatly with temperature ; the curves of adult HbCO, HbO_2 and MetHb all have a minimum at about 20°C. Foetal HbO_2 and MetHb behave as if their temperature solubility curves were similar to that of foetal HbCO (curve 5) but no quantitative data have been obtained for these two derivatives owing to a shortage in the supply of foetal blood. Qualitative observations on adult Hb crystallised from dilute sodium chloride solution (about 0·3 per cent) indicate that the solubility in this solvent increases rapidly with temperature.

DISCUSSION

No previous study of the solubility of the four main haemoglobin derivatives appears to have been made, although work has been done on the solubility of mammalian HbCO. The striking conclusion from this study is the contrast between Hb and the other three haemoglobin derivatives : the contrast is shown in both the human adult and the foetal haemoglobin series in their crystallography and in their solubility behaviour. This difference in behaviour of Hb from that of the other three haemoglobins cuts across the distinction made by L. PAULING and his co-workers[12, 13], between Hb and MetHb with their unpaired electrons and HbO_2 and HbCO with no unpaired electrons. It may be noted, however, that the solubility of MetHb lies closest of the three to the solubility of Hb.

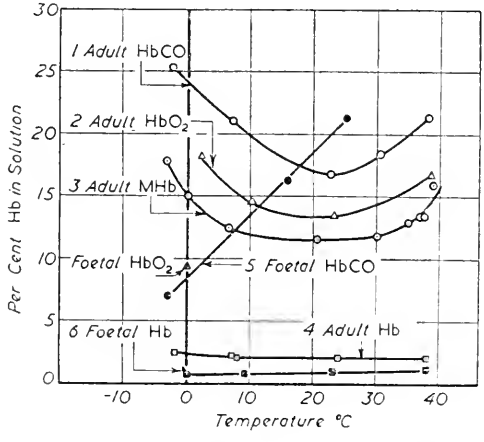


Figure 12. *Solubilities of adult and foetal haemoglobins as functions of temperature.*

A distinction between human adult and foetal haemoglobins is also clearly seen. They crystallize in different systems, and the difference between them is also evident from a comparison of the solubilities of their three derivatives HbCO, HbO_2 and MetHb. Adult HbCO, HbO_2 and MetHb have solubility-temperature curves, in 2M phosphate buffer *p*H 6·7, with a form often shown by proteins in strong salt solutions (*Figure* 12 curves 1–3). Foetal HbCO (HbO_2 and MetHb) has a solubility-temperature curve, in the same solvent, more characteristic of proteins in dilute salt solutions (*Figure* 12 curve 5). Adult and foetal Hb's are, however, alike in having very similar solubilities in this solvent ; both solubilities are unaffected by temperature.

It seems opportune to summarize here the differences which have so far been observed between human adult and foetal haemoglobins.

1. *Crystal form* : described here.

2. *Solubility behaviour* : described here.

3. *Electrophoretic mobility* : H. HOCH[14] has shown that between *p*H 7 and *p*H 8 human adult HbO_2 moves slightly faster than human foetal HbO_2. These more recent experiments do not confirm the results of M. A. ANDERSCH, D. A. WILSON and M. L. MENTEN[15] although their buffer conditions are reproduced exactly.

4. *Spectral absorption* : although the wave lengths of the visible and Soret absorption bands are identical in human adult and foetal HbO_2 and HbCO, the prominent fine structure band due to the trytophan in the protein appears at 289·8 mμ in foetal human haemoglobin, whereas human adult haemoglobin exhibits this band at 291·0 mμ, the position normal for most proteins. This is not a general difference between mammalian foetal and adult haemoglobins as sheep and rat foetal haemoglobins exhibit this band in the normal protein position, 291·0 mμ[16].

5. *Amino acid composition* : R. R. PORTER and F. SANGER[17] have shown that human adult haemoglobin contains 5 terminal valyl residues per molecule compared with 2·6 in human foetal haemoglobin, assuming a molecular weight of 66,000 in each case.

6. O_2 *dissociation* : in the red cell adult Hb has a lower affinity for O_2 than has foetal Hb. In dilute solution this difference is reversed[6].

7. *Resistance to alkali* : foetal haemoglobin is much more resistant to alkali than adult haemoglobin[18].

8. *Immunological behaviour* : R. R. DARROW, S. NOWAKOVSKY and M. H. AUSTIN[19] have shown that human adult and foetal haemoglobins are distinguishable immunologically.

9. *Molecular weight* : E. F. MCCARTHY and G. POPJAK[20] have suggested on a basis of osmotic pressure measurements at varying haemoglobin concentrations that sheep foetal haemoglobin has a

greater tendency to split into smaller molecular units than has sheep adult haemoglobin. H. GUTFREUND[21] has examined this property in human haemoglobins and reports that there is no difference between human adult and foetal haemoglobins in their tendency to split on dilution. He also reports that the molecular weight of human foetal haemoglobin, deduced from osmotic pressure measurements, is the same as that of the adult, 67,000. There is so far no satisfactory sedimentation and diffusion constant study of human foetal haemoglobin, but this is at present being carried out on the preparations described here, by R. CECIL on the Oxford ultracentrifuge.

X-ray data[11] show human adult HbCO, HbO_2 and MetHb to be orthorhombic and not tetragonal as stated by Drabkin[9, 10], though the difference in crystal measurements required by a tetragonal system is only very slight. It is unfortunate that human HbCO crystals did not give x-ray diffraction patterns which could yield further information about the molecule.

Human adult Hb crystallizes in the monoclinic system (and perhaps also in the orthorhombic system). Foetal HbCO, HbO_2 and MetHb crystallize in a system different from that of the adult derivatives, probably triclinic. Foetal Hb, like adult Hb, crystallizes in a system different from that of the corresponding HbCO, HbO_2 and MetHb.

The solubility data (*Figures* 9 and 10) given here do not suggest the presence of more than one component in HbCO crystallized from human adult blood. The variation in solubility of HbCO with ionic strength of phosphate buffer *p*H 6·7 (*Figure* 10 curve 1) is linear and is represented by Cohn's equation ($\log S = \beta - K_s^1 \, {}^{\Gamma}/_2$) for ionic strengths, ${}^{\Gamma}/_2 = 4 - 6$. This data agrees with that of Green, Cohn and Blanchard[7]. The discontinuity at ionic strength about ${}^{\Gamma}/_2 = 5{\cdot}5$ in the corresponding curve (phosphate *p*H 6·5) for human HbCO given by J. ROCHE, Y. DERRIEN and M. MOUTTE[22] could not be confirmed in the present work and may have been in part due to the formation of MetHb as their experiment was carried out at 22°C. These preparations of crystalline adult HbCO and HbO_2 also appear homogeneous by electrophoresis[23, 14] and in the ultracentrifuge[24].

Amorphous human HbCO has been found here to be more soluble than the crystalline material in potassium phosphate buffers at *p*H 6·7 and 0°C. Roche, Derrien and Moutte[22] have compared the variations in solubility with ionic strength for crystalline and amorphous HbCO of horse, dog and pig in phosphate buffer, *p*H 6·5 and 22°C. In each the amorphous preparation was more soluble than the crystalline. In ammonium sulphate, also, the amorphous material (HbCO of horse and rabbit) was more soluble than the crystalline, even after estimating a correction for differences in their *p*H and temperature.

The more theoretical aspects of the relation of crystallinity to solubility are discussed by G. S. HARTLEY[25].

The *p*H curve (*Figure* 11) is very flat between *p*H 6·5 and *p*H 7·1. This flatness is possibly to be expected where the protein is able to combine to such a large extent with ions, but it is difficult to be sure that the ionic strengths of the buffers are exactly comparable for all the *p*H's studied. The simple theory of phosphate dissociation[26] is probably not quite adequate for these very strong solutions.

The solubility of adult HbCO, HbO_2 and MetHb in strong salt solution (2M phosphate) decreases at first with temperature, then increases again. Adult Hb has no solubility-temperature coefficient in this solvent but in dilute sodium chloride solution the solubility increases rapidly with temperature. It is a property often observed in proteins that in strong salt solutions the solubility decreases as the temperature rises, while in salt free or in very dilute salt solution the solubility increases as the temperature rises. A. A. GREEN[27] claims to have crystallized human haemoglobin using this property. The solubility-temperature curve has therefore a different shape according to whether the solubility is measured in strong or in dilute salt solution. There appears to be a discontinuity between these two measurements for human adult HbCO which has no true solubility in phosphate buffers of ionic strength less than about $\Gamma/_2 = 4$ (*p*H 6·7). Horse HbCO has a true solubility[7] at $\Gamma/_2 = 2$.

Human adult HbCO crystals contain 58·6 per cent HbCO, the molecules are isodiametric and their arrangement in the lattice approaches that of close packed spheres, each with its sheath of bound water, the remaining 41.4 per cent being salt and water[28]. This sets an upper limit to the solubility in salt solutions, and it is difficult to see how Drabkin[9] could have obtained a 63 per cent HbO_2 solution. A solution 40 per cent saturated with HbCO is extremely viscous.

CONCLUSIONS

This work shows that in spite of the difficulties in obtaining solubility data from such viscous solutions, the results can lead to some useful classifications among haemoglobins and their derivatives. Human Hb is distinct from HbCO, HbO_2 and MetHb (both in the adult and in the foetal series) in crystal form and solubility behaviour and the same distinction is clear in these and many other properties between the adult and the foetal haemoglobins themselves.

We wish to thank the Bernhard Baron Memorial Laboratory, Queen Charlotte's Hospital, London for co-operation in supplying foetal blood and for some of the photographs, Dr. A. H. T. Robb-Smith, Department

of Pathology, Radcliffe Infirmary, Oxford and Dr. R. Barer, Department of Human Anatomy, Oxford, for other photographs. This research was supported by a grant to one of us (H.M.J.) from the Medical Research Council.

Received September 1948

REFERENCES

[1] HÜNEFELD, F. L. *Die Chemismus in der thierischen Organisation, Leipzig,* (1840) 160
[2] FUNKE, O. *Atlas of physiological chemistry*, supplement to Lehmann's *physiological chemistry*, (London, 1852) Plate X, Figs 1 and 6
[3] REICHERT, E. T. and BROWN, A. P. *The Crystallography of the Hemoglobins*, Carnegie Institute of Washington, Pub. No. 116 (1909) 317
[4] AMANTEA, G. *Ber. ges. Physiol.* 23 (1924) 167
[5] PERRIER, C. and JANELLI, P. *Arch. Fisiol.* 29 (1931) 289
[6] HAUROWITZ, F. *Hoppe-Seyl. Z.* 232 (1935) 125
[7] GREEN, A. A., COHN, E. J. and BLANCHARD, M. *J. biol. Chem.* 109 (1935) 631
[8] CANNAN, R. K. and REDISH, J., in MUDD, S. and THALHIMER, W. *Blood substitutes and blood transfusion* Springfield and Baltimore (1942) 147
[9] DRABKIN, D. L. *Amer. J. med. Sci.* 209 (1945) 268
[10] — *J. biol. Chem.* 164 (1946) 703
[11] PERUTZ, M. F. *Nature, Lond.* 160 (1947) 786
[12] PAULING, L. and CORYELL, C. D. *Proc. nat. Acad. Sci., Wash.* 22 (1936) 210
[13] CORYELL, C. D., STITT, F. and PAULING, L. *J. Amer. chem. Soc.* 59 (1937) 633
[14] HOCH, H. Unpublished work (1948)
[15] ANDERSCH, M. A., WILSON, D. A. and MENTEN, M. L. *J. biol. Chem.* 153 (1944) 301
[16] JOPE, E. M. This volume, p. 216
[17] PORTER, R. R. and SANGER, F. *Bio-chem. J.* 42 (1948) 287
[18] HAUROWITZ, F. *Hoppe-Seyl. Z.* 186 (1930) 141
[19] DARROW, R. R., NOWAKOVSKY, S. and AUSTIN, M. H. *Arch. Path. Lab. Med.* 30 (1940) 873
[20] MCCARTHY, E. F. and POPJAK, G. *Nature, Lond.* 159 (1947) 198
[21] GUTFREUND, H. Personal communication (1948)
[22] ROCHE, J., DERRIEN, Y. and MOUTTE, M. *C.R. Soc. Biol., Paris* 135 (1941) 1235
[23] MORRIS, C. J. O. R. Unpublished work (1946)
[24] CECIL, R. Unpublished work (1947)
[25] HARTLEY, G. S. *Quart. Rev. chem. Soc.* 2 (1948) 160
[26] GREEN, A. A. *J. Amer. chem. Soc.* 55 (1933) 2335
[27] — *J. biol. Chem.* 93 (1931) 495
[28] BOYES-WATSON, J., DAVIDSON, E. and PERUTZ, M. F. *Proc. roy. Soc., A.* 191 (1947) 83

A Solubility Study of Foetal and Adult Sheep Haemoglobin

M. J. KARVONEN*

The solubility of sheep foetal and adult haemoglobin was investigated in buffered ammonium sulphate solutions. The effect of three variables, pH, salt and protein concentrations, was tested. The results were interpreted as indicating that both foetal and adult Hb were composed of two nearly related components. The transition from the foetal to the adult type occurred during the last month of foetal life and the first month of post-natal life. The mixtures of the adult and foetal type Hb's showed solubilities which were more than additive. An approximate method was developed for assessing their composition.

DIFFERENCES exist between the solubilities of adult haemoglobin and foetal haemoglobin (Hb). G. ZANIER[1] in the course of haemolysis experiments observed, that foetal calf haemoglobin started to precipitate at lower NaCl concentrations than those needed for the Hb. of the adult cow. Recently J. WYMAN, J. A. RAFFERTY and E. N. INGALLS[2] have studied the salting out of foetal calves' Hb with strong phosphate buffers. The solubility of the Hb was investigated in a constant solvent, and the equilibrium was approached from the supersaturated side. Under the conditions of the study the foetal Hb was much more soluble than the adult. Both Hb's, however, showed a rather anomalous solubility ; a further increase of the protein concentration in the system beyond the onset of crystallization resulted in a great and rapid decrease in the solubility. This anomaly made it impossible to draw any conclusions as to the homogeneity either of the foetal or of the adult cow Hb.

The programme of the present work has been to follow the solubility of sheep Hb from early midfoetal up to adult life. The Hb of adult sheep has also been investigated. Both the ' variable solvent '—or salting out—and the ' constant solvent ' methods have been used. An attempt has been made to give the—often anomalous—results of the solubility measurements a physico-chemical interpretation and to correlate the findings with the physiology of the foetus.

* *Present address : Institute of Physiology, Helsinki University*

METHOD

Welsh sheep were used. Solubility experiments were performed (*a*) on samples from 24 foetuses, between the foetal age of 51 and 145 days ; (*b*) on 31 samples of lamb's blood, obtained from 9 lambs between birth and the 137th day of post-natal life ; (*c*) on samples from 18 adult sheep, the oldest being 21 years old.

The Hb solutions were prepared as 4 per cent carboxyhaemoglobin, by laking washed corpuscles with distilled water. Stroma was removed by filtering with kieselguhr, and by precipitating the non-Hb proteins with an ammonium sulphate concentration just below the onset of the precipitation of Hb. (The ionic strength of $(NH_4)_2SO_4 = 4{\cdot}5$.) The clear solutions were buffered by adding an equal volume of a $\Gamma/2 = 4{\cdot}5$ $(NH_4)_2SO_4$ solution, made to 0·4 M strength in buffer, in which the proportion of primary and secondary ammonium phosphate was varied according to the *p*H desired. The Hb was precipitated by adding weighed amounts of $(NH_4)_2SO_4$. All the operations were carried out at the temperature of $+ 1{\cdot}5 \pm 0{\cdot}3°C$, and the samples were kept under a CO-atmosphere.

It is generally recommended that the equilibration of protein solutions with the protein in the solid phase be approached from the supersaturated side. It turned out, however, that with the haemoglobins investigated, the contrary method, approaching the equilibrium from the undersaturated side, gave more satisfactory results. The haemoglobins had a tendency to form supersaturated solutions, and the tendency to supersaturation was the more pronounced the higher the proportion of the total Hb in solution. When the equilibrium between the precipitated Hb and the solution was approached from the undersaturated side, the results were less anomalous and more predictable. The Hb was precipitated by adding $(NH_4)_2SO_4$ to make its concentration $\Gamma/2$: 9·0, and the total ionic strength correspondingly 9·92. The salt was allowed to dissolve slowly, during several days. Then the Hb suspension was homogenized by stirring, and measured aliquots were pipetted into a series of test tubes. In a 'variable solvent' series the ammonium sulphate concentration was adjusted to values varying from $\Gamma/2$: 4·5 to 9·0, by adding equal volumes of buffered ammonium sulphate solutions of suitable strengths. In a 'constant solvent' series varying dilutions of the suspension were made in a series of tubes with equal electrolyte concentration. The protein concentration was always kept small—below 2 per cent—in order to minimize the disturbing effect of the uneven distribution of the electrolytes between the crystal water and the mother liquor. The equilibrations were carried on for a fortnight. The two phases were kept satisfactorily mixed by gentle stirring. After a fortnight, no

further increase of the haemoglobin concentration in the solution could be observed. Then the dissolved protein and the precipitate were separated, and the concentration of CO–Hb in the solution was measured colorimetrically.

A portion of the precipitate was examined using an ordinary microscope.

RESULTS

The effect of *p*H on the solubility of the Hb of a foetus, 100 days old, and on that of its mother was investigated. The equilibrium was approached both from the undersaturated and the supersaturated side. *Figure* 1 shows the results of the determinations. At *p*H 5·5 the solubilities of the two proteins are the same, at more acid *p*H's the foetal Hb is the more soluble, and at more alkaline *p*H's the adult Hb is more soluble than the foetal Hb. The shapes of the foetal and adult graph are widely different. At *p*H 7·2 the solubility of the maternal Hb is more than 20 times higher than that of the foetal Hb. The shape of the maternal graph is rather similar to the *p*H-solubility graph of adult horse Hb as published by S. P. L. SÖRENSEN and M. SÖRENSEN[3]. This *p*H-solubility graph is, however, by no means typical of all adult mammals, *e.g.* the corresponding graph for adult human Hb has a very different course. (Unpublished observations.)

In ' variable solvent ' experiments, the logarithm of the solubility of a pure protein is expected to be linearly related to the electrolyte concentration. Several samples of adult Hb were studied using this method, at two widely different *p*H's—*p*H 4·7 and 7·2. The solubility of the different samples was not always identical, and neither was the log solubility graph always strictly linear (*Figure* 2). Still, the graphs approached linearity. The same was found to be true of the samples obtained from foetuses younger than 120 days (*Figure* 3). However, after the 120th day of the foetal life and during the first month of post-natal life the solubility diagrams at varying electrolyte concentrations were largely irregular.

Theoretically, a solubility graph of the ' variable solvent ' type can be expected to deviate markedly from linearity, only if the components of a mixture have considerably different additive solubilities. If the solubilities of the components, however, overlap to a great extent, the deviation from linearity is difficult to observe. Therefore, both adult and foetal Hb were investigated also according to the ' constant solvent ' technique. It turned out that the solubility diagrams thus obtained in both cases were characteristic of a two-component system. *Figure* 4 shows the solubility diagram of the Hb of a foetus, 114 days old, at three different *p*H's. When the solubilities of the two com-

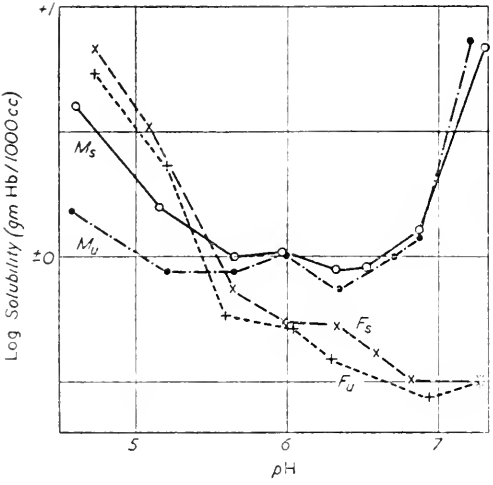
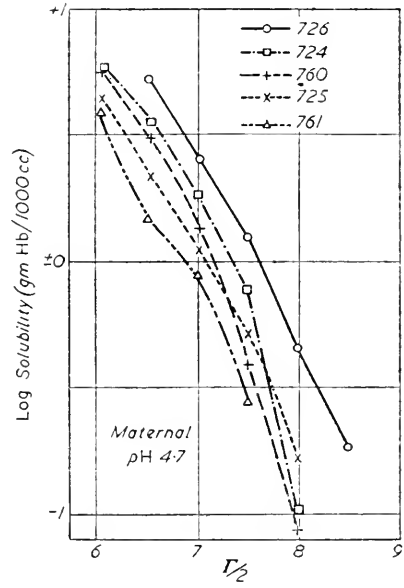


Figure 1. *The effect of varying* p*H on the solubility of the haemoglobins of a foetus,* 100 *days old, and on that of its mother.* M_u *: maternal, and* F_u *: foetal Hb, equilibrium approached from the undersaturated side,* $\Gamma/2 = 7{\cdot}26$. M_s = *maternal,* $\Gamma/2 = 7{\cdot}99$, *and* F_s = *foetal,* $\Gamma/2 = 7{\cdot}39$, *in both the equilibrium approached from the supersaturated side.*

Figure 2. *The solubility of five maternal samples of Hb at* p*H* 4·7.



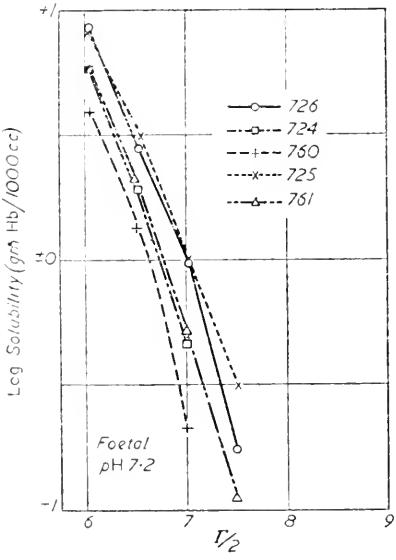


Figure 3. *The solubility of five foetal samples of Hb at* p*H* 7·2. *The foetal ages : No.* 724 = 74 *days, No.* 725 = 85 *days, No.* 760 = 114 *days, and No.* 761 = 115 *days.*

ponents are calculated according to the diagrams, it is found that they are in the following relation to each other :

*p*H	7·2	5·9	4·6
$\frac{\textit{Solubility of A}}{\textit{Solubility of B}}$	1/1·92	1/1·72	1/1·14

The *p*H-solubility graphs are, therefore, essentially parallel, and do not differ as much as, *e.g.*, those of the foetal and maternal Hb. When the composition of the mixture is calculated according to the three diagrams, the following percentages are obtained :

*p*H	7·2	5·9	4·6
Percentage of A	12%	19%	20%
Percentage of B	88%	81%	80%

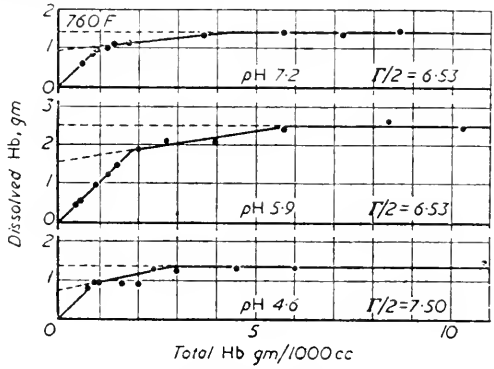


Figure 4. *The solubility of a sample of sheep foetal Hb in three solvents, with varying total Hb concentration. Equilibration from the undersaturated side.*

The agreement between these three values is to be regarded as satisfactory. That the proportion of the components is not essentially affected by great variations in the *p*H, makes it probable that the components are preformed and not artifacts.

Until the 120th day of foetal life the precipitate had been composed of one type of crystals only, greatly different from those of the adult. During the last month of the foetal life and the first month of post-natal life the precipitate became irregular, sometimes amorphous (possibly microcrystalline), and crystals of the adult type began to appear. Simultaneously with this change in the appearance of the solid phase, the solubility of the Hb samples increased. The solubility was often, however, much higher than could be accounted for by assuming, that foetal and adult Hb were present as a mixture, each component showing an additive solubility.

In order to investigate whether it was necessary to assume the presence of an unusually soluble new Hb, or whether the anomalously high solubilities were due to some interactions of the foetal and adult Hb's, artificial mixtures of adult and foetal Hb were made. It was found, that the anomalous solubilities observed in the samples from the transition period could be closely reproduced by making suitable artificial mixtures of foetal and adult Hb. The atypical solubility of the mixtures was paralleled by irregularities in the solid phase. There was, therefore, no necessity to introduce a new Hb, and, on the basis of the observed analogies, the transition could be simply ascribed to a gradual intermixture of the foetal with the adult type of Hb.

By using a suitable procedure, one could achieve a partial separation of the foetal and adult type Hb's from the transition samples, but completely pure fractions were not obtained with the applied methods.

As the artificial and naturally occurring mixtures of adult and foetal Hb showed interactions affecting their solubility, no simple solubility method could be used for estimating the percentage of the components. An attempt was made, however, to use the observed solubilities of the artificial mixtures at two widely different *p*H's for assessing, purely empirically, the approximate composition of the samples from the transition period. This method can by no means be regarded as an exact one, as neither of the components of the artificial mixtures evidently is a single substance. If the mixtures were composed of only two proteins, then theoretically, of course, even the anomalous solubilities ought to be thermodynamically strictly determined. Several series of artificial mixtures were made. On the

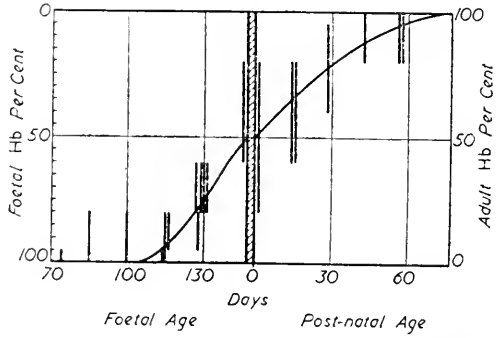


Figure 5. The approximate composition of the Hb during late foetal and early post-natal life, as based on the analogous solubility of the artificial mixtures. Each vertical line represents the limits of the composition of each sample.

basis of the solubility characteristics common to every series the approximate composition of the transition samples was determined. In *Figure* 5 the probable limits of the composition of each sample is indicated with a vertical line. The change from 100 per cent foetal to 100 per cent adult type Hb is passed within two or three months.

One lamb, whose Hb was followed from the age of 3 to 137 days, was an exception to the rest of the animals. In this lamb the transition could not be explained as a gradual intermixture of the foetal with the adult Hb. The transition obviously led to the production of a third type of Hb. At *p*H 4·7 both its solubility and the crystal habit corresponded to the adult type of Hb, whereas at *p*H 7·2 the final Hb had the properties of the foetal type of Hb. It apparently was not a mixture of these two substances. Further studies on this lamb were interrupted by its death, the cause of which remained undetermined.

DISCUSSION

The physico-chemical methods which have been used in the identification and purification of proteins have different degrees of selectivity. As to the homogeneity of a given sample of protein, only suitably conducted solubility experiments can be expected to give an ultimate answer. The electrophoresis and the ultracentrifuge can indicate the presence of a mixture, but, on the other hand, they never can exclude the possibility of its existence. The evidence of the present work suggests, that both foetal and adult sheep Hb are composed of two nearly related proteins. This finding, however, still requires to be confirmed by the separation of the pure components.

The equilibrium achieved between the dissolved protein and the solution was different when it was approached from either the supersaturated or from the undersaturated side. There obviously is in the Hb solutions some factor, which tends to stabilize the supersaturated state. The uneven electrolyte distribution between the crystal water and the mother liquor can hardly be the sole cause of this supersaturation. The finding, that the tendency to supersaturation was the less the higher the proportion of the total Hb in the precipitate, suggests that this supersaturation may be due to some 'protective colloid' which is precipitated with the Hb, but more rapidly than the Hb itself. This question, however, requires further research.

The mixtures of foetal and adult type Hb showed much higher solubilities than the sum of the solubilities of the components. This phenomenon of supersaturation occurred independently of the direction, from which the equilibrium was approached. This increase in the solubility can be understood as a manifestation of an increased

disorder in the solid phase, but the existence of some interactions in the dissolved phase can also not be excluded.

The long duration of the foetal-adult transition—about two months—makes it rather probable, that the transition means a change in the Hb synthesis, not a peripheral change in the circulating erythrocytes. This view is also supported by the finding that *in vitro* the mixtures apparently are quite stable.

The erythrocytes of the foetus are histologically different from those of an adult. The appearance of the adult type of Hb and the histological transition are not, however, strictly parallel. The transition of the blood picture from foetal to adult is a gradual change, which is in progress already before the appearance of the adult type of Hb.

During the main period of the foetal life, the liver is the most important site of blood formation. Bone marrow, however, attains an increasing importance as an erythropoietic organ towards the end of the foetal life. But again, there is no parallelism between the development of bone marrow and the appearance of the adult type Hb, because the bone marrow in the sheep foetus contributes increasingly to the erythropoiesis already from the age of approximately 60 days onwards. The causal mechanism of the transition remains an unsolved question.

SUMMARY

1 In adult sheep the haemoglobin (Hb) is salted out approximately according to Cohn's equation, but if a constant solvent solubility study is made by varying the concentration of the protein, the Hb behaves in a way, which suggests the presence of two probably nearly related components.

2 The same is true of the Hb of foetal sheep before the foetal age of about 115 to 120 days. The solubility of foetal Hb is, however, widely different from that of the adult Hb ; at *p*H 7·2 the adult Hb is about 20 times more soluble than the foetal one.

3 During the last month of the foetal life and the first month of the post-natal life the solubility of the Hb is analogous to the solubility of artificial mixtures made of adult and foetal Hb's. The solubility is generally higher than additive. The solubility of artificial mixtures can be used as a purely empirical basis for the approximate estimation of the percentage composition of the Hb during the transition period.

4 In exceptional cases the transition may result in the production of a third type of Hb.

5 The causative mechanism of the transition remains unknown.

The author wishes to express his gratefulness to the late Sir Joseph Barcroft, who at all stages of the present work gave the author encouragement and criticism. The author is also greatly indebted to Professor D. Keilin, F.R.S., to Professor F. J. W. Roughton, F.R.S., to Mr G. S. Adair, F.R.S., and to Dr D. B. Taylor, for their valuable advice on many points.

Received August 1948

REFERENCES

[1] ZANIER, G. *Arch. ital. Biol.* 25 (1896) 58
[2] WYMAN, J., RAFFERTY, J. A. and INGALLS, E. N. *J. biol. Chem.* 153 (1944) 275
[3] SÖRENSEN, S. P. L. and SÖRENSEN, M. *C. R. Lab. Carlsberg* 19 No. 11 (1933)

VIII

COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY OF OXYGEN CARRIERS

Chlorocruorin

H. MUNRO FOX

Chlorocruorin is a red-green dichroic respiratory protein only found dissolved in the blood plasma of certain marine worms. It is chemically closely related to haemoglobin. Spectroscopically its haem is nearer to that of cytochrome a *than to protohaem. Chemically it differs from protohaem by the oxidation of one vinyl group. Chlorocruorin has a low affinity for oxygen and a higher affinity for carbon monoxide than any haemoglobin.* Serpula *has both chlorocruorin and haemoglobin in its blood. Chlorocruorin has never been found within a cell; those animals which possess it in their blood have protohaem, not chlorocruorohaem, in their tissues.*

THERE exist four coloured proteins with a respiratory function which are found in the blood or body-cavity fluid of animals. These are haemoglobin, chlorocruorin, haemocyanin and haemerythrin. The first two are close chemical relatives. Chlorocruorin is dichroic, being red when concentrated, green when dilute. The only place in which it is found is in solution in the blood of certain marine polychaete worms, particularly of the group Serpulimorpha. This pigment received its very appropriate name from Ray Lankester in 1867. His pioneer studies established its similarity to haemoglobin, but from that time until 1925 chlorocruorin was almost completely neglected. In the latter year Sir Joseph Barcroft pointed out to me that there must be pigments among the invertebrate animals which are somewhat like haemoglobin but different from it. Stimulated by this, I went to the marine biological station at Roscoff in Brittany and started to investigate chlorocruorin in the blood of Spirographis (*Sabella spallanzanii*)[1].

Chlorocruorin, oxygenated and deoxygenated, has absorption spectra analogous to those of haemoglobin, but the bands are shifted towards the red end of the spectrum. Its derivatives, such as the haemochromogen and the porphyrin, likewise have bands whose axes have longer wave lengths. Spectroscopically, chlorocruorohaem (which Warburg subsequently misnamed ' Spirographishämin ') recalls the haem of cytochrome *a*, in that each of them forms haemochromogens with α-bands towards the red end of the spectrum. The pyridine-haemochromogen of chlorocruorohaem has its α-band at 583 mμ, that of cytochrome *a* at 587 mμ, while that of the protohaem of haemoglobin is at 557 mμ. Chlorocruoroporphyrin has been found

by H. FISCHER and C. VON SEEMANN[2] to differ from protoporphyrin only in one side chain of one of its 4 pyrrol rings, where a vinyl is oxidized to an aldehyde group. This constitution of chlorocruoroporphyrin was confirmed by H. FISCHER and K. O. DEILMANN[3], who synthesized it by the oxidation of one vinyl group of protoporphyrin.

Oxygen attaches itself to the iron of chlorocruorin in the same numerical atomic ratio as it does to the iron of haemoglobin : two atoms of oxygen to one of iron[4]. Chlorocruorin has a much lower affinity for oxygen than most haemoglobins. At 17°C and *p*H 7·7 the chlorocruorin of *S. spallanzanii* is 50 per cent in the oxy state at an oxygen pressure of 27 mm Hg[5]. At the same temperature and in the absence of carbon dioxide the oxygen pressures for 50 per cent oxyhaemoglobin are 0·6 mm Hg for *Chironomus riparius* and 3·1 mm Hg for *Daphnia magna*[6]. These are the highest and one of the lowest oxygen affinities of all blood haemoglobins. The Serpulimorpha are sluggish sessile animals and considering their mode of life they have a surprising quantity of chlorocruorin in their blood ; indeed, the oxygen capacity is greater than that of any other invertebrate blood, whatever its respiratory pigment. While the oxygen affinity of chlorocruorin is low, however, its affinity for carbon monoxide is higher than that of any haemoglobin[7].

Until lately it was thought that all species of the group Serpulimorpha have chlorocruorin in their blood. But this is not the case[8]. I have found chlorocruorin in 21 species of Serpulimorpha, belonging to 15 genera. One of these species is *Spirorbis borealis* ; but another species in the same genus, namely *S. corrugatus*, has haemoglobin instead of chlorocruorin in its blood, while a third one, *S. militaris*, has neither blood pigment. The three species live in the sea in similar situations and one can suggest no functional reason for the differences in respiratory pigment.

Moreover, in the genus *Serpula* the blood is greenish brown, not red or green. This is because it contains both chlorocruorin and haemoglobin. Here, for the first time, two respiratory pigments have been found in the blood of one animal. With the spectroscope three absorption bands are seen : α-oxychlorocruorin, with α- and β-oxyhaemoglobin ; the small β-band of the former pigment is hidden by α of the latter. Young individuals have more haemoglobin, older ones more chlorocruorin. Chlorocruorin, present only in two specialized groups of polychaete worms, may be looked upon as a biochemical mutation of haemoglobin. In this case the greater concentration of the latter pigment in young than in older *Serpula* would be a case of recapitulation.

There is only one genus in the Serpulimorpha which has a respiratory pigment in a cellular tissue. This is *Potamilla*, whose muscles are

coloured pink by haemoglobin. In the blood, as one might expect, there is chlorocruorin. No muscles or other cells of any animal have been found to contain chlorocruorin.

Most animal cells contain protohaem (Keilin). It is therefore of particular interest to know whether—apart from *Potamilla*—the tissue cells of animals with chlorocruorin dissolved in their blood contain chlorocruorohaem or protohaem. In *Sabella spallanzanii* and *Protula intestinum*, the biggest available animals with chlorocruorin in their blood, no chlorocruorohaem can be found in tissues: the muscles, eggs and sperm contain protohaem alone. Chlorocruorohaem must, of course, be synthesized somewhere to supply the blood, but we do not as yet know where.

Received July 1948

REFERENCES

[1] Fox, H. Munro *Proc. roy. Soc. B*, 99 (1926) 199
[2] Fischer, H. and von Seemann, C. *Hoppe-Seyl. Z.* 242 (1936) 133
[3] — and Deilmann, K. O. *Hoppe-Seyl. Z.* 280 (1944) 186
[4] Fox, H. Munro *Proc. roy. Soc. B* 115 (1934) 368
[5] — *Ibid* 111 (1932) 356
[6] — *J. exp. Biol.* 21 (1945) 161
[7] — *Nature, Lond.* 162 (1948) 20
[8] — *Nature, Lond.* 160 (1947) 825

The Haemoglobins of Ascaris lumbricoides *var* suis

H. E. DAVENPORT

Two distinct haemoglobins have been extracted from Ascaris lumbricoides. *Both have a very high oxygen affinity and neither can be completely deoxygenated* in vacuo. *The oxygen dissociation velocity was measured. Perienteric fluid haemoglobin in presence of* $Na_2S_2O_4$ *at p*H 6, 20·5°C *is half deoxygenated in* 150 sec. *compared with* 0·008 *sec. for sheep haemoglobin. With body wall haemoglobin the reaction is more rapid; at p*H 6, 3°C, $t_{50} = 80 \pm 10$ *sec. The reaction is accurately unimolecular and its rate independent of the concentration of the reducing agent. It is therefore a deoxygenation and not a true reduction. The deoxygenation velocity of perienteric fluid haemoglobin increases with p*H *between p*H 5 *and p*H 9 *and has a temperature coefficient of* 5. *Carbon monoxide dissociates from the haemoglobins more rapidly than oxygen;* 300 *times more rapidly from perienteric fluid haemoglobin at* 3°C, *p*H 6. *By its oxidative metabolism the parasite is capable of deoxygenating the body wall haemoglobin which may have some significance as a short period store of oxygen.*

THE presence of two distinct haemoglobins in *Ascaris lumbricoides*, the large roundworm of the pig, was recorded by D. KEILIN[1]. One haemoglobin occurs in the perienteric fluid and the other in the body wall of the parasite. They differ from each other, and from the haemoglobin of the host, in the position of their absorption bands. Perienteric fluid oxyhaemoglobin has the α band at 5784Å compared with 5798Å for the body wall pigment.

The nature of the metabolic processes in *Ascaris*, whether aerobic or anaerobic, has been for many years the subject of controversy. Recently H. LASER[2] has shown that the oxidative enzyme systems of the parasite are well adapted to function at low oxygen tensions. The possibility that the worm haemoglobins participate in supplying the low oxygen requirement appeared to justify a study of their properties.

The concentration of the haemoglobins is low and subject to considerable individual variation but the absorption bands of oxyhaemoglobin are always visible when worms are observed spectroscopically against a strong light. An average sample of the perienteric fluid

contains $3{\cdot}3 \times 10^{-5}$M haematin, representing as haemoglobin 2 per cent of the total protein. Individuals having higher or lower concentrations of haemoglobin in the perienteric fluid exhibit a parallel variation in the body wall haemoglobin concentration.

For a study of the properties of the haemoglobins they were extracted and partially purified. From the perienteric fluid, by fractionation with $(NH_4)_2SO_4$, preparations containing $2{\cdot}5 \times 10^{-4}$M haematin were obtained. Haemoglobin represented 12 per cent of the dry weight. Similar preparations were obtained from the body wall after the haemoglobin had first been extracted by soaking in distilled water.

In these concentrated solutions the two haemoglobins retained the characteristic difference in their absorption band positions but the spectra differed from mammalian oxyhaemoglobins in two important respects. The absorption bands are more diffuse and the density of the β band is greater than that of the α band. A similar type of spectrum has been reported by D. KEILIN and Y. L. WANG[3] for the oxyhaemoglobin they extracted from root nodules of leguminous plants.

In order to confirm that the pigments are true oxyhaemoglobins it was necessary to show that they possess the property of reversible oxygenation. Both however were found to have a remarkable resistance to deoxygenation when solutions were equilibrated against a frequently renewed vacuum. After equilibration for 3 hours at 20°C the body wall haemoglobin was 50 per cent deoxygenated but only slight evidence of deoxygenation could be observed in the perienteric fluid haemoglobin. On treating the solutions with sodium hyposulphite the two bands disappear and are replaced by a single band with the maximum at 555mμ. What is remarkable is that the change occurs slowly in contrast to the apparently instantaneous deoxygenation of other oxyhaemoglobins when they are treated with this reagent.

The kinetics of the dissociation of oxygen from haemoglobin was first investigated by H. H. HARTRIDGE and F. J. W. ROUGHTON[4] using sheep haemoglobin. The measurements have been extended by G. A. MILLIKAN[5, 6] to other vertebrate haemoglobins and by K. SALOMON[7] to two invertebrate haemoglobins. In every case the reaction was so rapid that the rapid flow technique devised by Hartridge and Roughton was necessary for measurements to be made. With the *Ascaris* haemoglobins, on the other hand, the slowness of the reaction made possible the use of a simple static method.

In presence of $Na_2S_2O_4$ *in vacuo* the reaction follows a strictly unimolecular course with both haemoglobins and its velocity is independent of the concentration of the reducing agent. By the

criteria of Hartridge and Roughton[4] direct reaction between the reducing agent and oxyhaemoglobin, if it occurs, must play an insignificant part in the overall reaction, which therefore has the characteristics of a deoxygenation and not of a true reduction. *Figure* 1 illustrates the result of a typical experiment using perienteric fluid haemoglobin. The time for half completion of the reaction (t_{50}) is 150 sec compared with 0·008 sec for sheep haemoglobin under the same conditions[4]. The reaction velocity of the body wall pigment occupies an intermediate position between these extremes. With a fresh preparation at 3°C, *p*H 6, t_{50} was 80 $\pm$ 5 sec but a decrease in the reaction velocity was observed in old preparations. No change of this kind could be detected with perienteric fluid haemoglobin and this pigment was used in the study of the effect of other factors on the deoxygenation velocity.

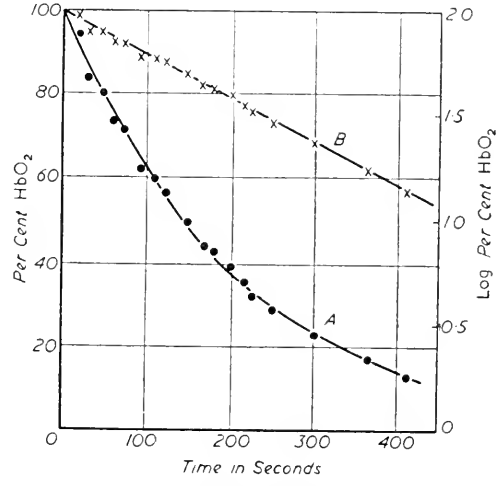


Figure 1. *Deoxygenation of* Ascaris *perienteric fluid oxyhaemoglobin in presence of* $Na_2S_2O_4$ in vacuo. *p*H 6, 20·5°*C.*

A—ordinates on left.
B—ordinates on right.

Hartridge and Roughton[4] have shown that the Bohr effect in the oxygen equilibrium curves of mammalian blood is the result of change in the dissociation velocity with change in *p*H. Thus with sheep haemoglobin at *p*H 5 deoxygenation proceeds about 8 times more rapidly than at *p*H 9. The influence of *p*H on the dissociation of oxygen from *Ascaris* perienteric fluid haemoglobin was found to be smaller but reversed in direction. At 20°C the reaction velocity at

*p*H 9 is double that at *p*H 6. R. M. FERRY and A. A. GREEN[8] have reported a reversal of the Bohr effect in mammalian bloods below *p*H 6·5 but no kinetic studies upon this phenomenon have been published.

Temperature change has a marked influence upon the reaction velocity. In a series of measurements between 4°C and 20°C a twentyfold increase in the reaction velocity was found to occur. The temperature effect follows the Arrhenius equation and has a temperature coefficient of 5 compared with 3·8 for sheep haemoglobin[4]. Extrapolation of the data to 38°C, the normal temperature of the environment of *Ascaris* gives $t_{50} = 10$ sec, a value far greater than the 0·0025 sec deduced by Hartridge and Roughton for sheep haemoglobin at mammalian body temperature.

The results so far described diverge widely in the magnitude of the time scale from those obtained with other haemoglobins but their validity was confirmed by a further series of experiments. According to J. B. CONANT[9] ferricyanide in presence of oxyhaemoglobin reacts only with dissociated haemoglobin. G. A. MILLIKAN[6] has used ferricyanide as a means of immobilising dissociated haemoglobin in his measurements of the dissociation velocity of muscle CO-haemoglobin. When ferricyanide replaced sodium hyposulphite in experiments with the *Ascaris* oxyhaemoglobins it was found that t_{50} was the same for both reactions. Control experiments showed that variations in the ferricyanide concentration had no effect upon the reaction velocity and that the magnitude of the back reaction of dissociated haemoglobin with liberated oxygen was insignificant.

Slow reactions of this kind are not without precedent in the gas relations of other haemoglobins. It was shown by Roughton[10] that the velocity constant for the dissociation of CO from sheep haemoglobin at *p*H 7 and 20°C is 0·044. By a coincidence this is also the value for the oxygen dissociation velocity constant of *Ascaris* perienteric fluid haemoglobin under the same conditions.

Carbon monoxide displaces oxygen very slowly from combination with the *Ascaris* haemoglobins. The spectra of the CO-haemoglobins (*Figure* 2) were measured in presence of $Na_2S_2O_4$ since rapid reversion to oxyhaemoglobin occurs if traces of oxygen are present. The CO-affinity of the haemoglobins is thus relatively low. Roughton[10] has shown that oxygen dissociates from sheep haemoglobin 10,000 times faster than CO but associates only 30 times more rapidly. The great relative affinity for CO, 250 times the affinity for oxygen, is thus principally the result of the low CO dissociation velocity. Using the ferricyanide method it was found that CO dissociates from the *Ascaris* haemoglobins more rapidly than O_2. With body wall haemoglobin,

under all conditions the reaction occurred too rapidly for measurement. With perienteric fluid haemoglobin at 3°C, *p*H 6, t_{50} is 10·3 sec, compared with 3,000 sec for the oxygen dissociation.

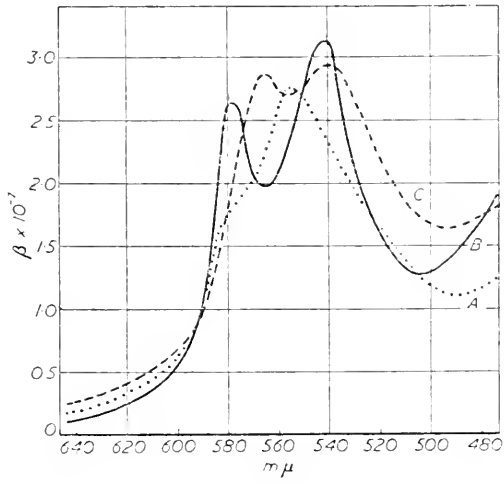


Figure 2. *Absorption spectra of* Ascaris *body-wall haemoglobin* (*A*), *oxyhaemoglobin* (*B*) *and CO-haemoglobin* (*C*).

$$\beta = \frac{2{\cdot}303}{c.d.} \cdot \log \frac{I_o}{I}$$

where c = *haematin concentration as g. mols./ml.*

d = *thickness of cell in centimetres.*

I_o *and* I = *Intensities of incident and transmitted light respectively.*

The impossibility of obtaining complete deoxygenation of the haemoglobins in the absence of a reducing agent prevented the use of a direct method for determining the oxygen equilibrium curves. R. HILL[11] has used haemoglobins of known oxygen affinities to measure the quantities of oxygen evolved when isolated chloroplasts were illuminated. The tensions of oxygen attained were low and a haemoglobin of high oxygen affinity, ox muscle haemoglobin, was used. Aqueous leaf extracts, shown by Hill to promote oxygen evolution from isolated chloroplasts, were found to bring about a slow deoxygenation of the *Ascaris* oxyhaemoglobins when they are incubated together in a vacuum. If chloroplasts were then added to the deoxygenated haemoglobin and illuminated a rapid and complete reoxygenation of the haemoglobin occurred. The progress of the reoxygenation was measured and compared with that of ox muscle

haemoglobin under the same conditions. The results of these experiments showed that, with an experimental error of 5 per cent in the method of measurement, equal increments of oxygen evolved produced equal increments of oxyhaemoglobin concentration up to complete saturation. The oxygen affinity of the *Ascaris* haemoglobins is therefore too great for the equilibrium curve to be measured within the limits of an experimental error of this magnitude.

In view of this extremely high oxygen affinity it is necessary to know, before possible functions of the haemoglobins can be suggested, whether the bound oxygen is available to the oxidative enzyme systems of the parasite. It was found, by experiments in which worms were subjected to anaerobic conditions, that a slow deoxygenation of the body wall haemoglobin occurs. No evidence was obtained for a similar deoxygenation of the perienteric fluid haemoglobin. It is therefore possible that the body wall haemoglobin acts as a short period store of oxygen available in conditions of extreme oxygen deficiency. The evidence for this is not, however, strong.

A haemoglobin having properties closely resembling those of the perienteric fluid haemoglobin of *Ascaris* has been observed in the perienteric fluid of *Strongylus spp.*, intestinal parasites of the horse. The deep red colour of these worms, commonly known as ' blood worms ', is due to high concentrations of this pigment. The aberrant properties of the *Ascaris* haemoglobins are not, however, shared by all nematode haemoglobins. *Nippostrongylus muris*, a small nematode parasite of the laboratory rat, contains high concentrations of a haemoglobin which, under conditions of oxygen deficiency, undergoes rapid deoxygenation *in vivo*. Extracts of the haemoglobin were found to have a high oxygen affinity. In dilute solution at 19°C, *p*H 8·2 the oxygen equilibrium curve is a rectangular hyperbola and the haemoglobin is half saturated with oxygen at a tension of less than 0·1 mm Hg, compared with 0·7 mm Hg for similar solutions of ox muscle haemoglobin.

Received September 1948

REFERENCES

[1] KEILIN, D. *Proc. roy. Soc. B.* 98 (1925) 312
[2] LASER, H. *Bio-chem. J.* 38 (1944) 333
[3] KEILIN, D. and WANG, Y. L. *Nature, Lond.* 155 (1945) 227
[4] HARTRIDGE, H. H. and ROUGHTON, F. J. W. *Proc. roy. Soc. A.* 104 (1923) 376
[5] MILLIKAN, G. A. *J. Physiol.* 79 (1933) 158
[6] — *Proc. roy. Soc. B.* 120 (1936) 366
[7] SALOMON, K. *J. gen. Physiol.* 24 (1941) 367
[8] FERRY, R. M. and GREEN, A. A. *J. biol. Chem.* 81 (1929) 175
[9] CONANT, J. B. *J. biol. Chem.* 57 (1923) 401
[10] ROUGHTON, F. J. W. *Proc. roy. Soc. B.* 115 (1934) 451
[11] HILL, R. *Proc. roy. Soc. B.* 127 (1939) 192

Oxygen and Carbon Dioxide Transport by Blood Containing Haemocyanin

H. P. WOLVEKAMP

A general survey of the properties of the haemocyanins in relation to their biological significance is given. Apart from results previously published by other workers in this field as well as by the author, some new data on the blood of Homarus *and* Cancer, *and on the respiration of* Sepia *are recorded.*

HAEMOCYANIN, the copper-containing blood pigment dissolved in the blood of the cephalopods, a number of gasteropods, the decapod Crustacea, Limulus and the scorpions, for a time excited the interest of biochemists and physiologists because oxygen dissociation curves of a simple hyperbolic shape had been obtained from experiments on solutions of some types of this blood pigment. For our understanding of the mechanism of oxygenation, however, later studies on haemoglobin, of which the chemical properties are much better known, have been much more illuminating. Now, though from a general biochemical point of view the study of haemocyanin perhaps has lost some of its attraction, it still presents a number of characteristics and offers some problems that will excite the interest of workers in the field of comparative biochemistry and physiology.

As my personal relation with physical chemistry proper rather resembles that of Moses with Canaan when he, standing on the mount of Nebo, was permitted to see the land of promise from afar but not allowed to go thither, I shall in the following confine myself mainly to the biological aspects of blood gas transport. Accordingly I shall only very briefly summarize some biochemical data.

The structure of the prosthetic group that can be split off by alkali is largely unknown. The copper it contains combines reversibly with oxygen in a ratio of two atoms of copper to one molecule of oxygen as has been ascertained by BEGEMANN[1] (1924), Redfield and his co-workers (1928) and by GUILLEMET and GOSSELIN[1] (1932).

MILLIKAN[1] (1933) found that oxygenation and reduction proceed with a velocity comparable to oxygenation and reduction of haemoglobin.

A true oxidation product, methaemocyanin can be formed, but this compound retains the power to combine reversibly with oxygen. Accordingly in gasometric determinations of the quantity of oxygen

reversibly bound, ferricyanide has to be replaced by potassium mercuric cyanide which gives with haemocyanin a compound named cyan-haemocyanin which does not combine with oxygen.

Carbon monoxide is bound but the affinity for this gas is very small.

Investigations chiefly carried out by THE SVEDBERG *et al*[2] and by J. ROCHE[3] with their co-workers on the molecular weight, the absorption spectra in visible and ultra violet light, the chemical composition, the titration curves and the position of the isoelectric point, indicate typical specific and even intra-specific differences between the haemocyanins.

Finally the mode of oxygen binding still shows characteristic specific differences when solutions of purified haemocyanin are investigated. The shape and position of the oxygen dissociation curves are strongly influenced by the presence of salts, and the reversible breaking up of the enormous molecules in definite *p*H ranges presents a complication not found in concentrated solutions of haemoglobin of vertebrates.

Every conceivable type of dissociation curve has been obtained by investigators working on haemocyanin. In the first place dialyzed solutions of haemocyanin of the edible snail yield a simple hyperbolic curve at different *p*H thus indicating that the prosthetic groups of each molecule have the same affinity for oxygen and do not mutually influence each other (STEDMAN and STEDMAN[1] 1928). The dissociation curve of the blood of the same animal, however is typically s-shaped (STEDMAN and STEDMAN[1] (1929), WOLVEKAMP and KERSTEN[4]). REDFIELD and Miss INGALLS[1] (1933) in a series of very careful experiments obtained several undulatory curves (*Figure 1*). The shape of the curves,

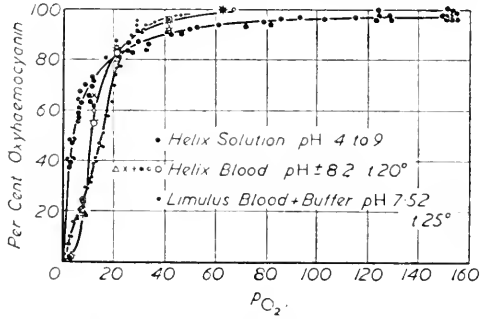


Figure 1. *Oxygen dissociation curves of a salt free solution of haemocyanin of the edible snail* (*Stedman and Stedman*[1]), *haemocyanin of the horse shoe crab* (*Redfield and Ingalls*[1]) *and the blood of the snail* (*Wolvekamp and Kersten*[4]).

which deviate from hyperbolas, was suggested by Redfield to be due to the blood pigment consisting of two or more components, each of which react with oxygen independently of the others according to Hill's equation, with different integral values of n. The idea that undulatory curves can best be explained by postulating the presence of more than one component seems plausible and is illustrated by an experiment of F. H. McCutcheon[5] who obtained undulatory dissociation curves from a solution containing haemoglobin of a tadpole and of an adult frog. Moreover this point of view is substantiated by the work of Svedberg *et al*[2], Roche[3] and Brohult & Borgman[6]. On the other hand the s-shape might as well be explained by assuming interdependence between prosthetic groups of one molecule, especially as the development of Adair's theory has in haemoglobin led to remarkable results.

Abandoning these interesting biochemical questions we shall now turn to the comparative physiology of haemocyanin. The affinity for oxygen of the haemocyanin in the blood of the king crab (Limulus) is very great. The oxygen dissociation curve of the blood of the snail Busycon (*Figure 5*) is also fairly steep. In both bloods the Bohr effect is reversed. Whilst the first fact certainly will enable the animals to acquire a fair supply of oxygen at low oxygen pressures provided, however, that their gills function effectively, the diffusion of oxygen into the tissues must be limited by the low pressure at which it is delivered.

It has sometimes been contended that a negative Bohr effect might enhance the oxygen uptake, assuming that in a habitat poor in oxygen considerable amounts of carbon dioxide might be present. Now A. Krogh[7] has pointed out that relatively high carbon dioxide tensions in natural water will only be found in a milieu where fermentative processes take place, because even in carbonate-free water the carbon dioxide tension would only rise to $\pm$ 5 mm if all the oxygen originally present had been converted into carbon dioxide by respiration. As Limulus (*Figure 5*) often burrows in the mud, the possibility at least of measurable CO_2 tensions occurring in the respiratory milieu must be admitted. The discharge of oxygen into the tissues must be still more impaired by the negative Bohr effect. Of course it might be argued that acquisition of a sufficient amount of oxygen by the gills must have precedence, though this seems to me rather far-fetched.

Turning to the properties of the blood of the edible snail (*Helix pomatia L*) we have to deal with a still more enigmatic case. As has been mentioned before, the oxygen dissociation curve is sigmoid in the virtual absence of carbon dioxide, but it becomes more or less hyperbolic at relatively low carbon dioxide pressures. It may be pointed out that this change of shape is not caused by any permanent change

in the physico-chemical properties of the blood, due to manipulations during the experiment, because the phenomenon is reversible (*Figure 2*).

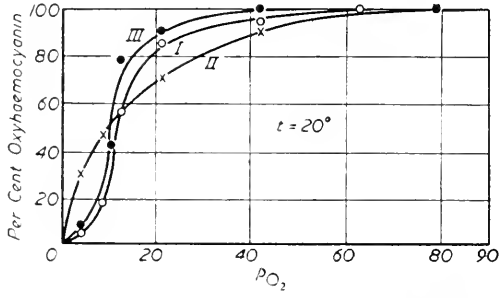


Figure 2. Abnormal Bohr effect in the blood of the edible snail (Wolvekamp and Kersten[4]). I—Dissociation curve in the virtual absence of CO_2. *II—at a* CO_2 *pressure of 23 mm. III—in the virtual absence of* CO_2. *The blood sample previously used for the determination of curve II was evacuated and used for obtaining curve III.*

It is a typical *p*H effect because identical dissociation curves are obtained after addition of dilute hydrochloric acid instead of carbon dioxide, provided the *p*H values are the same[4]. Now this fact according to current notions on the significance of the s-shape, ought to make this animal one of the most unhappy representatives of the animal kingdom because not only the discharge of oxygen into the tissues is interfered with but the acquisition of oxygen in the so-called lung, in which according to M. A. IJsseling[8] carbon dioxide tension may rise to relatively high values, especially during hibernation, must needs be lowered as well.

The oxygen dissociation curves of the blood of several decapod Crustacea have been determined as long ago as 1926 by Stedman and Stedman[1] and by Redfield, Coolidge and Hurd[1]. They are all steep, and occupy a similar though not identical position. Recently I have performed some experiments on the influence of temperature and *p*H on the oxygen binding of blood of lobster and crab. Previously, similar experiments on purified *solutions* of haemocyanin have been performed by Stedman and Stedman[1] and by Redfield and Ingalls[1].

The blood of the lobster (*Homarus vulgaris M.E.*) shows two peculiarities. In the first place at higher temperatures the inflection of the dissociation curve is much more marked. This, combined with the customary shift of the whole curve to the right, will greatly enhance the discharge of oxygen into the tissues at higher temperatures, a fact

of some interest in relation to the increase of metabolic rate at rising temperatures in poikilothermic animals. In the second place there is an enormous Bohr effect (*Figure 3*).

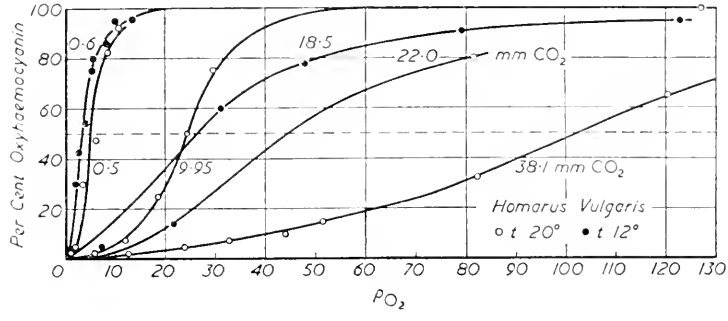


Figure 3. *Oxygen dissociation curves of the blood of the lobster.*

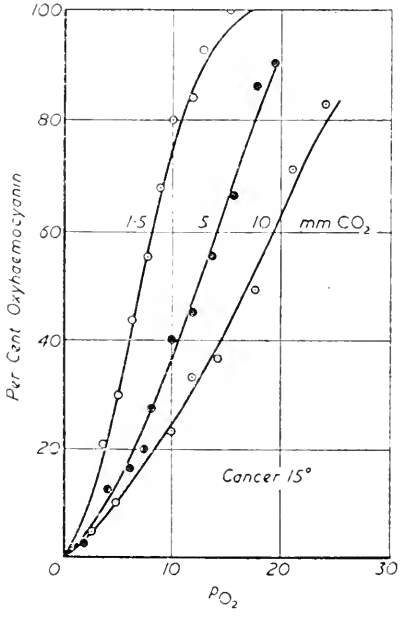


Figure 4. *Oxygen dissociation curves of the blood of the edible crab.*

The displacement of the dissociation curve of the edible crab (*Cancer pagurus L*) by carbon dioxide on the contrary, is comparatively small (*Figure 4*).

The crab is a very sluggish animal feeding on mussels. One is particularly struck by the extraordinarily slow movements of the powerful claw muscles. The lobster spends much of its life in hiding places but will, when capturing a prey or trying to escape some enemy, execute a series of vigorous quick movements, during which the oxygen consumption must rise considerably, and this call for extra oxygen

will certainly be dealt with in the main by the Bohr effect, as a fine regulation of the blood flow in animals with an open circulation system can hardly be expected.

Data on the gas-binding properties of the blood of four species of ink-fishes belonging to three biological types of very different anatomical build and different behaviour have been obtained, namely the American squid (*Loligo pealei*) by Redfield, Coolidge and Hurd[1] (1926) and by Redfield and Ingalls[1] (1933), the European squid (*Loligo vulgaris Lam.*) and the cuttle fish (*Sepia officinalis L.*) by Wolvekamp, Baerends, Kok and Mommaerts[9] and the octopus (*Octopus vulgaris Lam.*) by Wolvekamp[10].

The torpedo-shaped squid is a very active pelagic animal, swimming incessantly with the aid of its triangular lateral fins. The flattened cuttle fish will either swim about rather slowly with the aid of its fin seam, or lie on the bottom much as plaice or rays do. *Octopus* has a bag-like body and possesses no fins. It crawls about on the bottom with the aid of its arms but spends most of its time in some hiding place. Moreover all ink-fishes may execute backward movements by discharging a jet of water from the mantle cavity, by way of the so-called funnel. Squids and cuttle fish cover considerable distances during their migrations to and from their spawning places.

The oxygen dissociation curves of *Octopus* blood are, in the absence of carbon dioxide, very steep. The displacement of the curve at moderate carbon dioxide tensions is considerable. The affinity of the haemocyanin in the blood of *Sepia* is of the same order of magnitude as in the blood of *Octopus*, but the Bohr effect is much more pronounced. In the European squid the affinity for oxygen is somewhat lower and the dissociation curve is more inflected. There is a large shift to the right of the oxygen dissociation curve, even at very small partial tensions of carbon dioxide. The affinity for oxygen of the blood pigment of the American squid is small and the Bohr effect may be called excessive (*Figure 6*). It is fairly clear that the squids, and specially the American species, will be able to live in well aerated water only, but *Octopus*, which often hides in narrow caverns, can stand lower oxygen pressures. *Sepia* would occupy an intermediate position. On the other hand the enormous Bohr effect will greatly enhance the acquisition of oxygen by the tissues in the squid. Generally speaking there is a correlation between the gas-binding properties of the blood and the way of living.

Simple inspection of the blood vessels of the gills in an animal whose pallial cavity has been exposed by a slit in the mantle, while a strong jet of water is directed on the gills, shows that the dark blue blood leaving the gills has become colourless before returning through the

Figure 5. Some marine animals whose blood contains haemocyanin. 1 *Squid*, 2 *Cuttle fish*, 3 Octopus, 4 Fulgur (Busycon) perversum L., 5 *Edible crab*, 6 *King crab*.

branchial artery. Redfield and Goodkind[1] (1929) determined the gas content of arterial and venous blood of the squid and found that the blood delivers to the tissues practically all the oxygen acquired in the gills, while the shift in *p*H accounts for about 30 per cent of the total amount delivered.

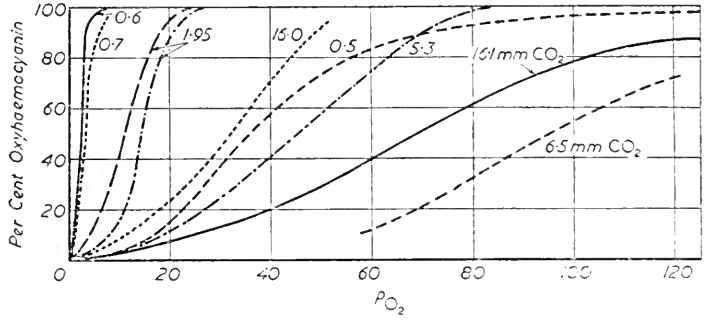


Figure 6. Oxygen dissociation curves of ink fishes
Octopus vulgaris . . . . . *t* 14° (*Wolvekamp*)
Sepia officinalis ———— *t* 14° —— —— *t* 20°
(*Wolvekamp, Baerends, Kok and Mommaerts*[6])
Loligo vulgaris —·—·—·— *t* 20° (,,)
Loligo pealei — — — — *t* 23° (*Redfield and Ingalls*[1])

The acquisition and distribution of oxygen in the cephalopods is furthered by a highly developed circulatory system and an efficient respiratory mechanism. The utilization of the oxygen dissolved in the respired water may amount to 80 per cent[11]. In collaboration with Mrs. J. Baerends-van Roon I determined the respiration volume of resting cuttle fish. The animals were clamped in a specially devised stand. A glass canula was tied into the funnel and connected with rubber tubing of a very wide bore in order to collect the total quantity of expired water. At a temperature of 15°–17°C animals of about 500 gm weight would respire 300–600 cm^3 of water in one minute.

We determined the oxygen consumption in well aerated water and found that animals of this size would consume about 100 cm^3 per kg per hour when at rest, that is about the same amount as used by some fresh water fish, while man at rest consumes twice as much. Redfield and Goodkind found for the American squid an oxygen consumption about six times higher than that found by us for *Sepia*.

By what means do *Sepia* and *Loligo* succeed in transporting these rather considerable quantities of oxygen to their tissues ? We have seen that the respiratory mechanism is efficient. Is the transporting system as efficient ? In the first place it must be pointed out that the oxygen capacity of the blood, though higher than that of other inverte-

brates with haemocyanin, is very low, the maximum value obtained by us for *Sepia* being 3 per cent. This means that at comparable saturation and unloading conditions the blood of *Sepia* transports only one sixth of the quantity of oxygen transmitted by human blood.

Further we ought to know the total quantity of blood. We can get from one *Sepia* of half a kilo weight about 20 cm^3 blood, but it is quite certain that we do not get all the blood contained in the circulatory system. There is another factor which ought to be taken into account. Comparative physiologists are in the bad habit of trying always to bleed their animals to the last drop, even by a lot of sucking and squeezing. I feel pretty sure that in doing so a not inconsiderable quantity of tissue fluid will pass over into the blood circulation and be drained off as well. This source of error would more or less counterbalance the first mentioned.

Even if it is assumed that the total quantity of blood is 50 per cent higher than the volumes obtained by bleeding the animals, we arrive at the conclusion that the quantity of blood per kg bodyweight is not higher, but if anything lower than in man. The low oxygen capacity must accordingly be compensated by a high circulation rate and by the properties of the blood-pigment, or by one of those factors. We do not know how far blood circulation may be adapted to the oxygen needs of the tissues, though some regulation of the blood circulation certainly occurs. On the other hand the blood leaving the tissues is always completely deoxygenated. In this respect the cephalopods compare unfavourably with mammals where in resting condition the arterial blood delivers no more than 30 per cent of its oxygen content. The rest forms a reserve upon which the tissues can draw during severe exercise by means of the Bohr effect combined with the opening of a great number of capillaries. In the ink-fishes the only means of discharging more oxygen into the tissues would seem to be a shortening of the respiratory cycle by speeding up blood circulation.

The carbon dioxide transport by blood containing haemocyanin shows some peculiarities worth mentioning. In the first place the blood does not contain carbonic anhydrase as was found by M. FLORKIN[12] and VAN GOOR[13]. The tissues, however, contain according to FERGUSON, LEWIS and SMITH[14] and to van Goor[13] varying quantities of this catalyst while the greatest activity of the enzyme is always found in the gills. Probably the carbon dioxide passes into the tissues in the form of carbonic acid, or bicarbonate ions together with H^+ ions will migrate into the blood, there to be counter-balanced by available base (the *p*H of the bloods in the absence of CO_2 lies at about 8·5). The reverse process will take place in the respiratory organs. Possibly complications occur in relation to ionic interchange due to osmotic regulation.

The Haldane effect is not very marked in the decapods, and is absent in the blood of the edible snail[15] (*Figure 7*). In the squid, however, the Haldane effect is very marked and is largely responsible for the carbon dioxide exchange in the gills (Redfield, Coolidge and Hurd[1]).

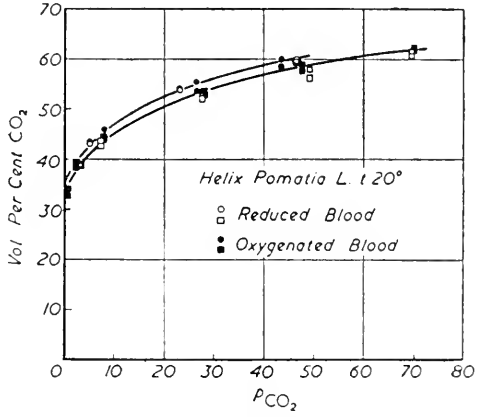


Figure 7. Carbon dioxide dissociation curves of the blood of the edible snail.

The question whether part of the carbon dioxide might be transported in the form of carbamate was investigated for the blood of the lobster,

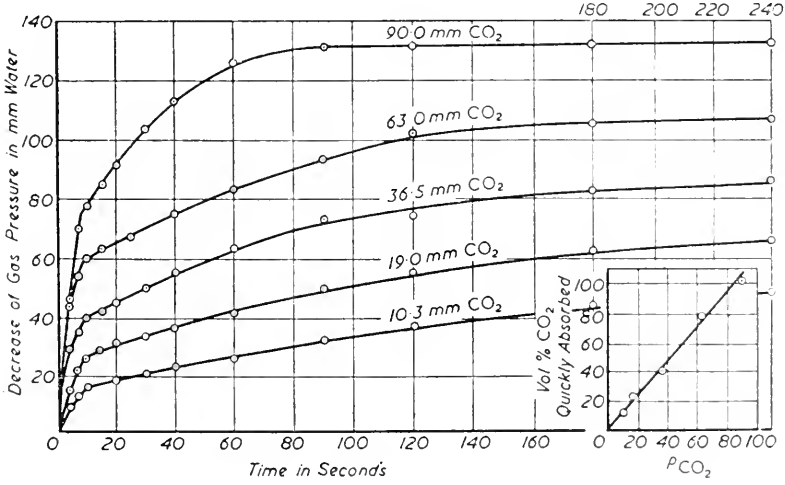


Figure 8. Absorption of carbon dioxide by the blood of the edible snail in the apparatus of Stadie and O'Brien[13] at different CO_2 pressures. The small graph gives the relation of the amount of CO_2 quickly absorbed with the CO_2 pressure in the gas mixture (Wolvekamp and Kruyt[15]).

the edible crab and the snail. The fact that the *p*H of the blood of these animals is higher than 8 when in equilibrium with atmospheric air, whilst the isoelectric point of haemocyanin lies between *p*H 4·7 and *p*H 5·2, would seem to favour the formation of this compound. This seems, however, not to be so for in collaboration with Kruyt[15] I found with the method of Stadie and O'Brien[16] that the amount of CO_2 quickly absorbed by the blood of the snail, the crab and the lobster does not surpass the quantities absorbed by water (*Figure 8*).

A comparison between the content of different amino acids in haemoglobin and in haemocyanin does not reveal as far as I can see very striking differences that would account for the absence of carbamate formation.

Table I. Content of Some Amino Acids in Round Numbers, based on a Table of Roche

	Hcy of Helix pomatia	*Hb of Homo*
Tryptophane	6·0%	2·5%
Cystine	2·0%	1·0%
Histidine	6·0%	8·5%
Lysine	7·5%	9·5%
Arginine	5·0%	3·5%
Tyrosine	4·5%	3·0%

Roche[3] from titration curves of haemocyanin of five species—amongst them the edible snail—confers to the haemocyanin the three *p*K values 6·6–7·0, 7·8–8·4 and $\pm$ 10·5.

As, however, the interpretation of those *p*K values in relation to the fact of the direct combination of CO_2 with the blood pigment has led in haemoglobin to controversies which are not yet solved (v. Roughton[17]), I do not think it advisable at the present to try to deduce any explanation, from these *p*K values and the table of amino acid contents, of the absence of direct binding of CO_2 by haemocyanin.

I should have preferred to present more well established facts and less speculations, but at the present our data on the gas binding properties of the blood of molluscs and crustaceans, fragmentary as they are, are still far ahead of our knowledge of their blood circulation and respiration, not to speak of the respiratory conditions in the natural habitat. It might be pointed out, on the other hand, that further experiments on cephalopods at least look promising.

It might also be objected that some of the gas binding properties of the blood, especially of the snail, might eventually prove to have no

biological significance at all, but personally I am more in sympathy with the opinion, put forward by Sir Joseph Barcroft in his admirable book *Features in the Architecture of Physiological Function* that, in the organism, a phenomenon is more likely to have a significance than not.

Received August 1948

REFERENCES

[1] The bibliography of papers published before 1933 may be found in the review of REDFIELD, *Biol. Rev.* 9 (1934) 175.
[2] SVEDBERG, THE and ERIKSSON-QUENSEL, I. B. *Biol. Bull.* 71 (1936) 489
[3] ROCHE, J. *Essai sur la biochimie générale et comparée des pigments respiratoires.* Masson, Paris, 1936
[4] WOLVEKAMP, H. P. and KERSTEN, H. J. *Z. vergl. Physiol.* 20 (1934) 702
[5] MCCUTCHEON, F. H. *J. cell. comp. Physiol.* 20 (1942) 393
[6] BROHULT, S. and BORGMAN, K. *The Svedberg 1884–30–1944.* Almqvist and Wiksell, Uppsala, 1945
[7] KROGH, A. *The Comparative Physiology of Respiratory Mechanisms.* University of Pennsylvania Press, Philadelphia, 1941
[8] IJSSELING ,M. A. *Z. vergl. Physiol.* 13 (1930) 1
[9] WOLVEKAMP, H. P., BAERENDS, G. P., KOK, B. and MOMMAERTS, W. F. H. M. *Arch. néerl. Physiol.* 26 (1942) 203
[10] — *Z. vergl. Physiol.* 25 (1938) 541
[11] HAZELHOFF, E. *ibid.* 26 (1938) 30 ›
[12] FLORKIN, M. *Arch. int. Physiol.* 40 (1935) 283
[13] GOOR, H. VAN *Enzymologia* 8 (1940) 113
[14] FERGUSON, J. K. W., LEWIS, L. and SMITH, J. *J. cell. comp. Physiol.* 10 (1937) 395
[15] WOLVEKAMP, H. P. and KRUYT, W. *Arch. néerl. Physiol.* 28 (1945) 620
[16] STADIE, W. C. and O'BRIEN, H. *J. biol. Chem.* 112 (1935) 723
[17] ROUGHTON, F. J. W. *Harvey Lect.* 39 (1944) 96

AUTHOR INDEX

This index does not include names cited in Part I : Tributes to Sir Joseph Barcroft